Journal of Global Biosciences
Peer Reviewed, Refereed, Open-Access Journal
ISSN 2320-1355
Volume 10, Number 12, 2021, pp. 9143-9148
Website: www.mutagens.co.in
URL: www.mutagens.co.in/jgb/vol.10/12/101202.pdf

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Research Paper

MOLECULAR DETECTION OF APICOMPLEXAN PARASITES IN ROAD-KILL VERTEBRATES OF AMRAVATI REGION

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Abstract

Current study was targeted to detect the Apicomplexan parasites infection in different vertebrate species of birds, reptiles, and mammals found in the Amravati region. Total 45 road-kill samples were collected from 18 different species from the region of Amravati city to investigate the parasitic infection. DNA, extracted from the samples was amplified using Apicomplexan and Hepatozoon specific primers. Out of 45 samples, 6 samples were found to be positive thereby confirming the prevalence of Apicomplexan parasites in the amplified samples. The result of this study has implication upon use of molecular diagnostics to investigate the occurrence of intracellular parasitic infections in economically important species as well in conservation biology.

Key words: Apicomplexa, Hepatozoon, PCR and 18s rRNA.

INTRODUCTION

The Hepatozoon are the members of large genus of Apicomplexan alveolates. There are about 300 known species of obligate intracellular parasites in this living world. Out of 300 species 46 infects animals, 120 infect reptiles, and rest infects birds and amphibians (Metzer et al., 2008). These parasitic protists are well known to cause hemolytic anemia and multiple organ dysfunctions in vertebrates. Most of these protists live in association with domestic animals like domestic cats, dogs, which are the popular pets in human culture since long time (Devada et al., 1996); (Smitha et al., 2003); (Priya et al., 2004). In India, out of the other infected species, *Babesia gibsoni* was the most common canine haemoparasite in Kerala since a few years (Augustine et al., 2017); (Jain et al., 2017). These parasites are able to cause diseases in vertebrates, which can be

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transferable to human beings. Well known pandemics like swine flu, Bird flu and COVID-19 are also zoonotic infections. To prevent such outbreaks, studies targeting the identification of parasitic protist in vertebrate species are important. Data generated during such studies provide foundation to develop vaccines and potential medicines against such diseases. This study comes under the field of molecular parasitology where molecular biology techniques like polymerase chain reaction are to diagnose the infection of parasite in the host species. We used polymerase chain reaction techniques as detection methods to detect the intracellular apicomplexan parasites. Polymerase chain reaction detects the presence of parasites and provides the concrete results by amplifying parasitic DNA. The conserved nature of gene provide excellent platform in the form of flanking ends which are used to design the primers for the PCR. In this study we used 18s rRNA specific detections primers, which are widely used marker to identify the infection of Hepatozoon (Kledmanee et al., 2009); (Kamani et al., 2013); (Rani et al., 2011a & 2011b); (Murugesan et al., 2017); (Singla et al., 2017); (Daskalaki et al., 2018). The slow evolving nature of 18s rRNA makes this loci as a marker of choice to identify and resolve deep phylogenies of parasitic protozoan. Nucleotide sequence of 18s rRNA is used as marker in identification of organism in closely associated vertebrate species to human beings from Amravati region. The findings presented in this paper can be consider as a baseline study in order to execute more advance studies using highly Sanger and NGS based techniques to provide more deeper insight.

MATERIALS AND METHODS:

We collected tissue as well as bloods clot samples of dead road kill vertebrate species. A total of 45 samples were collected from 16 different vertebrate species (Table 1). Tissue samples from the road kills were preserved in absolute alcohol prior to the DNA extraction. The blood samples were collected in 1.5ml tubes containing EDTA, while the blood clots were collected on blotting paper, preserved directly in zip-lock polyethylene bags. As all the samples used in the study originated from road-kill hence it do not come under the purview of animal ethics.

DNA Extraction and PCR amplification:

DNA was extracted using Nucleopore, Genetix Biotech's DNAsure tissue mini DNA extraction kit, following the protocols provided with the kit. The extracted DNA samples were stored at -20° C until use. The genomic DNA were subjected to selective amplification of the 18s rRNA gene fragment using highly specific primers. Detail

information of the primers used in this study is provided in the Table 2. PCR was performed in 10 μL of reaction mixture, comprising 3 μL of deionised water, 8 μL of DreamTaq Master Mix (Thermo Scientific, Waltham, USA), 1.5 μL of 2.5 mM MgCl2, 1 μL of 0.3% bovine serum albumin, 0.5 μL (5U/μL) Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, USA), 0.8μL of each primer, and 1 ul of concentrated genomic DNA. PCR cycling was performed in Veriti 96-well thermal cycler (Applied Biosystems, Waltham, USA). Total reaction mixture was amplified using optimised PCR conditions, in which the initial denaturation was carried out at 94 ∘ C for 5 min, followed 94 ∘ C of denaturation for 1 min, 50 s on 45 ∘ C for annealing of primers, followed by the extension on 72 ∘ C for 45 s and final extension at 72 ∘ C for 8 min. Total 35 cycles were ran to obtained ample amount of amplicon. Amplicons were tested using agarose gel electrophoresis in order to confirm the presence of the infection in the vertebrate species.

Separation and the visualization of amplicons:

The agarose gel electrophoresis technique was used for separation of the amplicons to identify the apicomplexan infection in the road-kill species. (Table 1). 2% agarose gel and Biotium Gel red dye was used to stain the amplicon to resolve the amplicon bands on Kodak Gel Logic 212, gel documentation system (Figure 1).

