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First report on molecular identification and fenbendazole resistance against *Baylisascaris transfuga* infection in *Melursus ursinus* (sloth bear)

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Summary

Based on morphometric findings and polymerase chain reaction amplification of internal transcribed spacer regions (ITSs),the worms and eggs retrieved from sloth bear (*Melursus ursinus*) kept at Mahendra Mohan Choudhury Zoological Park, Chhatbir (Punjab, India) were delineated as *Baylisascaris transfuga*. Therapeutic intervention with fenbendazole à 10 mg/ kg body weight for three consecutive days, associated with complete disinfection of the enclosures, proved to be effective for the first few days with the reduction of eggs per gram (EPG) of faeces from 2800 (zero day) to 400 (14.28 %), 700 (25 %) and 800 (28.57 %) on days 1, 4 and 7 post treatments, respectively. The intensity of EPG increased to 2400 (85.71 %) on day 21 post treatment with respect to day zero. A modified schedule with fenbendazole à 15 mg/kg body weight for first three days followed by 10 mg/kg body weight for next three days proved to be effective thus indicating the development of resistance. The study places on record the molecular characterisation of *B. transfuga* from sloth bears and development of resistance against fenbendazole.

Keywords: *Baylisascaris transfuga*; deworming; eggs per gram; ITSs; molecular identification; sloth bear

Introduction

The different species under *Baylisascaris* genus are relatively host specific nematodes of carnivores, omnivores, herbivores and rodents (Bauer, 2013). *Baylisascaris transfuga* is an ubiquitous nematode (Papini *et al.*, 1990; Foster *et al.*, 2004; Sato *et al.*, 2004), affecting various species of bears (Rogers & Rogers, 1976; Foster *et al.*, 2004; De Ambrogi *et al.*, 2011; Testini *et al.*, 2011). Various experimental trials to assess the potential of embryonated eggs of *B. transfuga* to infect accidental hosts had been proved to generate *visceral*, *neural*, or *ocular larva migrans* syndromes in different animals including mice (Papini *et al.*, 1994c, 1996), gerbils (Sato *et al.*, 2004), guinea pigs (Matoff & Komandarev, 1965), mongolian jirds (Sato *et al.*, 2004) and chickens (Papini *et al.*, 1993). In recent past, an outbreak of neurological disease in Japanese macaques, enclosed at proximity of the cages of American black bears have been reported in a zoological safari of Japan (Sato *et al.*, 2005). Due to the ability to infect the incidental hosts, the professionals dealing with the bears including zoo keepers, trappers and hunters are at constant risk of exposure to this parasite (Testini *et al.*, 2011). The presence of hardy cell wall and passage of large quantity of eggs with the faeces are the main reasons which render the infection of *B. transfuga* difficult to eliminate completely from the environment (Testini *et al.*, 2011). Excessive stress in captive conditions, results into heavy infection leading to clinical disease entity in the hosts and requires immediate therapeutic intervention.

The role of the benzimidazole anthelmintics have been established for the control of economically important gastrointestinal parasites of domesticated animals. But, the repeated indiscriminate use of these valuable anthelmintics has resulted in development of resistance (Sutherland *et al*., 1988). The resistance against domesticated animals have been reported from time to time (Sutherland & Leathwick, 2011). Recently, reports against captive animals including giraffe (Garretson *et al*., 2009) and alpacas (Galvan *et al*., 2012) are also documented. But, after thorough search of literature, authors were unable to find any such report in sloth bears.

The identification of the eggs based on morphology from the faecal samples corresponding to a particular species is cumbersome job, which requires skill, experience and expertise. Thus, various molecular techniques have been developed over the years for the identification of the eggs, larvae and adults of the parasites up to species level (Dangoudoubiyam *et al.*, 2009). Though various reports from India from time to time confronted the presence of

ascarids in sloth and Himalayan bears (Baylis & Daubney, 1922; Islam & Nashiruddullah, 2000; Nashiruddullah & Chakraborty, 2001; Singh *et al.*, 2009), but no study supported it with molecular based diagnostic confirmation. Thus, the present study was planned with the objective of PCR-based molecular characterisation of *B. transfuga* targeting internal transcribed spacer regions (ITSs) and therapeutic management with fenbendazole to control the parasite.

