MOLECULAR DETECTION OF *'MYCOBACTERIUM TUBERCULOSIS* COMPLEX' IN FAECAL SAMPLES OF CAPTIVE ZOO ANIMALS

Thesis

Submitted to the

Bihar Animal Sciences University, Patna

in partial fulfilment of the requirements for the degree of

MASTER OF VETERINARY SCIENCES

In

VETERINARY MICROBIOLOGY

By

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CERTIFICATE - I

This is to certify that the thesis entitled "**Molecular detection of *Mycobacterium tuberculosis* complex in faecal samples of captive zoo animals**" submitted in partial fulfillment of the requirement for the degree of **Master of Veterinary Science** in the discipline of **Veterinary Microbiology** of the faculty of Post - Graduate studies, Bihar Animal Sciences University, Patna, Bihar is the bonafide research work carried out by **Dr Bhartendu Vimal, Registration No.- VM0016/2019-20**, under my guidance and supervision and that no part of this thesis has been submitted for any other Degree or Diploma.

It is further certified that the assistance and help received during the course of this investigation and preparation of the thesis have been fully acknowledged.

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CERTIFICATE–II

This is to certify that the thesis entitled "**Molecular detection of *Mycobacterium tuberculosis* complex in faecal samples of captive zoo animals**" submitted by **Dr Bhartendu Vimal, Registration No-VM0016/2019-20,** to the Bihar Animal Sciences University, Patna, Bihar in partial fulfillment of the requirement for the degree of **Master of Veterinary Science** in the discipline of **VETERINARY MICROBIOLOGY** has been approved by the Advisory committee after an oral examination on the student in collaboration with an External Examiner.

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This thesis is
dedicated to
my loving family

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Date: 07-06-2022
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(Bhartendu Vimal)

CONTENTS

Abbreviations

%	Percentage
≤	Less than equals to
≥	More than equals to
°C	Degree centigrade
bp	Base pair
CZA	Central Zoo Authority
Da	Dalton
DNA	Deoxy-ribonucleic acid
dNTPs	Deoxy-nucleotide triphosphates
DW	Distilled water
et al	et alibi
g	gram
hr	hour
i.e.	That is
M	Molar
MDR	Multi Drug Resistance
mg	Milli gram
ml	Milli litre
mM	Milli mole
MTBC or MTC	*Mycobacterium tuberculosis* complex
Mw	Molecular weight
PCR	Polymerase Chain Reaction
pH	*-log* $[H^+]$ ion concentration
Pmol(pM)	Pico mole
rpm	Revolution per minute
TAE	Tris acetate EDTA
Taq	*Thermus aquaticus*
Tris	Tris hydroxyl methyl aminoethane
UV	Ultra voilet
v	Volts
w/v	Weight by volume
WHO	World Health Organization
µ	Micron
µg	Micro gram
µl	Micro litre
NTM	Non-tuberculous mycobacteria
RFLP	Restriction Fragment Length Polymorphism
ZN	Ziehl-Neelsen
LJ	Lowenstein-Jensen
MGIT	Mycobacteria Growth Indicator Tube
PBS	Phosphate Buffer Saline

