Original Research Article

Molecular Identification of *Oesophagostomum* spp. from Himalayan Grey Langur

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Nodular worm, *Oesophagostomum* spp. are most pathogenic strongyles of ruminants, pigs, non-human primates and other mammals. Transmission of *Oesophagostomum* parasite between humans and non-human primates are not frequent, however, it may occurs in areas where habitats are overlapped. The present study was carried out to explore the seasonal prevalence of strongyle infection in Himalayan grey langurs (*Semnopithecus ajax*) of Mid Himalayan range, Kumaon region, Uttarakhand over the period of six years (April 2011 to March 2017) with special emphasize on *Oesophagostomum* spp. The occurrence and intensity of infection was ascertained by qualitative and quantitative methods and results were compared with PCR method. The egg per gram (EPG) was determined by McMaster method from salt floatation positive samples. DNA was extracted from microscopy positive samples (*n*=50) and negative faecal samples (*n*=100); subsequently the DNA was subjected for amplification of Internal Transcribed Spacer-2 (ITS-2) of ribosomal DNA (rDNA) of *Oesophagostomum* spp. The overall incidence of strongyle infection based on microscopy was 12.82% (104/811). The mean EPG was varied between 171- 528±39. The genus specific PCR amplified single, 333bp *Oesophgostomum* spp fragment from genomic DNA. The PCR method amplified all the microscopy positive faecal samples, additionally it also revealed 29 positive out of 100 samples which were negative by microscopy. The overall prevalence of *Oesophgostomum* spp by PCR was 52.66 % (79/150), which indicates the higher sensitivity of the test than microscopy. The intensity of infection was more in monsoon and autumn months (from mid-August to November) and less positive cases were observed from winter and springs. The information gathered in this study revealed that *Oesophagostomum* is a common strongyles infecting Himalayan Grey Langur and it has zoonotic transmission potential.

Introduction

India is rich in unique and diverse wildlife. A number of factors threaten the existence of wild animals in this country, including parasitic diseases arising from gastrointestinal nematodiasis. The exchange of disease is a concern for wildlife conservation both outside

and inside the boundaries of parks and reserves. The presence of tourists and other personals has created a situation that may facilitate disease transmission between humans, livestock and wildlife (Simonetti, 1995; Butynski and Kalina, 1999). Anthropogenic disturbance is frequently speculated to influence the nature of parasite infections in wildlife (Greger, 2007) and concern over the potential of zoonotic disease transmission has grown with increasing human encroachment and population size (Chomel *et al.,* 2007; Goldberg *et al.,* 2007). The forested edges of primate habitat, especially those that border humanized landscapes, *i.e*., areas of intense human use such as villages or crop fields, provide opportunities for humans and non-human primates to come into close proximity.

Nodular worm infections, caused by *Oesophagostomum* spp are common in human and wild primates; mostly asymptomatic and clinical signs have only been recorded in captive settings (Stewart and Gasbarre, 1989). Studies have suggested that there was transmission of nodular worm among non-human primates and humans and thus primates poised a health risk to people or *vice versa* (Krief *et al.,* 2010; Ghai *et al.,* 2014). Among *Oesophagostomum* genus, *O. bifurcum*, *O. stephanostomum*, and *O. aculeatum*, are important due to their potential to infect humans (Ghai *et al.,* 2014). The ingestion of food with contaminated parasitic eggs and their 3rd stage larvae can cause infection (Soulsby, 1982). The major consequence of infection is development of nodules in the intestine due to development of third stage larva. The infection causes abdominal pain, anorexia, diarrhea and cachexia, and occasional death from peritonitis and intestinal occlusion (Soulsby, 1982; Taylor *et al.,* 2015). The eggs/infective third stage (L3) obtained after coproculture can be identified only to the genus level (Gasser *et al.,* 2009). Langurs are

common non-human primates in mid Himalayas; they feed on a variety of plant materials, periodic evacuation of parasites from digestive tract will be a common feature (Joseph *et al.,* 1999). Therefore, detailed survey is essential to understand the implication of gastrointestinal nematodiasis in langurs of mid Himalayan region. As of now, very little or no population data exist for nematode infections of free living langurs of mid Himalayan region. The paucity of data has necessitated this study with primary objective to provide data on gastrointestinal strongyles of langurs with special emphasize on *Oesophagostomum* spp of the cited region.

