MOLECULAR DETECTION OF BACTERIA OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX (MTBC) AND *MYCOBACTERIUM AVIUM* COMPLEX (MAC) IN INDIAN GREY MONGOOSE (*Herpestes edwardsii*)

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(15-02MS-005)

DISSERTATION

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DECLARATION

I hereby declare that this dissertation titled **"Molecular detection of bacteria of *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium avium* complex (MAC) in Indian Grey Mongoose (*Herpestes edwardsii*)"** is a bonafide record of research work done by me during the course of my Master's research program and that the dissertation has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title of any other University or Society.

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1. INTRODUCTION

Pathogens that are transmitted between environment, animals (wild or domestic) and humans introduce major challenges in animal and human health (Verma *et al.*, 2013). Tuberculosis (TB) is an ancient as well as emerging disease affecting both human and animal populations. It is a cryptic disease which takes many years to manifest clinical signs in animals. Infected animal can shed bacteria for long periods. Transmission occurs through direct and indirect routes of infection (Miller *et al.*, 2015). According to World Health Organisation, in 2010, one-third of the human population was infected by TB (Verma *et al*., 2014).

Tuberculosis is caused by acid fast, gram positive bacteria known as the *Mycobacterium tuberculosis* complex (MTBC). The MTBC consists of genetically closely related pathogens that can cause TB in humans and animals. The MTBC encompasses the human modified pathogens *M. tuberculosis*, *M. africanum* and other members of TB pathogen such as *M. canettii, M. bovis*, *M. caprae*, *M. pinnipedii*, *M. microti*, *M. orygis*, *M. suricattae*, Dassie bacillus and *M. mungi* that have been reported in a wide range of mammalian species. The MTB complex can infect captive wildlife species whereas the susceptibility, pathogenicity and immune response towards mycobacterial infection vary widely between mycobacteria and host animal species. The diagnostic tools used in domestic animals exhibit moderate performance (Lecu *et al*., 2011). The most studied TB pathogen is *M. bovis* which causes bovine tuberculosis in domestic and wild animals. Very little is known about the MTBC diversity in wildlife (Coscolla *et al.*, 2013).

The *Mycobacterium avium* complex (MAC) consists of *M. avium* (with subspecies *M. avium* subspecies *avium* (MAA), *M. avium* subspecies *paratuberculosis* (MAP), *M. avium* subspecies *silvaticum* (MAS) and *M. avium* subspecies *hominisuis* (MAH) (Turenne *et al.*, 2008). It is a very slow growing, acid fast, non-pigmented bacteria with lipid-rich cell wall due to which MAC has the ability to resist the adverse environmental condition. *Mycobacterium avium*

subspecies *paratuberculosis* causes Johne's disease in most of the ruminant species. They are transmitted through faecal oral route and cause Crohn's disease in humans (Erume *et al.*, 2001).

Indian Grey Mongoose belongs to the family *Herpestidae* under the order Carnivora. Diseases like Hepatitis E (Li *et al*., 2006), leptospirosis (Alexander *et al*., 2010) and salmonellosis (Miller *et al*., 2015) have already been reported in mongoose. Mycobacterium tuberculosis complex infection in Banded Mongoose (*Mungos mungo*) in Africa has been reported (Alexander *et al*., 2002 and Bruns *et al*., 2017) whereas the transmission of disease among the species still remains a hypothesis. With the exception of a report of rabies in Kannur District of Kerala (Jayson and Govind, 2014), reports on the detection of infectious agents in mongoose in India is scarce. There are no reports on TB among mongoose in India. There have been earlier cases of tuberculosis among captive wild animals at the Zoological Garden, Thiruvananthapuram.

The present study was attempted to test for the presence of MTBC and MAC in Indian Grey Mongoose dwelling in the premises of Zoological Garden, Thiruvananthapuram.

The following was the objective of the study

1. To detect the presence of bacteria of MTBC and MAC organisms in free ranging Indian grey mongoose by polymerase chain reaction (PCR).

2. REVIEW OF LITERATURE

2.1. TUBERCULOSIS IN FREE-RANGING WILD ANIMALS

Briones *et al*. (2000) reported the infection of *Mycobacterium bovis* in free ranging Iberian Lynx (*Lynx pardina*) for the first time. The author pointed out that the carnivores may get infected through consuming the naturally MTB affected ungulates.

Alexander *et al*. (2002) studied the outbreak of *Mycobacterium tuberculosis* in the free ranging banded mongoose (*Mungos mungo*) in Africa and reported the possibility of disease transmission from humans to wildlife by their close contact.

Palmer *et al*. (2002) identified that free ranging White-tailed Deer are susceptible to *M. bovis* and that increase in population of deer increases deer to deer contact that in turn leads to transmission of tuberculosis.

Naranjo *et al*. (2008) reported that European Wild Boar is a natural reservoir of *M. bovis* and they are able to transmit diseases to other wildlife species through direct and indirect contact.

Alexander *et al*. (2010) identified *M. mungi*, a newly identified species of mycobacterium in Banded Mongoose that live in close contact with humans.

Balseiro *et al*. (2011) reported the prevalence of *M. bovis* in Eurasian badgers (*Meles meles*). The author reported the transmission of disease from Badgers to cattle and vice versa.

Chu *et al*. (2011) figured out that MTB affects free ranging wild deer as well as deer species in farms. This study was based on two native deer species, *Cervus unicolor swinhoei* (Formosan Sambar, Sambar) and *C. nippon taiouanus* (Formasan Sika, Sika) in central Taiwan in which *M. bovis* and *M. tuberculosis* were detected.

Coscolla *et al*. (2013) reported *M. tuberculosis* strain isolated from wild Chimpanzee, which is very closely related to human associated lineage 6 than to the other classical animal associated MTBC strains.

Parsons *et al.* (2013) reported the presence of tuberculosis in free ranging Meerkats *(Suricata suricatta)* in South Africa. They used IS*6110* based PCR for the detection.

Barasona *et al*. (2015) documented the natural shedding of *Mycobacterium tuberculosis* complex in free ranging Eurasian Wild Boar (*Sus scrofa*) and its role as TB maintenance host in the environment by different routes.

A broad idea about tuberculosis in South African wildlife population was given by Miller *et al*. (2015). *M. bovis* has a wide host range which infects many endangered species that lead to their extinction in the wild. Increased habitat-loss causes spill over of disease in wildlife-livestock-human interfaces.

Bruns *et al*. (2017) reported the presence of *Mycobacterium bovis* in free ranging Banded Mongoose and the route of *M. bovis* transmission from other infected badgers and baboons.

Thapa *et al*. (2017) discussed the emergence of *M. orygis* in endangered species like rhinoceros, Bengal Tigers and elephants in South Asia. The authors also gave information about different MTB complex affected in wildlife population.