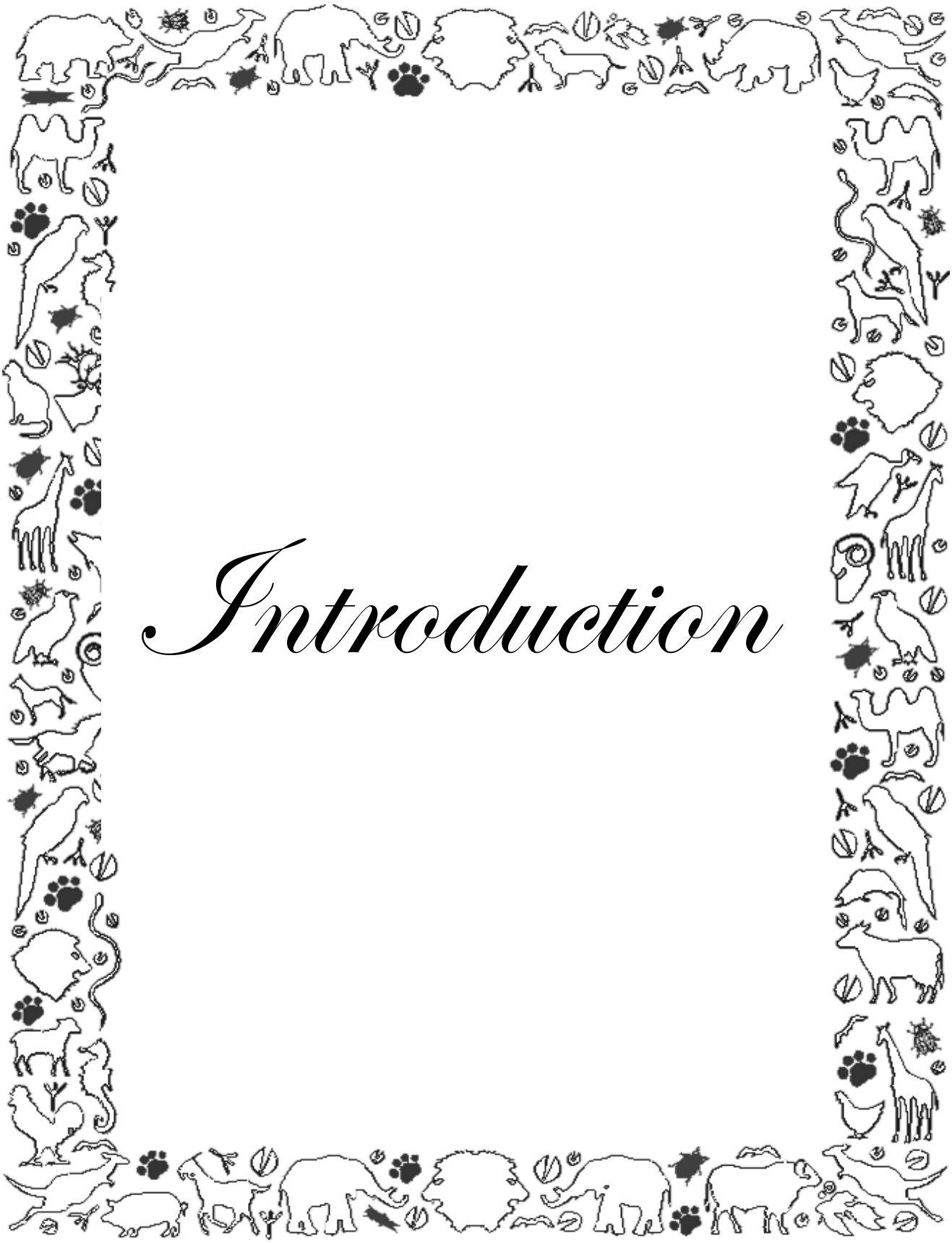
List of Tables, Figures, Plates

Abstract

The current study aimed to determine the occurrence of *Mycobacterium tuberculosis* complex (MTBC) in faecal samples from captive zoo mammals and birds. A total of 114 faecal samples including 84 faecal samples from captive wild mammals and 30 samples from captive wild birds by non-invasive method. In the current investigation, specimens were processed using the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method in order to recover acid-fast bacilli (AFB), culture, and extract bacterial DNA. The ZN method of staining was performed to identify acid fast bacilli. Qiagen QIAamp DNA Stool mini kit was used in this study to extract DNA from faecal samples of captive wild animals. The m-PCR assay was used to detect *Mycobacterium tuberculosis* complex species. Forward and reverse primers for the *hsp*-65 and *esat*-6 genes were individually amplified in m- PCR assay to identify primer pairing. The m-PCR positive samples were cultured to reconfirm the MTBC. Direct m-PCR on 114 faecal specimen-derived DNA yielded positive results in 10 (8.77%) samples, demonstrating simultaneous amplification of the two targets *hsp*-65 (441bp) and *esat*-6 (320bp). None of the 30 faecal specimens collected from captive wild birds tested positive for the m-PCR assay. The comparison of the findings of m-PCR, MGIT, and LJ medium revealed that the sensitivity and yield of m-PCR positive MTBC recovered from either the BACTEC system or a solid medium (LJ) were comparable and equivalent. The data of five mammalian orders (*Proboscidea*, *Artiodactyla*, *Carnivora*, *Perissodactyla*, and *Primates*) was examined. The results of the m-PCR and culture assays showed that the frequency of MTBC was highest in taxonomic order *Proboscidea* (50.00%), followed by *Artiodactyla* (20.00%), and *Carnivora* (3.33%). *Perissodactyla* and *Primates* have not been found to have MTBC. Further examination of the data revealed that m-PCR detected positive for MTBC bacteria in 08 (16.67%) faecal samples from herbivorous mammals and 02 (15.38%) omnivores. Herbivores had the highest overall frequency of MTBC bacteria among mammals, followed by omnivores.

The study found that MTBC in the faeces of 10 different species of captive wild mammals maintained at the Sanjay Gandhi Biological Park, Patna could be identified by m-PCR and cultivated using MGIT and LJ media.