Materials and Methods

Sample collection

The present study was carried out on free range langurs in the Kumaon ranges of Himalaya (29°28'N and 79°39'E, 7500 feet above mean sea level). A total of 811 relatively freshly voided faecal samples of langurs were collected over the period of six years (April 2011 to March 2017) in all seasons [spring (March to June), monsoon (July to September), autumn (October to November) and winter (December to February)]. The samples were collected randomly from both sex and different age group of langurs. The samples were collected non-invasively in labeled containers from the forest and immediately transferred to laboratory. Since identity of the animals was not possible, samples from the same animals may be collected more than one times. Therefore, the number of sample sizes was probably more than the number of animals.

Parasite examination

The occurrence and intensity of infection was determined by qualitative (salt floatation) method. The positive samples were subjected

for modified McMaster quantitative method for quantification of parasitic eggs (Soulsby, 1982; Fowler, 1986). The microscopy positive (*n*=50) and negative faecal samples (*n*=100) were subjected for DNA isolation using QIAamp DNA stool mini kit (Qiagen, Germany) following manufacturer's instructions. The genomic DNA was subsequently used for polymerase chain reaction (PCR) to identify *Oesophagostumum* spp.

Polymerase chain reaction

The primers for internal transcribed spacer -2 (ITS-2) with upstream flanking region about 117 bp of ribosomal DNA (rDNA) were self-designed using published sequences of *Oesophagostomum* spp (Oeso forward: 5' TCG ACT AGC TTC AGC GAT G 3'; Oeso reverse: 5'CCA AAG CAT TCT TAG TCG CT 3'). PCR was carried out in thin wall PCR tubes in 25 µl reaction volume. The PCR mixture consisted of 12ng of DNA template, 12 pmoles of each primer (Oeso forward and Oeso reverse), 1.5 mM $MgCl_2$, 200 µM of each dNTPs, and 2.5 µl of 10x Taq DNA polymerase buffer and 1 U of Taq DNA polymerase (Fermentas, Germany). The volume of the reaction was made up to 25 µl with autoclaved milli-Q water. Polymerase chain reaction was performed with the following conditions. Initial denaturation at 94ºC for two minutes was followed by 36 cycles each at 94ºC for 30 s, 53ºC for 30 s and 72ºC for 30s. This was followed by 5 min final extension at 72ºC. The amplicons were electrophoresed using 2% agarose gel and the DNA migration and resolution pattern was examined by UV transillumination technique and the picture was documented by photography.

Statistical analyses were performed using the Chi test for analysis (Snedecor and Cochran 1994).

Results and Discussion

The overall incidence of strongyle infection based on qualitative egg analysis on 811 samples was 12.82% (*n*=104) (Table 1). The mean egg per gram (EPG) was varied between 171- 528±39. The EPG was started appearing from Mid-April and maintained in various ranges up to November.

The peak EPG was observed during and after monsoon. Effect of the year on the prevalence of strongyle or *Oesophgostomum* spp was found to be non-significant. It was observed that the prevalence of the strongyle or *Oesophgostomum* spp was fairly comparable for all the six years of the study.

However, effect of season on the prevalence of strongyles was found to be highly significant (*p*=0.00) (Table 2). Prevalence was almost negligible (2.5%) in the winter months (December to February), followed by spring (March to June) (5.64%) by microscopy. Significantly higher (21.22%) prevalence of strongyle spp was observed in the monsoon *i.e.* from July and September followed by in autumn (9.38%) *i.e.* October to November.

The genus specific PCR amplification on ITS-2 specifically amplified single, *Oesophgostomum* spp fragment from genomic DNA isolated from positive and negative faecal samples by microscopy.

The amplicon size was 333bp (Figures 1 and 2), which including ITS-2 (216 bp) and flanking region of about 117 bp upstream to ITS-2. The specific PCR amplified all the microscopy positive faecal samples, which indicating positive detection of *Oesophgostomum* spp.

The PCR also showed 29 out of 100 samples, which were negative by microscopy (Table 3). The overall prevalence of *Oesophgostomum* spp by PCR was 52.66 % (79/150).

Table.1 Prevalence of strongyle infection in langurs by microscopy

Year (April to March)	No of samples screened	Percentage positive for Strongyle spp in qualitative method
2011-2012	194	11.34% (*n*=22)
2012-2013	186	13.97% (*n*=26)
2013-2014	168	13.69% (*n*=23)
2014-2015	93	12.9% (*n*=12)
2015-2016	91	13.18% (*n*=12)
2016-2017	79	11.39% (*n*=09)
Total	811	12.82% (*n*=104)

Table.2 Seasons wise prevalence of Strongyle spp by microscopy

Year (April to March)	No of samples screened	Percentage positive for Strongyle spp in qualitative method
Spring	195	05.64% (*n*=11)
Monsoon	344	21.22% (*n*=73)[**]
Autumn	192	09.38% (*n*=18)[**]
Winter	80	02.5% (*n*=02)