Materials and methods

Collection of the samples

The faecal sample of 13 bears (8 Himalayan black bears and 5 sloth bears), kept at Mahendra Mohan Choudhury Zoological Park, Chhatbir, Punjab, India were screened seasonally (from January 2013 to January 2014) at regular basis for any parasitic infestation. All the bears (7 males and 6 females) were kept individually in separate enclosures with wooden floors. Despite of proper management by zoo authorities including anthelmintic therapy, one sloth bear (*Melursus ursinus*) showed reoccurrence of nematode infection as observed by continuous presence of ascarid eggs in the faeces. For molecular identification of the parasite, the eggs were collected from the faeces before and after deworming and the adult worms were retrieved after deworming of the infected individual with fenbendazole (Fenomar®, Virbac, India). The worms after complete washing with normal saline were kept in 70 % ethanol until further processing. The faecal samples of all the bears 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 morphometric analysis targeting the size of the eggs was performed as per Kazacos and Turek (1983). The length and breadth of the eggs was depicted in micrometers and was expressed considering mean ± standard deviation.

Morphological analysis of the worms

For detailed examination male and female adult worms (n=5 each), based on size differences were selected and were subjected to morphometric studies. The length and width of the worms were measured in millimetres (mm) and were expressed considering mean ± standard deviation. Final confirmation was made based on morphological structures of worms after clearing in lactophenol (Zajac, 1994).

Genomic DNA extraction from adult worms

The genomic DNA was extracted from adult worms with QIAamp tissue kit (Qiagen, Hilden, Germany) by following manufacturer's instruction with slight modification. The worm was kept at -80 °C for 6 hours before starting the procedure. The worms were mechanicallly disrupted by using sterile pestle-mortar. The disrupted worms were kept in lysis (AL) buffer overnight at 56 °C. The final elution of the genomic DNA was carried out by using 100 µl of elution (AE) buffer.

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

The genomic DNA's extracted from the adult nematodes and eggs were used in PCR to amplify the internal transcribed spacer regions (ITS-1 and ITS-2), by using the primers (forward: 5'-ACT GCT GTT TCG AGA CCT TTC GAG-3' and reverse: 5'-TAG CAC CTT CTT TGG ACT ATA GCC-3') (Testini *et al.,* 2011) for the detection of *B. transfuga*. The PCR protocol for amplification of the templates was performed in a 25 µl of total reaction volume with following conditions in sequential order: (i) Initial denaturation (94 °C for 12 min.), (ii) Denaturation (30 cycles of 94 °C for 30 sec.), (iii) Annealing (58 °C for 45 sec.), (iv) Extension (72 °C for 45 sec.), and (v) Final extension (72 °C for 7 min.). For complete confirmation of the eggs belonging to the same parasite species, the eggs collected from the faecal sample on day 1 post deworming were also subjected to the DNA extraction and PCR amplification process. Negative faecal sample from another bear, kept at Tiger safari in Ludhiana, screened twice served as a negative control. The DNA from the eggs of *Toxocara canis* was also run simultaneously as a control to rule out the presence of any other related species. The positive products obtained were sent for custom sequencing to Xcleris Genomics, Ahmedabad, India.

The identity of the sequences and homology was confirmed after comparing the product sequences with the sequences available in GenBank, by using Basic Local Alignment Search Tool (BLAST 2.2.22) (Testini *et al.*, 2011). To ensure open reading frame and to exclude pseudogenes individual ITSs sequences were translated into amino acid sequences and were then analysed using MEGA 5.0 (Molecular Evolutionary Genetic Analysis) software (Tamura *et al.*, 2011) for phylogenetic analysis.