Further investigation is recommended to establish the presence of MTBC and/or the gene abundance as a function of species and dietary patterns.



Introduction

Chapter 1 Introduction

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis*, which are aerobic, non-spore forming, acid fast bacilli having rod shaped morphology. It is non-motile and gram positive in nature. Due to presence of high lipid and mycolic acid in their cell wall, a special staining named Ziehl-Neelsen (ZN) is used for their identification. The *Mycobacterium tuberculosis* complex (MTBC) consists of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG (Bacillus, Calmette, and Guérin), *M. africanum*, *M. microti*, *M. mungi*, *M. canetti*, *M. caprae*, and *M. pinnipedii* (Orgeur and Brosch, 2018) and has a zoonotic importance. It causes chronic inflammatory disease affecting humans, domestic animals, birds, and wildlife. (Poirier *et al*., 2019). Many species of wild animals such as badgers, badgers, coyotes, raccoons, hares, rabbits, hedgehogs, brush tail possums, coatis, capybaras, lions, deer, elk, wild boars, foxes, primates and pinniped (grey seal) (Matos *et al*., 2014; Gortazar *et al*., 2012; Barnett *et al*., 2013) are supposed to be susceptible with MTBC species either through "maintenance hosts" or "spillover hosts" (Bailey *et al*., 2013). Spillover transmission of MTBC to other wildlife species and domestic livestock animals has been already reported in South African Buffalo (Michel *et al*., 2010) and lions (Viljoen *et al*., 2015). It has been detected in Asian elephant in Nepal (Paudel *et al*., 2014), wild elephant in Srilanka (Perera *et al*., 2014), captive sloth bear in Karnataka, India (Shylaja *et al*., 2021) Apart from that Indian elephants have also been found seroreactive to TB antigen (Abraham *et al*., 2008). Besides of that, it is estimated that one third of total human population is infected with MTBC (WHO report, 2019).

Many factors are responsible for MTBC transmission in animals like number of infected animals, susceptible hosts, routes of infection, anatomical location and lesions of disease, structure of tuberculosis lesions, pathogen shedding, and the infective dose (Corner, L.A. 2006). Environmental contamination plays also an important role in MTBC transmission (Ghodbane *et al*., 2014). Direct oral and or nasal transmission is the most common route of infection (Morris *et al*., 1994). Indirect transmission mainly occurs sharing of resources like water and feeding areas (Palmer *et al*., 2004). Despite decades-long eradication campaigns, BTB is still very prevalent in some European countries including Spain, where wildlife reservoirs of *Mycobacterium bovis* have been confirmed (Bravo *et al*., 2019; Fellag *et al*., 2019).

To control any disease, diagnosis at earliest plays an important role. In wild and domestic animal most of the diagnosis of MTBC has been conducted by post-mortem examination and tubercular lesions of lungs. However, few reports are available on ante-mortem detection of MTBC infection (Lekko *et al*., 2020). Diagnosis in wildlife is a prime step in disease control and management but is also essential in the evaluation of surveillance strategies, in pathogenesis, epidemiological and transmission studies as well as in the assessment of the efficacy of vaccination trials. However, diagnosis in wildlife is challenging due to capture and restraint difficulties inherent to wildlife collection of samples, frequent lack of gold standard diagnostic techniques, lack of knowledge about the true infection status, difficulty in interpretation and conducting experimental studies, as well as limited financial resources. Nevertheless, MTBC in wildlife is an active area of research. Serological tests are especially useful in wildlife because they are economically attractive, technically easy, enable large-scale surveillance and can be applied both in live or dead animals and, in the latter, in combination with pathology. In farmed wildlife, combinations of cellular and humoral tests could enhance diagnostic accuracy (Thomas *et al*., 2021). For epidemiological investigations and the diagnosis of tuberculosis in wild animals, many techniques have been developed, including immunological, serological, and molecular biology techniques but culture remains the gold standard technique for the diagnosis of tuberculosis mycobacteria infections. The lymph nodes are the most frequently used sample for the research of mycobacteria in animals, but this invasive sampling made by well trained staff, is almost limited to dead animals.

Therefore, the analysis of faeces is an emerging method in wild animals (Biswas *et al*., 2019) on the model of what has been reported for the routine diagnosis in human patients (Fellag *et al*., 2019; Gaur *et al*., 2020).