Zachariah *et al*. (2017) confirmed the presence of *M. tuberculosis* in three wild Asian Elephants by means of PCR. The authors also pointed out that the spill over of the disease is from humans to elephants.

2.2. TUBERCULOSIS IN CAPTIVE WILD ANIMAL SPECIES

Lantos *et al*. (2003) diagnosed *Mycobacterium bovis* subspecies *caprae* in captive Siberian Tiger.

Zlot *et al*. (2016) detected *Mycobacterium tuberculosis* in captive Asian Elephants in North America. Culturing trunk secretion from elephants confirmed the infection.

Hedau *et al*. (2016) found TB infected carcass of a captive Sloth Bear from Maharaj Bag Zoo. Based on history, clinical signs, gross findings, impression smears

and histopathological findings, they confirmed that the infected animal died due to tuberculosis.

2.3. TUBERCULOSIS IN LIVESTOCK

Smith *et al*. (2005) reported that the high declining rate of cattle herds in UK was due to bovine tuberculosis acquired from badgers.

Boland *et al*. (2009) studied the reduced milk production in bovine tuberculosis infected cattle herds in Ireland. The authors also pointed out that milk production was very low in TB reactor cows than in non-reactors.

Monki *et al*. (2014) studied the occurrence of intestinal tuberculosis in horses with chronic diarrhoea and weight loss.

2.4. EVIDENCES OF ZOONOTIC IMPORTANCE OF TUBERCULOSIS

Cosivi *et al*. (1998) studied the significance of infection by *Mycobacterium bovis* in developing countries. The authors also reported that bovine TB and human immunodeficiency virus infections are common in developing countries. Very close physical contact between humans and infected cattle and consumption of milk contaminated by *M. bovis* has long been considered as the main routes of TB transmission from animals to humans.

Srivastava *et al*. (2008) studied the presence of *Mycobacterium tuberculosis* in the cattle farms of North India. The authors established that the source of infection may also be from humans.

Anaelom *et al*. (2010) discussed the anthropogenic significance of bovine tuberculosis in developing countries. The authors also reported that the use of cattle products such as milk and meat from affected animals leads to tuberculosis.

Bilal *et al*. (2010) reported the infection of bovine tuberculosis in humans from cattle by means of aerosol route and also from unpasteurised milk of bovine TB affected animals.

Murphree *et al*. (2011) reported that *M. tuberculosis* was transmitted from captive elephants to humans by means of respiratory route.

Singh *et al*. (2011) reported that *M. avium* subspecies *paratuberculosis* (MAP) was able to infect humans through faecal-oral route from Johne's disease infected animals.

Muller *et al*. (2013) studied the occurrence of *Mycobacterium bovis* and *Mycobacterium caprae* among human populations. The authors also observed that the pathogen transmission was due to close contact with infected cattle and by the consumption of unpasteurised milk and meat.

Singh *et al*. (2016) documented the present status of MAP in domestic animals and humans in India. The authors also discussed its importance in human health.

2.5. MOLECULAR DETECTION OF MYCOBACTERIUM TUBERCULOSIS COMPLEX BACTERIA USING PCR

Boddinghaus *et al*. (1990) developed 16SrRNA based PCR for the rapid detection of mycobacteria.

Eisenach *et al*. (1990) reported the fast diagnosis of *Mycobacterium tuberculosis* from sputum using IS*6110* based PCR.

Shawar *et al*. (1993) studied the isolation of *Mycobacterium tuberculosis* based on IS*6110* PCR results from clinical samples.

Mulcahy *et al*. (1996) observed the detection of *Mycobacterium tuberculosis* based on IS*6110* PCR.

Coros *et al.* (2008) reported that MTBC comprises of the species *M. tuberculosis, M. africanum, M. bovis, M. bovis* Bacillus Calmette-Guerin*, M. cannetti, M. caprae, M. microti,* and *M. pinnipedi.* IS*6110* is found exclusively within the MTBC.

2.6. MOLECULAR DETECTION OF *MYCOBACTERIUM AVIUM* COMPLEX BACTERIA USING PCR

Vary *et al*. (1990) used IS*900* based PCR for detection of *M. paratuberculosis* in cattle faeces.

Macgregor *et al*. (1999) developed PCR method to detect *Mycobacterium avium - M. intracellulare* complex using 16S rRNA genes.

Aravindhan *et al*. (2007) studied the diagnosis of MAC using PCR based techniques.

Shin *et al.* (2010) developed a PCR based on IS*1311* to detect all members of the MAC. *Mycobacterium avium* subsp. *avium, M. avium* subsp. *hominissuis, M. avium* subsp. *paratuberculosis, M. avium* subsp. *silvaticum* and *M. intracellulare* are the species included under the MAC.

Singh *et al*. (2011) detected *Mycobacterium avium* subspecies *paratuberculosis* DNA using IS*900* and IS*1311* PCR.

Monki *et al*. (2014) identified the existence of *M. avium* subspecies in horses by means of multiplex PCR assays and restriction fragment length polymorphism (RFLP) methods.

2.7. MOLECULAR DIAGNOSIS OF MTBC AND MAC FROM FAECAL SAMPLES

Erume *et al*. (2001) extracted DNA from faecal samples for the detection of *Mycobacterium avium subsp. paratuberculosis* using IS*900* - based PCR.

Balamurugan *et al*. (2006) evaluated the diagnosis of intestinal tuberculosis in humans by testing patient's faces by PCR targeting IS*6110*.

Wolf *et al*. (2008) studied the diagnosis of pulmonary tuberculosis using stool samples employing IS*6110* based PCR.

Gopinath *et al*. (2009) developed multiplex PCR assay for the detection of *Mycobacterium tuberculosis* and *M. avium* complex from different clinical samples from humans.

Ramdass *et al*. (2010) detected *M. tuberculosis* in human faecal sample by PCR targeting IS*6110*.

Welday *et al*. (2014) suggested that DNA for checking pulmonary tuberculosis can be extracted from stool samples using QIAGEN stool minikit.

Salgado *et al*. (2015) reported the presence of *Mycobacterium avium* subsp. *paratuberculosis* in free ranging Pudu (*Pudu puda*). They detected MAP from their faeces based on IS*900* PCR.

Wolf *et al.* (2015) used IS*6110* based PCR for non-invasive *Mycobacterium tuberculosis* detection in faecal samples from primates.

Singh *et al*. (2016) diagnosed MAP from the faeces of cattle and the isolation was based on IS*900* element.

Stewart *et al*. (2017) developed a non-invasive method by using immunochromatographic lateral flow device (LFD) to detect *M. bovis* from faeces.

2.8. BEHAVIOURAL AND ECOLOGICAL ASPECTS ON MONGOOSE

Choudhury *et al*. (2016) reported the status of Indian Grey Mongoose, its ecology and conservation threats.

Kalle *et al*. (2012) documented the feeding habit of mongoose in Mudumalai Tiger Reserve by examining their faeces. They feed on reptiles, birds, shrew, rodents and even fruits and vegetables.