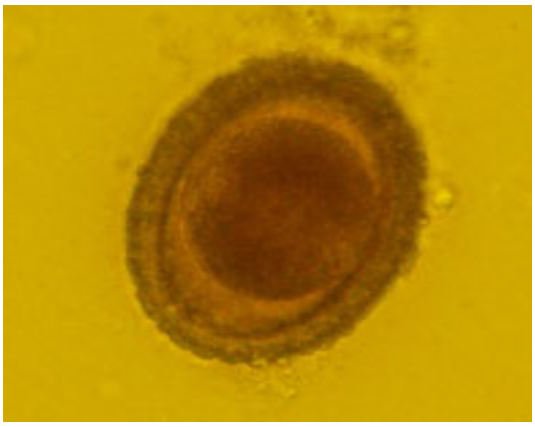

Fig. 1. Photomicrograph of Unembryonated *B. transfuga* egg

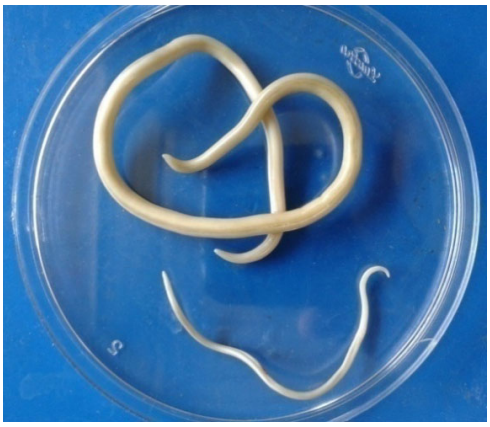

Fig. 2. The adult male (78 mm) and female (175 mm) *B. transfuga* worms collected from sloth bear

Therapeutic management

Baylisascaris transfuga infection was managed with fenbendazole (Fenomar®, Virbac, India) à 10 mg/ kg/ body weight *per os* for three consecutive days. Quantitative evaluation *i.e.* eggs per gram (EPG) of faeces was determined by using McMaster technique (Soulsby, 1982). Drug schedule was originally carried out for three days. Due to the failure in complete expulsion of the worms this was modified and an extended treatment schedule for six days was selected. The initial treatment with fenbendazole à 15 mg/ kg body weight for first three days was extended for another three days with a dose rate of 10 mg/kg body weight.

Results

Based on microscopical examination, only one sample was found positive for ascarid eggs. All other 12 samples were found negative for any parasitic egg or cyst. After deworming, nematode worms were also recovered from the infected individual and rest of the bears were observed negative. On detailed microscopic morphological examination, brown ascarid eggs appeared rounded to oval with thick, pitted cell wall and measured morphometrically (n=10). The egg size was 81.78 ± 10.90 µm (65.8 – 94.0 µm) × 65.8 ± 9.90 µm (47.0 – 75.2 µm) (length × breadth) (Fig. 1).

The adult worms of *B. transfuga* were identified on the basis of gross and microscopic morphological examination. Grossly, the size of the male worms (n=5) were measured 79.2 ± 11.3 mm (64 – 94 mm) × 1.5 ± 0.3 mm (1.2 – 1.8 mm) (length × width), whereas, female parasites (n=5) were measured 162.8 ± 18.7 mm (138 – 183 mm) × 2.3 ± 0.5 mm (1.7 – 2.9 mm) (length × width), respectively (Fig. 2). The distinctive morphological features included three developed triradiate lips (one dorsal and two subventral) with cervical alae and filariform oesophagus (Fig. 3) in adult parasites of *B. transfuga*. The caudal ends of the male parasites were slightly rolled with presence of large number of precloacal papillae (48 – 70) (Fig. 4), with prominent cloacal opening and tail knob (Fig. 5), whereas female posterior end had a blunt tail.

An 1177 base pair product was obtained after amplification of the DNA extracted from the eggs and the adults. Similar size products were also obtained for the DNA templates of the eggs retrieved after deworming, confirming the infection of sloth bear with *B. transfuga* only (Fig. 6). The similarity between the *B. transfuga* sequences obtained in the present study with the sequences available in GenBank targeting ITSs by Arizono *et al.* (2010) (AB571304.1), Testini *et al.* (2011) (HM594951.1) and Franssen *et al.* (2013) (KC543489.1) approached 99, 97 and 97 per cent, respectively (Fig. 7) by applying sequence diversity calculations targeting intra-population diversity. All other 12 samples found negative by classical parasitological technique were also confirmed negative by PCR. The primers were not able to amplify the DNA retrieved from the eggs of *Toxocara canis.*