Presently, various techniques are commonly employed for diagnosis of MTBC like tuberculin test used for the diagnosis of *M. tuberculosis* (https://www.cdc.gov/tb/topic/testing/default.htm). Culture of mycobacterium is considered as gold standard test (OIE, 2019). ZN staining from the affected lesion or sputum for the presence of rod shaped acid fast bacilli(AFB), histopathological diagnosis, molecular diagnosis through RT- PCR (Real Time Polymerase Chain Reaction) and PCR (Polymerase Chain Reaction) using primer and probes target specific gene of *Mycobacterium*.

Wildlife being precious and their conservation is crucial for their role in the ecosystem. An early detection of MTBC could be instrumental in order to save the life of wild animal and to check the spread of infectious particle from animal to animal and human. Detection of *M. tuberculosis* by tuberculin test requires restraining of domestic animals but these invasive techniques is almost impossible for wild animals.

Secretion of *M. tuberculosis* in faeces has been reported by earlier workers (Dron-In *et al*., 2020; Hamzah, 2019; Wolf *et al*., 2015) and their detection in faeces of captive wild animals could be a sort-in-hand for early detection, transmission and prevention of animals.

Confirmation of presence of MTBC can be done by ZN staining, culturing, PCR and RT-PCR technique. Culturing can be done either in conventional LJ (Lowenstein-*Jensen*) media (Kassaza *et al*., 2014) or more sensitive and less time consuming MGIT (Mycobacteria Growth Indicator Tube) liquid media in automated system BACTEC MGIT 960 (Siddiqi *et al.*, 2012). Modern molecular diagnostic method like PCR and RT-PCR plays important role in detection of MTBC more accurately and it has more sensitivity and specificity. All the Mycobacterial strains can be identified by using *hsp*65 encoding gene (Varma-Basil *et al*., 2013). Further, Mycobacteria and NTM (Non-tuberculous mycobacteria) can be easily differentiated by *hsp*65 gene by PCR-RFLP (Restriction Fragment Length Polymorphism) molecular tools using specific restriction endonuclease enzyme (Macente *et al*., 2013). This technique has 100 % sensitivity and 93.1 % specificity contrary to culture and microscopy methods (Bannalikar *et al*., 2006).

The techniques are absolutely non-invasive and could be done without panicking animals. As very few research findings have been reported so far for identification of *Mycobacterium bovis* in faecal samples from the captive wild animals. (Hamzah, 2019).

However, no report has been found to best of our knowledge regarding detection of MTBC in faecal sample of wild animals. Keeping in view, the necessity of detection of *Mycobacterium* species involved in causation of tuberculosis, the present study has been designed to develop and validate the rapid and accurate method of detection of presence of tuberculous Mycobacterium (MTBC) by non-invasive technique.

This study will help to make a strategy to develop diagnosis method for wild animals. In the given perceptions, present work has been undertaken with the following objectives.

Objective:

1. To determine the occurrence of *Mycobacterium species* directly in faecal samples of captive wild animals by polymerase chain reaction(PCR).
2. To isolate the *Mycobacterium* species in artificial culture media (MGIT and LJ media).
3. To confirm the isolates for *Mycobacterium tuberculosis complex* (MTBC) by ZN staining and MPT-64 antigen card test.

Review of Literature

The *Mycobacterium tuberculosis* complex (MTBC) is a group of *Mycobacterium* spp. that are genetically related to each other and often criticised for causing tuberculosis in both humans and animals. The various species that comes under the umbrella of MTBC include *M. tuberculosis, M. africanum, M. orygis, M. bovis, M. microti, M. canetti, M. caprae and M. pinnipedii* (Orgeur and Brosch, 2018). The conserved signature indels exclusively present in diverse proteins of MTBC forms the basis for distinguishing it from all other bacteria and also forms the basis for functional and diagnostic studies (Gupta, 2018). The members of MTBC are Gram positive, aerobic, rod-shaped bacteria non-motile and non-spore forming that contains mycolic acid in their cell wall, rich in lipids and DNA with a high guanine plus cytosine content (61–71%), and extreme hydrophobicity, are closely related to the waxy coating of the bacteria. By preventing nutrient intake, these presumably contribute to the slow growth of bacteria. They are acid-fast bacteria that stains well with ZN stain.

Lekko *et al.,* (2020) reviewed the various diagnostic methods for diagnosing tuberculosis such as the single intradermal tuberculin test, the gamma-interferon test, granulomatous lesions, serology for antibody detection, smears of the lesions and histopathological analysis. Isolation of Mycobacterium is considered as gold standard test because of high sensitivity and specificity. The World Health Organization, 2018 highlights the potential of Next-generation sequencing (NGS) in rapid diagnosis of drug resistant tuberculosis. The NGS overcomes the challenges associated with conventional phenotypic and molecular testing by providing rapid and detailed genomic information. Malone and Gordon, 2017 highlights the events in the evolution of MTBC based on phylogenetic analysis of species-specific variations and pointed out the close genetic relationship between human and animal adapted species. Captive animals may act as a potential source for spreading tuberculosis in human population due to chances of direct contact with public, through biological vectors and absence of sanitary barriers including precautionary hygiene and sanitary vigilance (Pereira *et al.,* 2018).