Rajashekara *et al*. (2015) recorded the population status of Indian Grey Mongoose (*Herpestes edwardsii*) and the habitat disturbance in Bengaluru region.

Shekhar (2008) studied the behavioural activities of two Ruddy Mongooses (*Herpestes smithii)* in Panna National Park, Madhya Pradesh. The author also explained the swimming capability of mongoose and that they feed on river invertebrates like crabs and snails.

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Glassware and Labware

The glassware used in this study for various purposes were obtained from Borosil (India) and labware from Tarsons (India). Filter tips were used for all pipetting applications.

3.1.2 Buffers and Reagents

i) Tris borate EDTA (TBE) buffer (5X) (for agarose gel electrophoresis)

Tris base : 54 g

Boric acid : 27.5 g

0.5 M EDTA (pH 8.0) : 20 ml

The pH of the stock solution was adjusted to approximately 8.3 and filtered through a 0.45 µm membrane filter. The working solution (0.5X) was made by diluting the stock solution as and when required.

ii) Ethidium bromide (1 per cent)

Ethidium bromide (Sigma) : 10 mg

Nuclease free water : 1 ml

The solution was mixed and stored at 4ºC. A concentration of 0.5 µg/mL was used in preparing agarose gel.

3.1.3 Molecular kits

Kit used for DNA extraction was QIAamp DNA Stool Mini Kit from Qiagen (Germany).

3.1.4 Equipment used

Mastercycler Nexus gradient thermal cycler (Eppendorf, US), biological safety cabinet Class II B2 (Esco, Singapore), gel documentation system (Syngene, U.K.), electronic weighing balance (Sartorious, USA), spinwin microfuge (Bangalore Genei,

India), vortex mixer (REMI equipments, India), laminar air flow system (Labline, India), water bath (Labline, India), submarine horizontal electrophoresis system (Biotech, India) and micropipettes (Finpippette, Finland and Eppendorf, USA).

3.1.5 Primers

Specific primers of PCR amplification of genes of mycobacteria were synthesised by IDT (Integrated DNA Technologies, USA)

Table 1. Details of PCR primers used for detection of bacteria of mycobacterium, MTBC and MAC

SET	OLIGO NAME	SEQUENCE 5'- 3'	PRODUCT SIZE (bp)	REFERENCE
1	Myco16S rRNA F	ATAAGCCTGGGAAACTGGGT	484	Shin *et al.* (2010)
	Myco16S rRNA R	CACGCTCACAGTTAAGCCGT		
2	MTB1 F	CCTGCGAGCGTAGGCGTCGG	123	Eisenach *et al.* (1990)
	MTB2 R	CTCGTCCAGCGCCGCTTCGG		
3	MAC1 F	GCGTGAGGCTCTGTGGTGAA	608	Shin *et al.* (2010)
	MAC2 R	ATGACGACCGCTTGGGAGAC		

3.1.6 Samples

Faecal samples for the study were collected from free ranging Indian grey mongoose (*Herpestes edwardsii*) in and around the Zoological Garden, Thiruvananthapuram. Positive samples for *M. avium* subspecies *paratuberculosis* and *M. phlei* were obtained from Tamil Nadu University of Veterinary and Animal Sciences, Chennai.

3.2 METHODS

3.2.1 Study area

The study was conducted at the Zoological Garden, Thiruvananthapuram. The zoo is located in the heart of the Thiruvananthapuram City, which is located at 8.5˚N 76.9˚E the west coast near the Southern tip of mainland India. Surrounded by many residential areas, administrative buildings and zoo had total of 106 captive wild animals consisting of herbivores, carnivores, primates, reptiles and avian species. For this study faecal samples were opportunistically collected from the free ranging Indian Grey Mongoose (*Herpestes edwardsii*) present in the Zoological Garden, Thiruvananthapuram.

3.2.2 Sample collection

A total of 21 faecal samples were collected in 2 ml collection tubes (Tarsons) and kept at 4°C till analysis.

3.3 EXTRACTION OF DNA

Extraction of DNA from faecal sample was attempted by three methods: using phenol – chloroform method, SDS - Proteinase K - phenol chloroform method and using commercial kit (QIAamp DNA Stool Mini Kit). For standardization, the bacteria (*M. phlei*) were added to faecal sample and a 10 per cent (weight/volume) suspension was prepared and then subjected to DNA extraction by the three methods.

3.3.1 DNA extraction by phenol chloroform method

Total DNA was extracted by phenol chloroform method as described by Sambrook and Russell (2001) with slight modifications. Briefly, 200 µl of faecal sample suspension was taken and mixed 200 µl of tris saturated phenol (TSP) (pH 7.8) and centrifuged at 12,000 *g* for 3 min. The aqueous phase was carefully pipetted and transferred to another tube and 100 µl of TSP and 100 µl of chloroform were added, mixed and centrifuged as above. The upper phase was pipetted out into a fresh tube and 200 µl of chloroform was added, mixed well and centrifuged. The upper phase was again transferred to a fresh tube and 20 µl of 3M sodium acetate and 1 ml of absolute ethanol were added and mixed gently and kept at -20°C overnight for DNA precipitation. After the overnight precipitation step, the tube was brought to room temperature and centrifuged at 12,000 *g* for 15 min to pellet the DNA. The supernatant was decanted and the pellet was washed in 200 µl of 70 percent ethanol at 12,000 *g* for 3 min. The alcohol was decanted and the DNA dried in a dry bath at 90°C. The dried DNA was reconstituted in 30 µl of nuclease free water (NFW) (Life technologies, USA) and stored at -20°C.

3.3.2 DNA extraction by SDS - Proteinase K - phenol chloroform method

In this modified phenol chloroform method, 200 µl of fecal sample suspension was taken and mixed with 20 µl of 10 percent SDS and 10 µl of proteinase K (20 mg/ml) (Qiagen, Germany) in a microfuge tube and incubated at 60°C for 1 h. After incubation, 200 µl of tris saturated phenol (TSP) (pH 7.8) was added, mixed and centrifuged at 12,000 *g* for 3 min. Rest of the steps were as described in step 3.3.1.