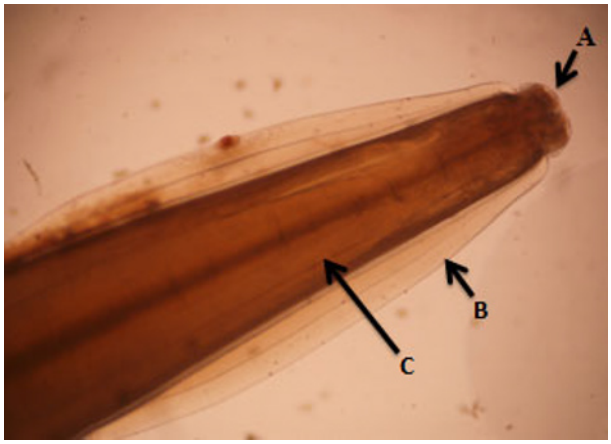

Fig. 3. Photomicrograph of anterior end of *B. transfuga* showing triradiate lips (A), cervical alae (B) and oesophagus (C)

The bear showed high EPG (2800) for *B. transfuga* eggs before the therapeutic intervention. The regular intervention strategy *i.e.* deworming with fenbendazole à 10 mg/kg body weight for 3 consecutive days proved little effective with decreased EPG values, ranging between 400 – 800 at days 1, 4 and 7 post treatment with a decrease in 85.73 %, 75.0 % and 71.43 % on days 1,4 and 7, respectively. But, an increased EPG value of 2400 (6 times) was recorded at

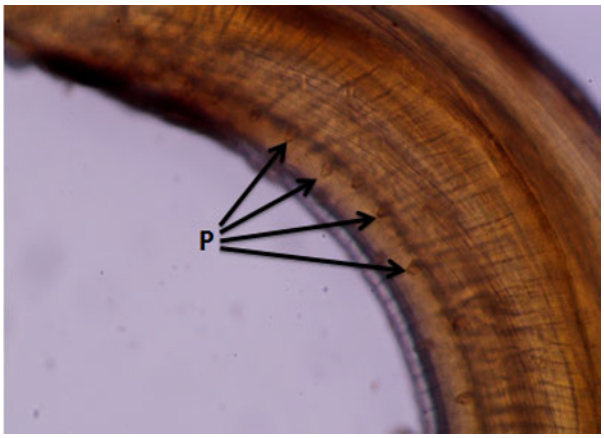

Fig. 4. Photomicrograph of the curved caudal end of male *B. transfuga* with precloacal papillae (P)

day 21 post treatment with respect to day 1. Therefore, a modified schedule was opted with an increased dose and period of treatment *i.e.* for 6 consecutive days with a dose rate of 15 mg/kg body weight for the first three days followed by 10 mg/kg body weight for the next three days. With an increased treatment period schedule the EPG values were observed zero at days 1, 4, 7 and 21 post treatment (after the last day of treatment).

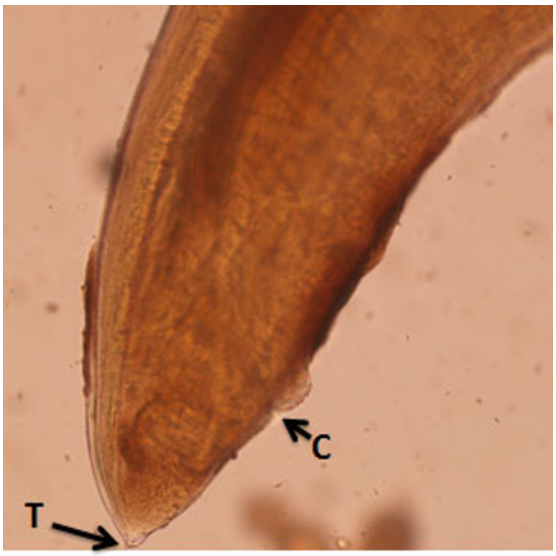

Fig. 5. Photomicrograph of posterior end of male *B. transfuga*: cloacal opening (C) and tail knob (T)