Prevalence of MTBC infection in wild animals

Barasona *et al.,* (2017) studied the persistence of environmental *Mycobacterium tuberculosis* complex (MTBC) in water resources and possibility of cross-species transmission at livestock/wildlife interface. They found 55.8% of mud samples and 8.9%

of water samples positive for MTBC which may possibly spread the infection at livestock/wildlife interface.

Gormley and Corner (2018) highlighted the role of wild animals in the epidemiology of tuberculosis and the role of stakeholders in the control and elimination of tuberculosis.

Maciel *et al.,* (2018) in their study evaluated 80 wild boars out of which 27.9% showed histological changes reflecting tuberculosis and 31.3% were found positive for the presence of *M. bovis* genome which suggest the possibility of these animals serving as Mycobacterium reservoir.

Rosenbaum *et al.,* (2015) evaluated 220 buccal swabs from 16 species of free-ranging old-world monkeys and observed in 30 (13.6%) of the samples positive for *IS6110* gene sequence suggesting the presence of *Mycobacterium tuberculosis* complex in non-human primates.

Diagnostic methods employed for detection of MTBC

Many wildlife species are involved in the transmission and maintenance of tuberculosis. Therefore, the diagnosis of such infection is of paramount importance because it supports screening, epidemiological investigation in addition to ensuring the effectiveness of control strategies.

Abaye *et al*., 2017 detected *Mycobacterium tuberculosis* in the stool of HIV positive individuals (humans) who was negative for sputum smear microscopy and culture. So, it strongly supports that faecal sample examination may be very important aspect for the detection of *Mycobacterium species*. (It supports that faecal sample examination may be an important aspect for the detection of *Mycobacterium* species).

de Jesús Beleño-Sáenz, 2021 analysed the faecal samples of free-ranging wild boar with an electronic nose system, which identifies the volatile organic compounds emitted from faeces to detect tuberculosis.

Dhama *et al.,* 2011 reviewed tuberculosis in birds by *Mycobacterium avium* infections. He observed that lesions in lungs are not commonly found in birds. Granulomatous lesion without calcification is commonly found. Tubercular nodules can be observed in liver, spleen, intestine and bone marrow. Modern molecular techniques like

PCR-RFLP and gee probe may be useful for rapid diagnosis and speciation of mycobacteria subspecies.

Dron-In *et al*., 2020 confirmed that *M. caprae* can shed with the faeces of naturally infected red deer (*Cervus elephus*). In total (n= 2806) red deer only 2% (n=55) were found to positive for *M. caprae* by pathological examination, PCR and culturing of the affected organ material. In one red deer, *M. caprae* is isolated from the heart sac as well as from the faeces. Whole genome sequencing (WGS) also confirmed that both strains were clonally related.

Dwyer *et al.,* 2020 reviewed the current diagnostics regarding the epidemiology of TB in African rhinoceros where they found blood-based immunoassays as effective but of limited value due to its inability for distinguishing between subclinical and active infections.

El Khechine *et al.,* 2009 hypothesized that swallowed MTBC organisms could be detected in stool samples and observed sensitivity and specificity of 37.5 % and 100 % respectively on microscopic examination of stools.

Hamzah, 2019 detected *Mycobacterium bovis* from faeces of zoo animals by culture and ZN method. Also, and concluded that bovine tuberculosis may be dangerous re-emerging disease and *Mycobacterium bovis* as potential reservoir for zoonosis.

Mesman *et al.,* 2019 throws light on the importance of stool in the diagnosis of tuberculosis in children due to less availability of sputum required for conventional tests. They tested the stool samples with the TruTip close amplification system and optimized the assay to improve the sensitivity of test with low bacterial load.