**: *significant at P≤0.01;*

Table.3 Results of PCR amplification for *Oesophgostomum* spp

	No of samples screened	Percentage positive for Strongyle spp in qualitative method	Percentage positive for *Oesophgostomum* spp in PCR
Microscopy positive	50	50	50
Random microscopy negative samples	100	0	29

Fig.1 and 2 PCR amplification of ITS-2 and upstream flanking region about 117 bp of *O. stephanostomum*
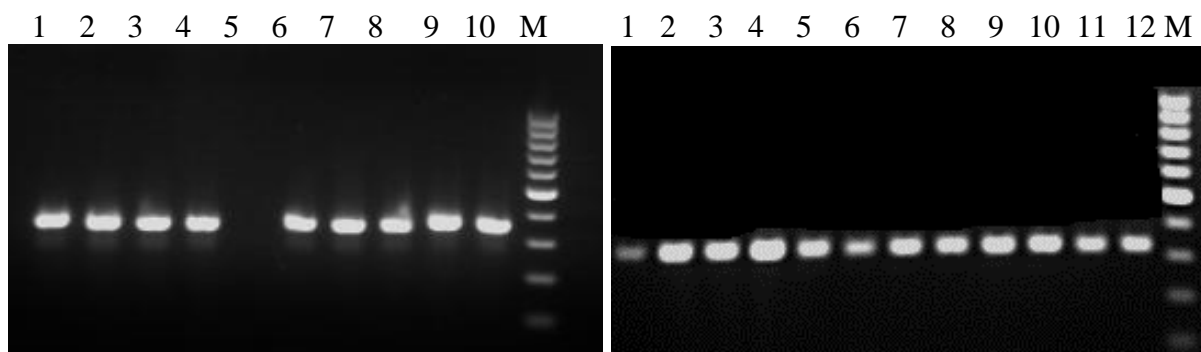
Lane M: 100 bp DNA ladder
Lane 1-4 and 6-10 in left gel is *Oesophgostomum* ITS-2, 333bp
Lane 5 – in left gel negative control
Lane 1-12 in right gel is *Oesophgostomum* ITS-2, 333bp

The present study mainly focused on prevalence of *Oesophgostomum* spp in langurs by specific PCR method, therefore no attempt was made for specific diagnosis for other strongyle species. There are also chances of mixed infections in langurs.

In the present study, we determined strongyles infection by microscopy and nodular worm infection by PCR in langurs in Kumaon region of Uttarakhand. The results revealed that first evidence of *Oesophagostomum* spp infection present in langurs of mid-Himalayas in Kumaon region by molecular method. The microscopy results revealed the prevalence of strongyles were lowest in the winter months followed by spring that might be due to hypobiosis of larvae in the host thus, less number of infective larvae in the pasture. However, significantly higher prevalence of strongyles was observed in the monsoon and post monsoon seasons. This pattern can be assigned to variation in the rainfall and temperature in the weather that favors the spurt of infective larvae in the environment (Soulsby, 1982; Taylor *et al.,* 2015). Limited number of study has been conducted in langurs pertaining to gastrointestinal strongyles. Earlier studies reported that strongyles, in which *Oesophagostomum* spp are common in Nilgiris langurs of Tamil Nadu (Rajendran *et al.,* 2004; Tiwari *et al.,* 2017*). O. bifurcum, O. stephanostomum* and *O. aculeatum* are most important species of non-primates and also human (Stewert and Gasberre, 1989; Gasser *et al.,* 2009; Ghai *et al.,* 2014). The present study a genus specific PCR was standardized to identify *Oesophagostomum* spp and compared with microscopy using same samples. The results of microscopy indicated that this test significantly under estimated the infection and in other hand PCR method proved more sensitive. As many as 29 negative cases out of 100 by microscopy were detected *Oesophagostomum* spp positive by PCR. Ghai *et al.,* (2014) also applied microscopy and PCR for diagnosis of different *Oesophagostomum* spp of primates including human and found that PCR is more sensitive than microscopy. Previous studies reported *Oesophagostomum* spp infection in primates from Uganda (Gillespie *et al.,* 2004, 2005) and its potential zoonotic importance (Ghai *et al.,* 2014; Cibot *et al.,* 2015). The study concluded that *Oesophagostomum* spp are common but highly neglected and under estimated infection in non-human primates of India. The langurs of mid Himalays move freely in human dwelling and also share same habitats with human pose severe zoonotic risk. The present study findings enlighten further to determine this infection in other primates of the country in details, due to increased proximity between non-human primates and humans. The further study is warranted on rDNA gene sequencing and phylogentic analysis of *Oesophagostomum* spp from non-human primates and human to ascertain zoonotic importance.

Acknowledgement

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