Discussion

A detailed discussion has been made regarding the parasites of different species of bears by Rogers and Rogers (1976) in their review. Recently, baylisascariosis has been incriminated as unusual roundworms infection of animals and humans (Bauer, 2013). In India, a very limited study has been conducted on *B. transfuga i.e.* also on morphological identification. Thus, the present study targeted the morphological and molecular identification of this detrimental bear nematode. The morphometric observations of the eggs are in concordance with the morphometric values reported by Sprent (1968), Papini *et al.* (1990) and Testini *et al.* (2011). Although, the morphological appearance and morphometric description of the eggs substantiates the identification of the parasite but due to the earlier report of incidence of *Toxocara canis* and *T. mystax* in bears (Couturier, 1954) necessitated the use of molecular techniques for specific identification and confirmation of the eggs of *B. transfuga*.

The morphological observations of adult male and female *B. transfuga* parasites were in corroboration with the observation of Testini *et al.* (2011), who recovered the same parasites from polar bears.

The product size of 1177 bp obtained after PCR amplification was in concordance with the study of Testini *et al.* (2011). The genetic markers targeted ITSs, showed marked interspecific differences with *B. procyonis* species (JQ403615.1), *B. columnaris* species (KC543484.1, KC543485.1, KC543486.1, KC543487.1, KC543488.1) and *B. schroederi* species (JN210911.1, JN210912.1) reported from Norway, the Netherlands and China, respectively (Fig. 7) on application of sequence diversity calculations. There was pronounced similarity with the sequences available in GenBank targeting ITSs of *B. transfuga*. Franssen *et al.* (2013) carried out the study on *B. transfuga* parasites recovered from brown bear and sloth bear; whereas, Testini *et al.* (2011) recovered the parasites from polar bears, which showed greater homology with the parasites recovered from sloth bears in the present study. The complete phylogenetic analysis of the sequences showed that the strain recovered in this study is most closely related to the parasite strain recovered from pigs (not from bears) which were delineated as *B. transfuga* by the authors (Arizono *et al.*, 2010). In order to check the specificity of the primers, simultaneous amplification of DNA extracted from the eggs of *T. canis* was carried out through PCR, which showed negative results (Fig. 6).

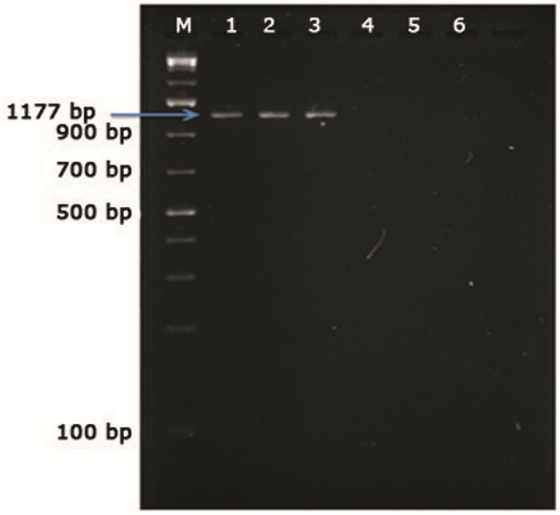

Fig. 6. PCR amplification targeting ITSs: M – 100bp plus Gene Marker, 1 – PCR product of adult worm, 2 – PCR product of eggs collectedbefore deworming, 3 – PCR product of eggs collected after deworming, 4 – Negative control, 5 – Negative control containing PCR products of *Toxocara canis* eggs, 6 – Non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