RESULTS AND DISCUSSION

Polymerase chain reaction was able to amplify only 6 samples from of 45 samples in 3 species. Thus, this study confirms the low frequency of infection in the species of Amravati region. Expected length of apicomplexan amplification was found in 2 samples of road-kill dogs, 1 each in wild and domestic cats. Similarly, amplification were detected in two avian samples. Though sequencing was not performed but occurrence of family/genus specific apicomplexan species such as *Hepatatzoon canis* and *Trypanosoma avium* species cannot be ruled out (Smitha et al., 2003); (Merino et al., 2014). Very few studies have been carried out so far from India to detect these parasites in vertebrates (Singh et al., 2012); (Bhattacharjee et al., 2013); (Senthil Kumar et al., 2009). The protocols developed in study will be applicable to identify intracellular parasitic infections in range of vertebrate species. This pilot study can be extended to more species important from economic (domestic) as well as conservation (wild) point of view.

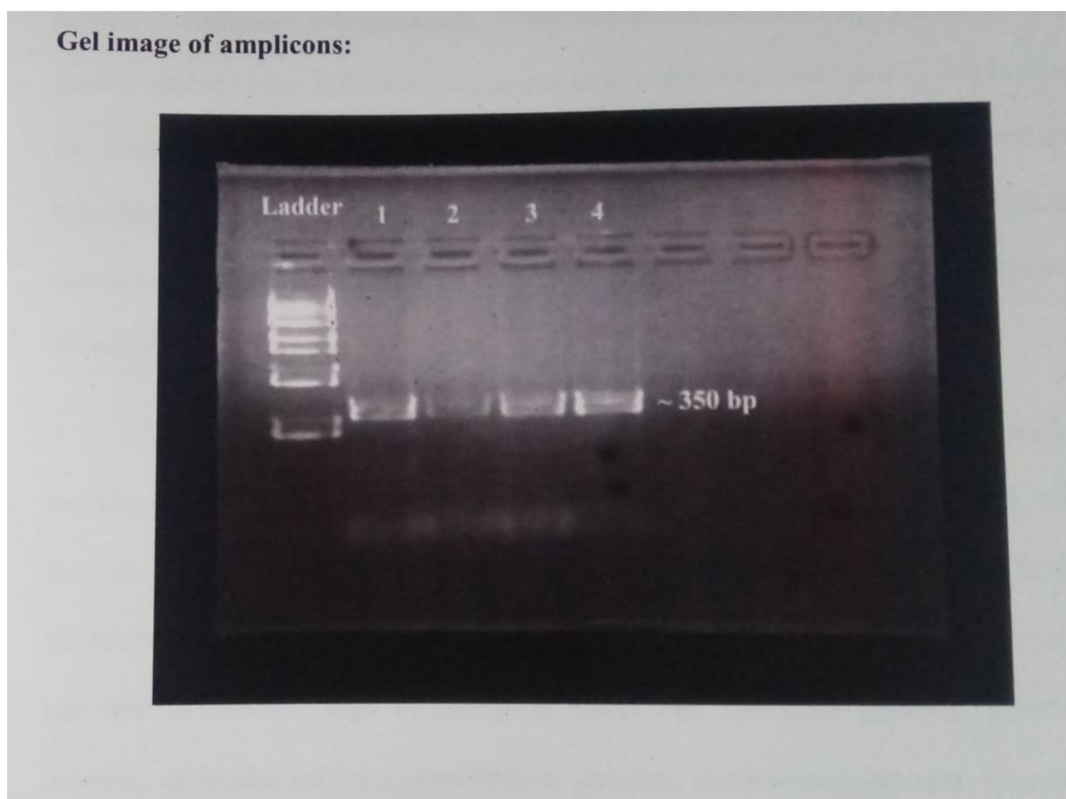

Figure 1. Amplicons of the apicomplexan 18s rRNA loci, amplified in the DNA samples of the vertebrate species. The presence of 350 kb white DNA bands indicating the infection of the apicomplexan species in corresponding DNA samples.

Table 1. Detail information of the samples used in this study:

No	Animal name	No of samples
1	Frog	3
2	Wild cat	2
3	Domestic cat	1
4	Mongoose	3
5	Squirrel	5
6	Donkey	1
7	Langur	1
8	Gecko	1
9	Eagle	1
10	Small Indian Civet	2
11	Rat	4
12	Dog	8
13	Snake	6
14	Pigeon	1
15	Garden Lizard	2
16	Crow	4
	Total	45

Table 2. Detail information of the primers used in this study to amplify the 18s rRNA gene of the apicomplexan species.

Sr. No	Primers	Primer sequence	Annealing temperature, (Tm)
1	HEP300F	GTTTCTGACCTATCAGCTTTCGACG	65.8 ∘C
	HEP300R	CAAATCTAAGAATTTCACCTCTGAC	60.9 ∘C
2	HEM01	TATTGGTTTTAAGAACTAATTTTATGATTG	59.8 ∘C
	HEM02	CTTCTCCTTCCTTTAAGTGATAAGGTTCAC	68.0 ∘C
3	Hep Car F	ACTGCAAATGGCTCATTAAAACA	57.5 ∘C
	Hep CarR	CTGGAATTACCGCGGCTG	58.4 ∘C
4	Hep RARF	GATACCGTCGTAATCTCTACCAT	61.1 ∘C
	Hep RARR	AAAGATTACCTAGACCTGRCG	57.4 ∘C
5	HepIABF	ACTGCTCTATCAGCCAGTGA	58.4 ∘C
	Hep IABR	ACGAACCCTTTAACAGCAACAG	60.3 ∘C

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