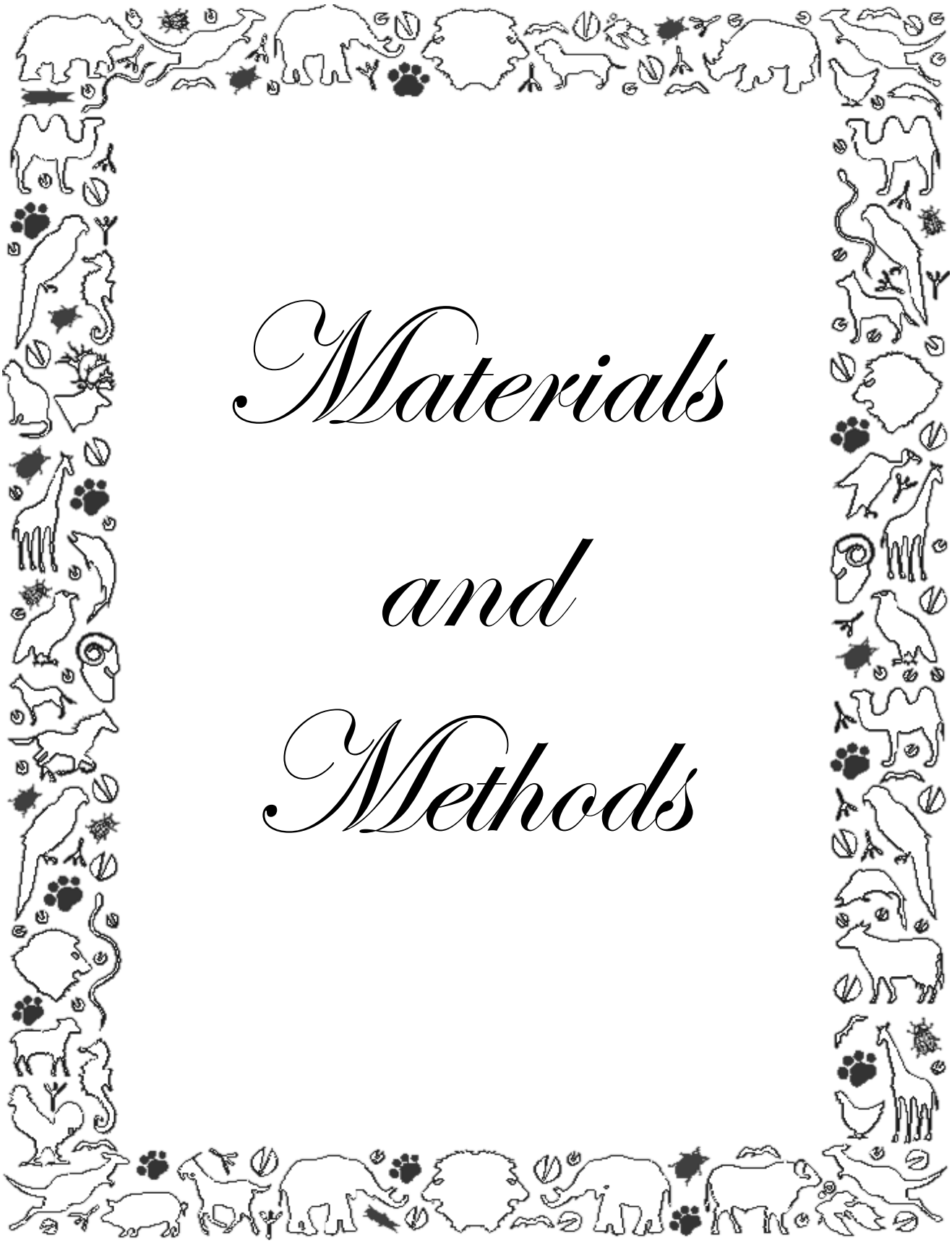
Sattar *et al*., 2021 detected *Mycobacterium avium complex* (MAC) from faecal sample of birds (chicken and captive birds) by using PCR and culture.

Thomas *et al.,* 2021 gives an overview of the factors related to environment, host, sampling, and diagnostic techniques which can affect test performance. The serological tests relatively inexpensive and easy to perform in wildlife as it facilitates large-scale surveillance in both ante- and post-mortem. They also pointed out cost effectiveness and accurate results as the limitations of diagnostic tests for wildlife TB diagnosis.

Veeraselvam *et al.,* 2015 collected 42 fresh faecal samples from apparently healthy sloth bears kept under captivity and found 5 samples positive for *M. bovis pnc*A gene.

Viljoen *et al*., 2019 investigate the suitability of single intradermal cervical test(SCIT) in lions of Kruger National Park in which specificity was found to be low with 54 % false positive results.

Wolf *et al.,* 2015 used as non-invasive method from faecal samples for detecting tuberculosis in primates by PCR amplification of insertion element *IS6110* of *M. tuberculosis*. He concluded that IS6110 detection is advantageous for its MTBC specificity.


Materials
and
Methods

Chapter 3 Materials and Methods

3.1 Site of the study

This study was performed at the Department of Veterinary Microbiology, Bihar Veterinary College, Bihar Animal Sciences University, (BASU), Patna, Bihar, India in collaboration with Molecular Medicine Laboratory, Department of Microbiology, All India Institute of Medical Sciences (AIIMS), Bhopal.