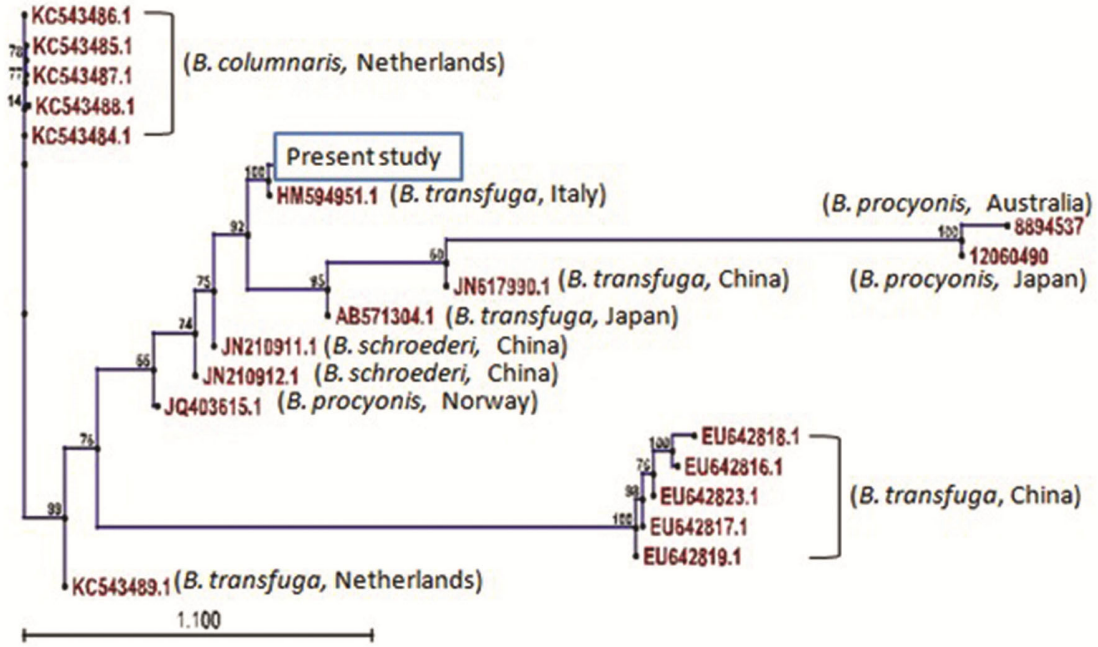

Fig. 7. Phylogenetic relationship of *Baylisascaris transfuga*, recovered from *Melursus ursinus*, and related species of *Baylisascaris* genus (The values/ numbers at the nodes of the branches depict confidence level)

The parasite under study possesses a direct life cycle and the mature females are capable of passing 10,000 to 20,000 eggs per gram of faeces, leading to heavy contamination of the environment (Papini *et al.*, 1994). Hence the parasite is considered to be difficult to eliminate from the environment. We have seen by *in vitro* developmental study on the eggs of this parasite that they require around 12 – 15 days to become infective when optimum temperature and humidity is provided (Moudgil *et al.*, 2014). Similarly, Papini *et al.* (1994b) observed the development of *B. transfuga* eggs to infective stage on days 11 and 12 post incubation in 0.1 N sulphuric acid at room temperature. The present study revealed that three-day treatment schedule with benzimidazole (fenbendazole) is not sufficient to eliminate the parasite from the host as indicated by increase in EPG values at Day 21. This observation could be considered as treatment failure either due to development of tolerance or resistance, which could be attributed to regular use of fenbendazole as an anthelmintic in the bears for a long time with a lower dosage regimen *i.e.* 7.5 mg/ kg body weight or ineffective drug dosage as the bear failed to consume the whole therapeutic dose. Papini *et al*. (1994a) successfully used mebendazole against *B. transfuga* infection in polar bears. The benzimidazole resistance has been repeatedly reported in domestic animals (horses, sheep, goats, pigs, cattle) (Gerwert *et* al., 2002; Owen *et al.*, 2008; Sutherland & Leathwick, 2011) and recently in some zoo animals such as alpacas (Galvan *et al*., 2012) and giraffe (Garretson *et al*., 2009). This is the first report of benzimidazole (fenbendazole) resistance against *B. transfuga* in sloth bears. The enteric invasion of the definitive host by *B. transfuga* appears less pathogenic, unless heavy infection leads to serious complications, involving obstruction of the intestines (Bauer, 2013). The prophylactic and therapeutic management of this parasite requires special attention due to availability of large number of the eggs in the environment of positive individuals. The resistance against benzimidazoles develop in stages and in the initial phase the individual parasites respond to repeated doses of the drug (Coles *et al*., 2001), as it has been observed in the present study. Thus, further study with another group of anthelmintic could help to overcome the resistance against *B. transfuga* in sloth bears.

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