3.3.3 Isolation of DNA from stool for pathogen detection QIAamp DNA Stool Mini Kit (Qiagen, Germany)

Fecal sample of 180–220 mg was weighed and transferred into a 2 ml microcentrifuge tube and the tube was placed on ice. Then 1.4 ml Buffer ASL was added to each sample. The tubes were continuously vortexed for one min or until the fecal sample was thoroughly homogenized. Further the suspension was heated for five minute

at 70°C and vortexed for 15 sec. The sample was centrifuged at 12,000 *g* for one min to pellet fecal particles. From the centrifuged sample 1.2 ml of the supernatant was pipetted into a clean 2 ml microcentrifuge tube and discarded the pellet. To each sample one InhibitEX tablet was added and vortexed continuously for one minute or until the tablet was completely suspended. Suspension was incubated for one minute at room temperature, to allow inhibitors to adsorb to the InhibitEX matrix. Firstly, the samples were centrifuged at 12,000 *g* for three minute to pellet inhibitors bound to InhibitEX matrix. The supernatant was pipetted into a new 1.5 ml microcentrifuge tube and the pellet was discarded and again centrifuged the sample at 12,000 *g* for three min. 15 µl of proteinase K was added into a new 1.5 ml microcentrifuge tube. 200 µl of supernatant was pipetted from the above step into the 1.5 ml microcentrifuge tube containing proteinase K. Buffer AL of 200 µl was added into the microcentrifuge tube containing the supernatant and protinaseK and vortexed for 15 sec. Then incubated at 70°C for 10 min. Then 200 µl of ethanol was added (96–100 percent) to the lysate, and mixed by vortexing. The lid of a new QIAamp spin column was labelled and placed in a 2 ml collection tube. The complete lysate was carefully applied from the above step to the QIAamp spin column without moistening the rim. Then the cap was closed and centrifuged at 12,000 *g* for one minute. The QIAamp spin column was placed in a new 2 ml collection tube, and discarded the tube containing the filtrate. The QIAamp spin column was carefully opened and 500 µl of Buffer AW1 was added. Cap was closed and centrifuged at full speed for 1 min. The QIAamp spin column was placed in a new 2 ml collection tube, and discarded the collection tube containing the filtrate. Carefully opened the QIAamp spin column and 500 µl of Buffer AW2 was added. The cap was closed and centrifuged at full speed for 3 min.The collection tube containing the filtrate was discarded. The QIAamp spin column was placed in a new 2 ml collection tube and discarded the old collection tube with the filtrate and centrifuged at 12,000 *g* for 1 min. The QIAamp spin column was transferred into a new, labelled 1.5 ml microcentrifuge tube. Carefully open the QIAamp spin column and 200 µl Buffer AE was pipetted directly into the QIAamp membrane. At last, the cap was closed and incubated for 1 min at room temperature, then centrifuged at full speed for 1 min to elute DNA. Pipetted out the whole elute in a new 1.5ml of tubes and stored at -20˚C until further analysis.

3.4 PCR amplification

3.4.1 Detection of mycobacteria by 16S rRNA PCR

The extracted DNA from all the 21 samples collected during the course of study were tested for the presence of mycobacteria by the conventional PCR as described by Shin *et al.* (2010). Samples were subjected to PCR to detect 16S rRNA gene of mycobacteria. The primers used in this study are presented in Table 1 (Set 1).

For amplification, the 20 µl of PCR comprised of 10 µl of 2X EmeraldAmp GT PCR Mastermix containing *Taq* polymerase (TaKaRa), 2 µL of each 10 pmol of forward and reverse primers, 3µl of template DNA and rest NFW to make up the volume. Along with this, a positive control (*M. phlei*) and negative control was included. The PCR cycling parameters were as follows: initial denaturation at 95˚C for 5 min, followed by 35 cycles of denaturation at 95˚C for 60 sec, annealing at 60˚C for 40s, and elongation at 72˚C for 35 sec and a final extension at 72˚C for 10 min.

3.4.2 Detection of MTBC by PCR

The presence of genome of bacteria belonging to the *M. tuberculosis* complex in the samples was tested by employing PCR to detect IS*6110* in these bacteria as described by Eisenach *et al*. (1990). The details of primers are given in Table 1 (Set 2). The 20µl of reaction mix consisted of 10 µl of 2X EmeraldAmp GT PCR Mastermix containing *Taq* polymerase (TaKaRa), 2 µL of each 10 pmol of forward and reverse primers, 3µl of template DNA and rest NFW. The cycling conditions for amplification were 95°C for 30 sec (initial denaturation), 35 cycles of 94°C for 30 sec (denaturation), 68°C for 30 sec (annealing) and 72°C for 30 sec (polymerization) there after single cycle at 72°C for 10 min (final extension).

3.4.3 Detection of MAC by PCR

The presence of genome of bacteria belonging to *Mycobacterium avium* complex in the samples was tested by employing PCR to detect IS*1311* in these bacteria as reported by Shin *et al*. (2010). The details of the outer and inner set primers

are given in the Table 1 (Set 3). The 20 µL reaction mixture comprised of 10 µL of 2X EmeraldAmp GT PCR Mastermix containing *Taq* polymerase (TaKaRa), 2 µL each of 10 pmol forward and reverse primers, 3 µL of template DNA and rest NFW to make up the volume. Along with this, a positive control (*M. paratuberculosis*) and negative control were included. The PCR cycling parameters were as follows: initial denaturation at 95˚C for 5 min, followed by 35 cycles of denaturation at 95˚C for 60 sec, annealing at 60˚C for 40s, and elongation at 72˚C for 35 sec and a final extension at 72˚C for 10 min.

3.5 Gel electrophoresis of PCR product

Agarose gel electrophoresis was performed for separating the PCR product prior to visualization under UV light. The agarose gel of 1.5 per cent concentration was prepared in 0.5X TBE buffer. Agarose was weighed and dissolved in 0.5X TBE buffer by heating and was cooled to 50°C. Ethidium bromide was added to a final concentration of 0.5 µg/ml to the melted agarose. The gel was poured into the gel tray after sealing ends with tape and placing the comb. After the gel had set, the comb was removed and gel was placed in the electrophoresis apparatus. The 20 µL of the PCR product was loaded into the wells. Six microliters of 100 bp DNA ladder (Bangalore Genei, India) was loaded in one of the wells as the molecular weight marker. Electrophoresis was carried out at 100V until bromothymol blue dye had migrated to more than two-thirds of the gel. The gel was visualized to identify the size of the product and photograph was taken in a gel documentation system under UV illumination.

4. RESULTS

4.1 Sample collection

In this study, 21 faecal samples were collected from the free ranging Indian Grey Mongoose (*Herpestes edwardsii*) inhabiting the Zoological Garden, Thiruvananthapuram. Faecal samples were opportunistically collected from the zoo premises.

In this study, three methods were evaluated for extraction of DNA from a mixture of stool and *M. phlei,* namely phenol - chloroform method, SDS – Proteinase K - phenol - chloroform method and kit method. The DNA extracted by these methods was used to perform the PCR for mycobacteria using the 16S rRNA primers as described in 3.4.1. The DNA isolated by the SDS - Proteinase K - phenol - chloroform method and kit method yielded specific positive bands of almost the same intensity whereas the DNA extracted by phenol - chloroform method gave a band of slightly lesser intensity (Plate1). The kit method was used for DNA extraction from the samples collected during the study.