Faecal samples of various species of animal were collected from the Sanjay Gandhi Biological Park (SGBP), Patna following standard procedure.

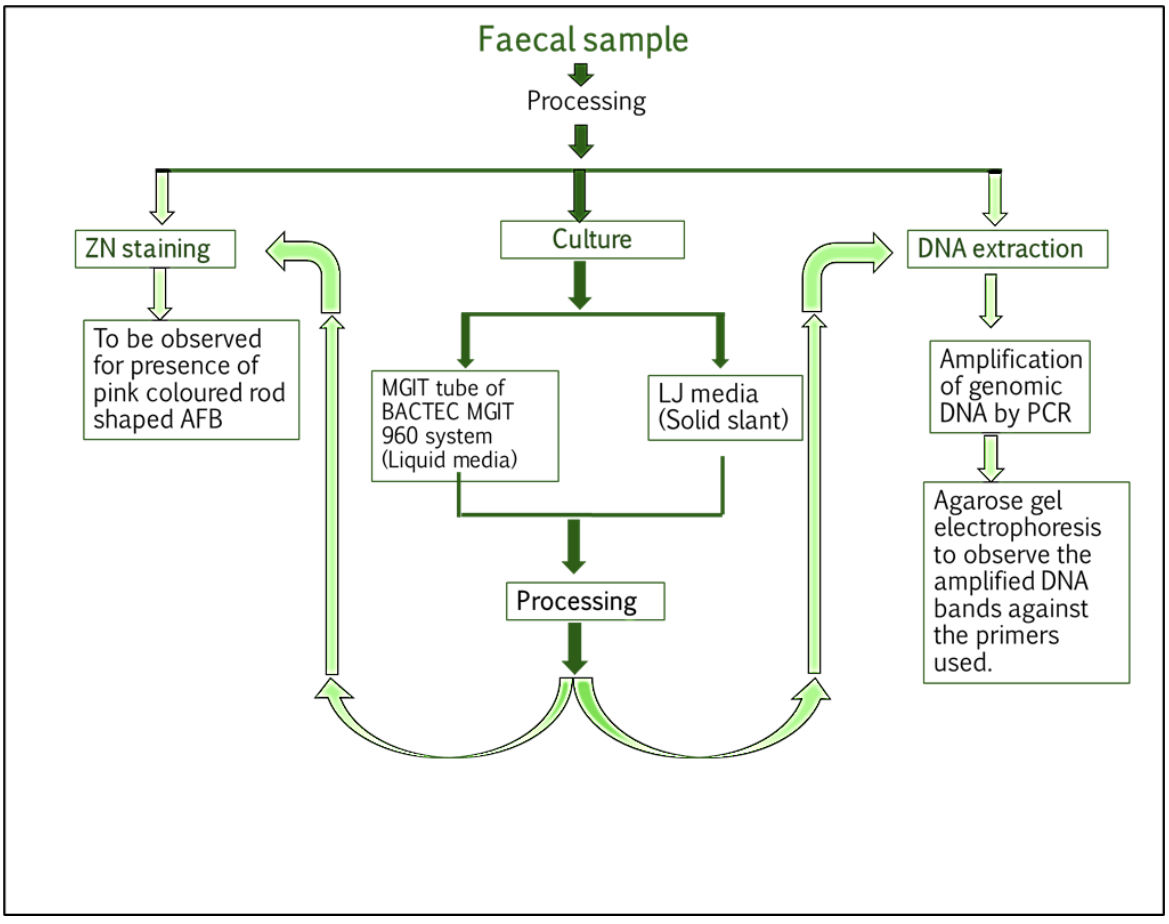


Figure 1: Plan and outline of investigation

3.2 Captive wild animals selected for study:

The study population comprised of 25 species of wild mammals and 22 species of wild birds maintained at Sanjay Gandhi Biological Park, Patna. The order wise species of animals is depicted in Table 1, 2, 3 and 4.