4.2 Detection of mycobacteria by PCR targeting 16S rRNA gene

Of the 21 samples tested, 16 were found positive in the PCR for 16SrRNA gene of mycobacteria. Amplicon of 484 bp generated by the PCR reaction were obtained on agarose gel electrophoresis. The positive control used was *M. phlei* which also gave a single band in the PCR (Plates 2).

4.3 **Detection of MTBC by PCR**

The clinical samples were subjected to conventional PCR for MTBC targeting IS*6110*. Of the 21 samples was tested no samples were found to be positive for MTBC (Plate 3).

4.4 **Detection of MAC using PCR**

Of the 21 samples, none were found to be positive to MAC targeting IS*1311*. The positive control used was *M. paratuberculosis* that gave a single specific band of 608 bp (Plate 4).

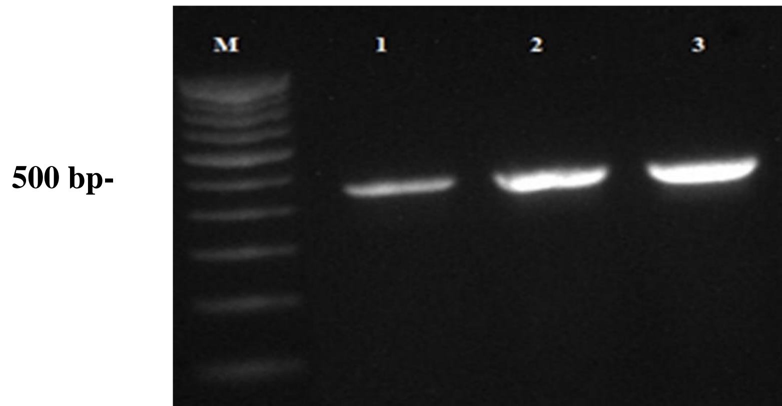

Plate 1: Agarose gel showing 484 bp amplicons generated by targeting 16S rRNA gene of mycobacteria

Lane M: 100 bp DNA Ladder

Lane 1: PCR amplicon obtained using DNA extracted by phenol - chloroform method

Lane 2: PCR amplicon obtained using DNA extracted by SDS - Proteinase K - phenol - chloroform method

Lane 3: PCR amplicon obtained using DNA extracted by kit method

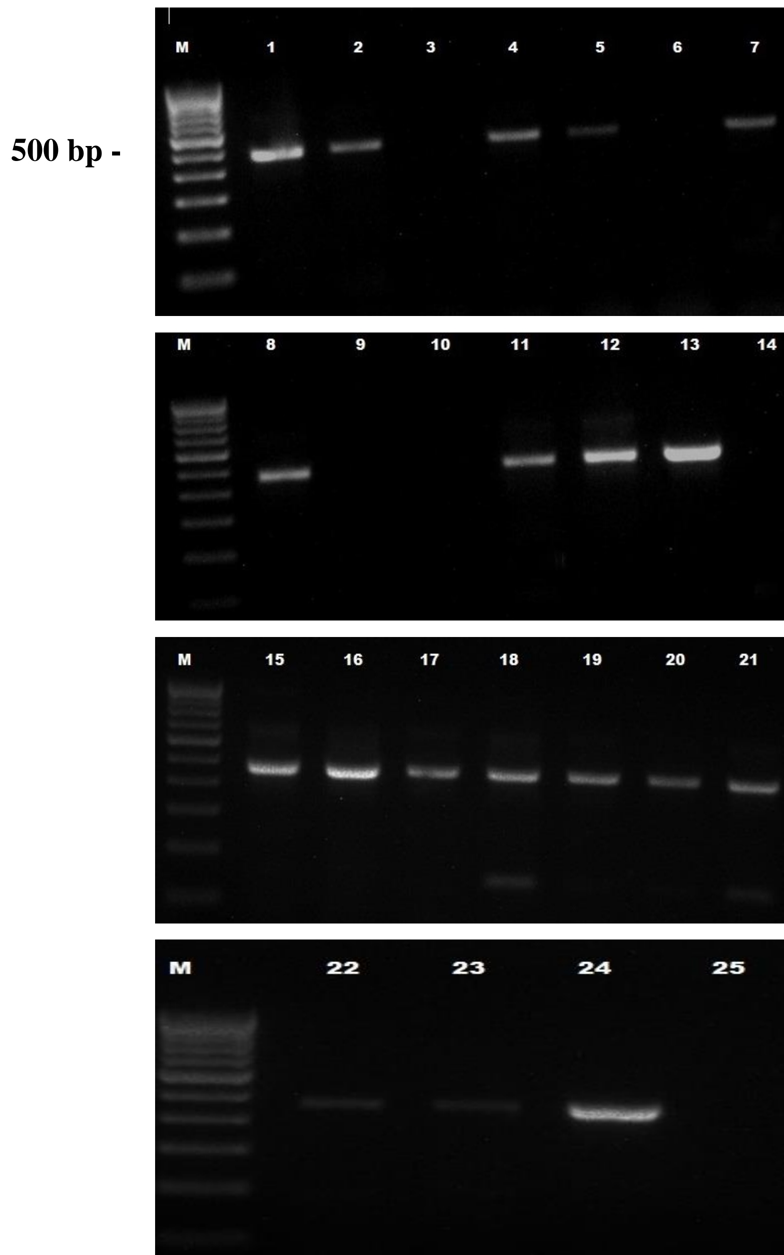

Plate 2: Agarose gel showing 484 bp amplicons generated by targeting 16S rRNA gene of mycobacteria

Lane M	: 100 bp DNA Ladder
Lanes 1 to 12 & 15 to 23	: Samples
Lane 13 & 24	: Positive control (*M. phlei*)
Lane 14 & 25	: No template control

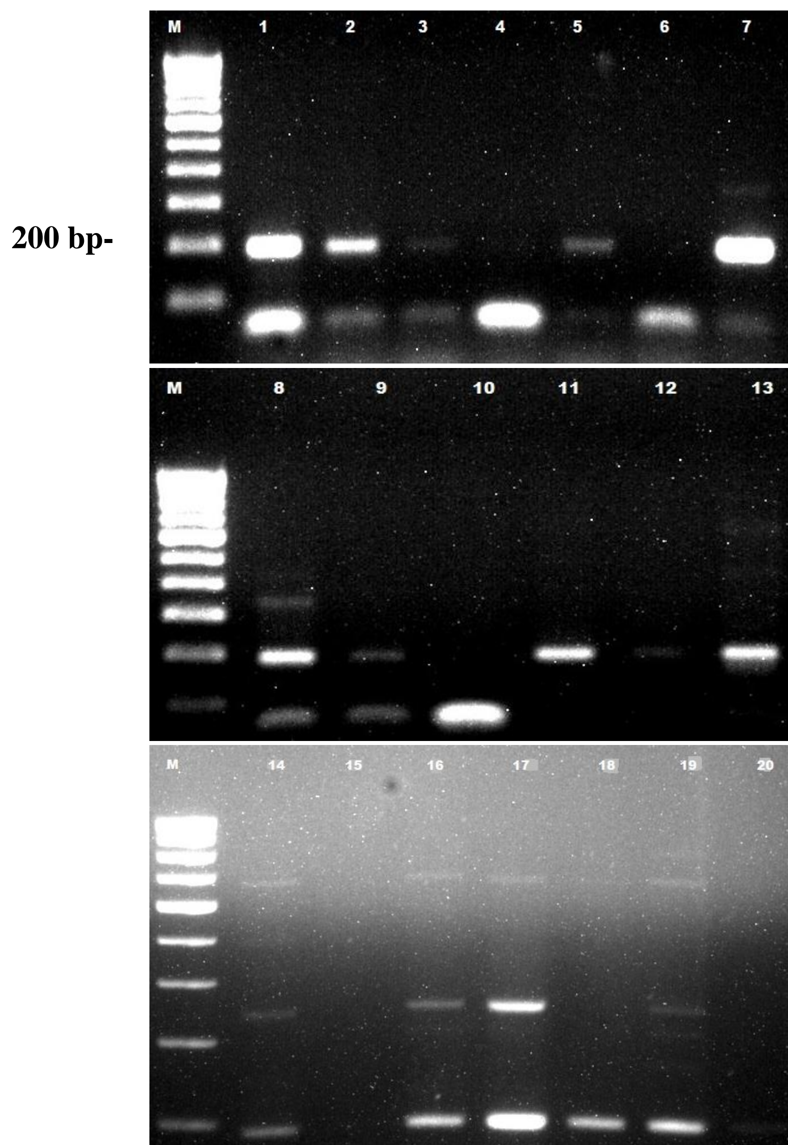

Plate 3: Agarose gel showing non-specific amplicons generated in PCR targeting IS6110 gene of MTBC

Lane M	: 100 bp DNA Ladder
Lanes 1 to 19	: Samples
Lane 20	: No template control

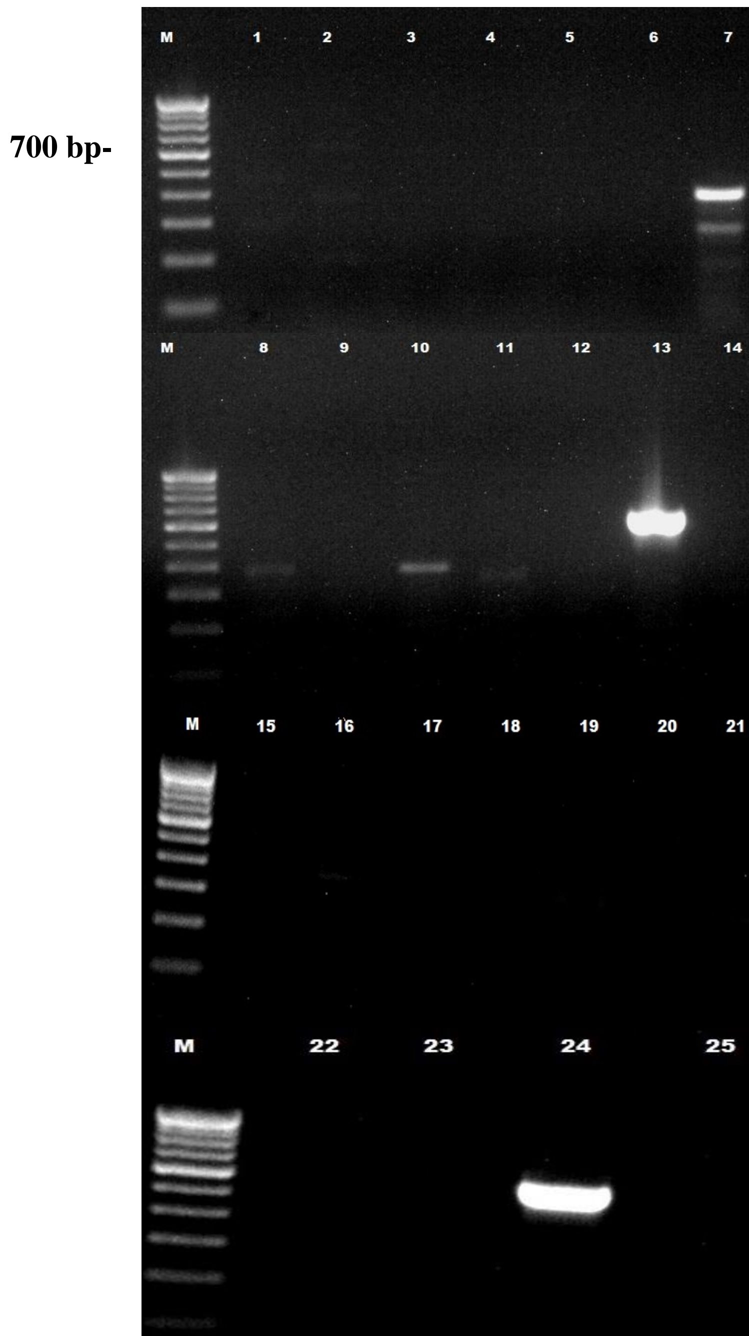

Plate 4: Agarose gel showing non-specific amplicons generated in samples tested by PCR targeting IS1311 gene of MAC

Lane M : 100 bp DNA Ladder
Lanes 1 to 12 & 15 to 23 : Samples
Lane 13 & 24 : Positive control (*M. paratuberculosis*)
Lane 14 & 25 : No template control

5. DISCUSSION

The Indian Grey Mongoose (*Herpestes edwardsii*) comes under the order Carnivora, and is mainly native to Southern Asia. It has been recorded in varying types of habitats from thorn forests, dry forests, cultivated lands, urban areas up to Himalayas. It comprises more than 30 species. They live inside hollow tree trunks, or by digging hole in the ground. Mongooses are good climbers and swimmers (Shekhar *et al.*, 2008). They live in small packs of related individuals and are extremely social and diurnal animal. This omnivorous scavenger preys on rodents, lizards, birds' eggs and a variety of invertebrates (Choudhury *et al.*, 2016). They even opportunistically feed on fruits (Kalle *et al.*, 2012). The life span of this species is up to 15 to 20 years in the wild.

As per the IUCN status the current population of Indian Grey Mongoose is stable (Least Concern). According to Indian Wildlife Protection Act (1972), this species is listed in Schedule II part IV. Their population is not quantitatively monitored in any country because they are mainly found in human settlement areas depending on the availability of food and shelter. Peoples from Northern part of India used to capture this species for skin for making painting brush and their meat is consumed by several tribes.

In some islands like Hawaii, Caribbean, Japan and West Indies mongooses are exotic species. They were introduced from India in 1910 for controlling venomous snakes and rats to protect agricultural land as a form of pest control. Unfortunately, the number of mongooses and their living area increased due to the absence of natural predators in these islands. Mongooses feed on other terrestrial animals and birds living on the islands. Their high population density and affinity towards human dwelling areas make this species a public threat. Introduced species are responsible for biodiversity loss and can play a major role as the reservoir of many zoonotic pathogens in many countries. For example the Small Indian Mongoose *(Herpestes auropunctatus)* serves as a reservoir of Salmonella species in West Indies (Miller *et al.* 2015). Also

Hepatitis E virus has been detected in Small Asian Mongoose *(Herpestes javanicus)* in Japan (Li *et al.* 2006).

Reports on tuberculosis in mongoose (Herpestidae family) are not common. However, three studies (Alexander *et al.*, 2002, Alexander *et al.*, 2010, Bruns *et al.*, 2017) have described the prevalence of tuberculosis in mongoose. There is also a report of TB infection with M*ycobacterium suricattae* in Meerkats (*Suricata suricatta*), also a member of the Herpestidae family. The occurrence of TB in meerkats was reported to be due to anthropogenic exposure (Parsons *et al.*, 2013).

It is difficult to detect TB in animals based on clinical signs as specific signs are not exhibited by the infected animals. Hence, molecular level diagnosis is very essential for confirmation. Usually TB is detected in mongoose at the time of post mortem examination which is further confirmed by molecular methods.

In this study, a sensitive molecular diagnostic test, PCR, was performed on DNA extracted from faecal samples of mongoose. Faecal sample DNA was extracted using Qiagen Stool Minikit. The study was conducted in three steps, where in the extracted DNA was first subjected to PCR to detect 16S rRNA of any mycobacteria. Then the DNA was tested in a PCR to detect mycobacteria of the MTBC and in the third step, it was tested for mycobacterial of MAC.

5.1 Detection of mycobacteria by 16S rRNA gene PCR

In this study, PCR targeting 16S rRNA gene of mycobacteria was conducted to detect mycobacteria in the faecal samples. Twenty one samples were tested and 16 (76.19 percent) were found positive. Amplicons of 484 bp in size, were obtained on agarose gel electrophoresis. Positive control also showed the specific band of 484 bp. Boddinghaus *et al.* (1990) developed 16S rRNA based PCR for the rapid detection of mycobacteria. Shin *et al.* (2010) reported that PCR based on primers targeting 16SrRNA gene were suitable for the detection of mycobacteria in faecal samples.

5.2 Detection of MTBC by IS*6110* based PCR

In this study, all the 21 samples were tested for MTBC by conventional PCR using primers targeting IS*6110* using primers published by Eisenach *et al*. (1990) Out of the tested samples, none of them were found to be positive. The use of IS*6110* based PCR as a test for detecting MTBC has been reported by many researchers. Ramdass *et al.* (2010) and Balamurugan *et al.* (2006) used primers that amplified a 123 bp fragment of IS*6110*. Many researchers reported that IS*6110* based PCR was effective in detecting MTB and other intestinal and pulmonary tuberculosis from clinical samples (Wolf *et al.,* 2015; Ramdass *et al.,* 2010; Pulimood *et al.,* 2008; Balamurugan *et al.,* 2006).

5.3 Detection of MAC by IS*1311* based PCR

All the 21 samples were also tested for MAC by conventional PCR targeting IS*1311*. None of the samples were found to be positive. Specific amplicons of 608 bp in size were obtained in the positive control.

Shin *et al.* (2010) reported that the PCR based on primer targeting IS*1311* with 608 bp sequence were suitable for the detection of *M. avium* subspecies members.

Singh *et al.* (2012) reported that PCR amplification of mycobacterial DNA was very sensitive and reliable compared to culture and smear methods. The PCR helps to detect whether the positivity is due to MTBC or other non-tuberculous infections. The efficiency of PCR amplification mainly depends on the appropriate DNA target sequence. In this study, we targeted the 16S rRNA gene for mycobacteria, IS*6110* for MTBC and IS*1311* for MAC. The PCR for detecting 16S rRNA gene has a broader spectrum of detecting all mycobacterial including non-tuberculous and non-pathogenic species, while that for IS*6110* has a specificity for MTBC and the one for IS*1311* could detect all MAC species.

In the study, 16 samples (76.19 percent) were found to harbour DNA for mycobacteria and none of the samples were positive for mycobacteria of the MTBC or MAC. This may be due to the presence of saprophytic non-pathogenic mycobacteria seen in the soil which is ingested during foraging. Soil is easily contaminated by water or by animal faeces. Mycobacterium can survive in the soil for a longer period in

association with amoeba or other protozoa. Some of the fast growing soil mycobacteria identified are *M. fortuitum, M. tokaiense*, or *M. austroafricanum* and *M. heidelbergense* (Hruska *et al.* 2012).

6. SUMMARY

Tuberculosis is a bacterial disease that is seen in humans and animals. The diseases has also been reported from a number of species of wild animals, both free ranging and in captivity. There are very limited reports of the occurrence of TB in mongoose. A study was conducted to detect the presence of MTBC and MAC organisms in free ranging Indian Grey Mongoose in and around Zoological Garden, Thiruvananthapuram. Since it is difficult to confirm TB in animals, a sensitive molecular biological test, PCR, was used in the study.

The study was conducted on DNA extracted from faecal samples of the animals. For extraction of total DNA from faecal sample, three methods were tried viz., phenol – chloroform method, SDS - Proteinase K - phenol chloroform method and using QIAamp DNA Stool Mini Kit. Since there was not much difference among the results obtained in PCR when DNA extracted by the three methods were used as template, the kit method was used for DNA extraction throughout the study.

In the study, 21 faecal samples were tested by PCR for detection of any mycobacteria coming under MTBC and those coming under MAC. Of the 21 samples 16 (76.19 percent) were positive for mycobacteria. None of the samples were positive for mycobacteria coming under MTBC or MAC. The results of the study indicate that the mongooses in the area are free from mycobacteria of the MTBC and MAC.

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MOLECULAR DETECTION OF BACTERIA OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX (MTBC) AND *MYCOBACTERIUM AVIUM* COMPLEX (MAC) IN INDIAN GREY MONGOOSE (*Herpestes edwardsii*)

BINDYA A

(15-02MS-006)

Abstract of Dissertation Submitted in Partial Fulfillment of the Requirement for the Degree of

MASTER OF SCIENCE

(Wildlife Studies)

Faculty of Veterinary and Animal Sciences

Kerala Veterinary and Animal Sciences University

2017

CENTRE FOR WILDLIFE STUDIES

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY

POOKODE, WAYANAD, KERALA, INDIA

ABSTRACT

In India, TB is a highly prevalent disease, which causes high mortality among humans. This study was undertaken to detect the presence of MTBC and MAC organisms in free ranging Indian Grey Mongoose (*Herpestes edwardsii*) by polymerase chain reaction (PCR). Twenty one faecal samples were collected from free-ranging mongoose in Zoological Garden, Thiruvananthapuram. The DNA from the faecal samples was extracted by Qiagen stool minikit. The extracted DNA was subjected to PCR to detect any mycobacterium (PCR targeting 16S rRNA gene), MTBC (PCR targeting IS*6110* and MAC (PCR targeting IS*1311*). Out of 21 samples, 16 (76.19 percent) were found positive for mycobacteria. None of the samples were positive for bacteria belonging to MTBC and MAC. Therefore it was concluded that the mongooses in the selected area are free from any pathogenic mycobacteria of MTBC and MAC.

Acad. Form No. 19

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY

Faculty of College of Veterinary and Animal Sciences

PROGRAMME OF RESEARCH WORK FOR DISSERTATION FOR MASTERS DEGREE

(Vide Rule 25(b) of Post Graduate Regulations 1998)

1. Title of Dissertation

Molecular detection of bacteria of *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium avium* complex (MAC) in Indian grey mongoose (*Herpestes edwardsii*)

2a. Title of departmental/KVASU research project of which this forms a part

NA

2b. Code No. if any, and order by which the departmental/KVASU research project is approved

NA

3a. Name of the student

Bindya A.

3b. Admission No.

13-02MS-005

4a. Name of the Major Advisor (Guide)

Dr. Chintu Ravishankar

4b. Designation

Assistant Professor

Department of Veterinary Microbiology

College of Veterinary and Animal Sciences,

Pookode

5. Objectives of the study

To detect the presence of MTBC and MAC organisms in free ranging Indian grey mongoose by Polymerase Chain Reaction (PCR).

6. Practical/Scientific utility

Indian grey mongoose or common grey mongoose (*Herpestes edwardsii*) belongs to the family Herpestidae under the order Carnivora. They have long speckled grey fur with a long tail, small head and four short clawed limbs. Mongooses have a wide natural distribution, occurring from Turkey and the Arabian Peninsula to Bangladesh, India and SriLanka. Other than their native ranges, these animals have been introduced to the West Indies, Hawaii, Jamaica, Cuba and Puerto Rico to control poisonous snakes and rats for improving agriculture. However, these animals have increased in number and cause damages to poultry and other small mammals.

Studies have shown that Banded mongooses *(Mungos mungo)* are a major zoonotic reservoir of various viral, bacterial and parasitic diseases all around the world, especially in the introduced places. Cases of diseases like human tuberculosis (Alexander, 2002), Hepatitis E (Li*et al*., 2006), leptospirosis (Alexander *et al*., 2010), salmonellosis (Miller *e tal*. 2015) and Bovine tuberculosis (Bruns *et al*., 2017) have already been reported in mongoose. With the exception of a report of rabies in Kannur district of Kerala (Jayson and Govind, 2010), reports on the detection of infectious agents in mongoose in India is scarce. Tuberculosis is a cause of significant morbidity and mortality in both domestic and wild animal's worldwide.The Indian Grey mongoose lives and survives better in close contact with humans, domestic animals as well as captive wild animals in zoos. The proposed study will help to understand whether free ranging Indian grey mongooses harbour *Mycobacterium* spp. belonging to the MTBC and MAC.

7. Important publications on which the study is based

Eisenach *et al.* (1990) described a PCR based on insertion sequence 6110 (IS*6110*) to detect *Mycobacterium tuberculosis*.

Alexander *et al.* (2002) reported an outbreak of *Mycobacterium tuberculosis* in free ranging banded mongoose and suricates in Botswana. The author pointed out that this pathogen was transmitted from humans to the wild animals through their excretions in the environment.

Coros *et al.* (2008) the MTBC comprises of the species *M. tuberculosis, M. africanum, M. bovis,* Bacillus Calmette-Guerin, *M. cannetti, M. caprae, M. microti,* and *M. pinnipedi.* IS*6110* is found exclusively within the MTBC.

Shin *et al.* (2010) developed a PCR based on IS*1311* to detect all members of the MAC *M. avium* subsp. *avium, M. avium* subsp. *hominissuis, M. avium* subsp. *paratuberculosis, M. avium* subsp. *silvaticum* and *M. intracellulare* are the species included under the MTC.

Wolf et *al.* (2015) used IS*6110* based PCR for non-invasive *Mycobacterium tuberculosis* detection in fecal samples from primates.

Choudhury (2016) reported the status of Indian Grey Mongoose, its ecology and conservation threats.

8. Outline of the technical programme

Fresh fecal samples will be collected and DNA will be isolated using QIAamp DNA stool mini kit as per the manufacturer's recommendations.

Detection of the presence of any *Mycobacterium* spp. in fecal sample will be carried out by amplification of 16S rRNA region as described by Shin *et al*. (2010).

Detection of MTBC and MAC organisms will be carried out by PCR using IS*1311* and IS*6110* genes as described by Eisenach *et*

al. (1990) and Shin *et al*. (2010) respectively.

8. Main items of observation to be made:

1. Presence of *Mycobacterium* spp. in faecal samples from Indian Grey Mongoose by PCR
2. Presence of MTBC organismsin faecal samples from Indian Grey Mongoose by PCR
3. Presence of MAC organismsin faecal samples from Indian Grey Mongoose by PCR

10. Facilities

a. Existing facilities:

PCR and other lab equipments required for the work.

b. Additional facilities required:

Primers, collection tubes, PCR tubes and DNA Stool Mini Kit.

11. Duration of study: One semester

12. Financial estimate:

DNA Stool Mini Kit: Rs.20,000

Primers : Rs. 10,000

Travelling : Rs. 1200

Total : Rs. 31,200

13. Signature of student

14. Signature of major advisor

Place: Pookode

Date:

15. Name, designation and signature of members of the Advisory Committee

1. Dr. George Chandy (Course Director)

Officer in Charge,

Centre for Wildlife Studies,

College of Veterinary and Animal Sciences, Pookode.

2. Dr. Chintu Ravishankar (Major Advisor)

Assistant Professor

Department of Veterinary Microbiology

College of Veterinary and Animal Sciences, Pookode.

3. Dr. Abdul Azeez C.P. (Member)
Assistant Professor,
Centre for Wildlife Studies,
College of Veterinary and Animal Sciences, Pookode.

Appendix I

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Appendix II

Time frame of Work

Semester IV

1. Field observations

2. Standardization of diagnostic tests

3. Collection of samples from field

4. Testing of samples by PCR

5. Interpretation of results

6. Dissertation writing

CERTIFICATE

Certified that the research project has been formulated observing the stipulations laid down under the Prevention of Cruelty to Animals Act (Amendment, 1998).

Place: Dr. Chintu Ravishankar

Date: Guide

CURRICULUM VITAE

Name of the Candidate	:	Bindya A
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Publication Made	:	Nil
Membership in Professional Bodies	:	Nil