Table 1: Stratification of captive wild mammals selected for collection of faecal samples

Sl.No.	Order	Species of Mammal	Scientific name
1.	*Carnivora*	Himalayan Black bear	*Ursus thibetanus*
		Sloth bear	Melursus ursinus
		White tiger	*Panthera tigris* subsp. *tigris*
		Bengal tiger	*Panthera tigris* subsp. *tigris*
		Asiatic lion	*Panthera leo* subsp. *persica*
		Leopard	*Panthera pardus*
		Hyena	*Hyaena hyaena*
		Wolf	*Canis lupus*
2.	*Proboscidea*	Elephant	*Elephas maximus*
3.	*Perissodactyla*	One-horned Rhinoceros	*Rhinoceros unicornis*
		Zebra	*Equus quagga boehmi*
4.	*Artiodactyla*	Hippopotamus	*Hippopotamus amphibious*
		Giraffe	*Giraffa camelopardalis*
		Indian Gaur	*Bos frontalis gaurus*
		Hog deer	*Axis porcinus*
		Swamp deer (Barahsigha)	*Rucervus duvaucelii*
		Chetal (Spotted deer)	*Axis axis*
		Sambar deer	*Cervus unicolor*
		Barking deer	*Muntiacus muntjak*
		Black buck	Antilope cervicapra
		Sangai (Manipuri) deer	*Cervus eldi thamin*
		Nilgai - Blue Bull	*Boselaphus tragocamelus*
5.	*Primate*	Rhesus monkey	*Macaca mulatta*
		Chimpanzee	*Pan troglodytes*
		Langur (Baboon)	*Presbytis entellus*

Table 2: Captive wild birds selected for collection of faecal samples

Sl.No.	Order	Species of birds	Scientific name
1.	*Accipitriformes*	Vulture	*Gyps himalayensis*
		Kite-Pariah/Black Kite	*Milvus migrans*
2.	*Anseriformes*	Duck Brahmin(Ruddy Shelduck)	*Tadorna ferruginea*
3.	*Bucerotiformes*	Hornbill	*Anthracoceros malabaricus*
4.	*Casuariiformes*	Emu	*Dromaius novaehollandiae*
		Cassowry	*Casuarius casuarius*
5.	*Columbiformes*	Common Emerald dove	*Chalcophaps indica* subsp. *indica*
6.	*Galliformes*	Lady Amherst's pheasant	*Chrysolophus amherstiae*
		Silver pheasant	*Lophura nycthemera*
		Golden pheasant	*Chrysolophus pictus*
		Peafowl	*Pavo cristatus*
7.	*Gruiformes*	Crane Common	*Grus grus*
		Crane Sarus	*Grus antigone*
8.	*Passeriformes*	Finch Zebra	*Taeniopygia guttata*
		Hill myna	*Gracula religiosa*
9.	*Psittaciformes*	Medium sulphur crested cockatoo	*Cacatua galerita elonora*
		Scarlet macaw	*Aro macao*
		Macaw Red & Green	*Spix's macaw*
		Illiger's Macaw	*Primolium maracana*
		Parrot	*Psittacula krameri manillensis*
		Budgerigar	*Melopsittacus undulatus*
10.	*Struthioniformes*	Ostrich	*Struthio camelus*

Table 3: Categorization of captive wild mammals based on their feeding habit

Carnivore	Herbivore	Omnivore
Asiatic Lion	Barking deer	Chimpanzee
Bengal Tiger	Black Buck (Krishna mrig)	Himalayan Black Bear
Hyena	Chital (Spotted deer)	Langur
Leopard	Elephant	Rhesus Monkey
White Tiger	Giraffe	Sloth Bear
Wolf	Hippopotamus	
	Hog deer	
	Indian Gaur	
	Nilgai - Blue Bull	
	One horned Rhinoceros	
	Sambar deer	
	Sangai (Manipuri) deer	
	Swamp deer (Barasingha)	
	Zebra	

Table 4: Categorization of birds based on their feeding habit

Carnivore	Herbivore	Omnivore
Kite-Pariah/Black Kite	-	Budgerigar
Vulture	-	Cassowary
-	-	Common Emerald dove
-	-	Crane Common
-	-	Crane Sarus
-	-	Duck Brahmin (Ruddy Shelduck)
-	-	Emu
-	-	Finch Zebra
-	-	Golden pheasant
-	-	Hill myna
-	-	Hornbill
-	-	Illiger's Macaw
-	-	Lady Amherst's pheasant
-	-	Medium Sulphur crested cockatoo
-	-	Ostrich
-	-	Parrot
-	-	Peafowl
-	-	Red & Green Macaw
-	-	Scarlet macaw
-	-	Silver pheasant

3.3 Materials

3.3.1 Source of faecal sample:

Altogether 84 samples were collected out of 25 species of captive wild animals whereas 30 specimens were obtained from 22 species of captive wild birds. Species wise details of samples has been presented in Table 5 and 6. This study includes 114 faeces samples collected from captive wild animals and birds between December 2020 to January 2022.

Table 5: Species wise distribution of faecal sample collected from captive wild mammals

Sl. no.	Mammals	Total no. of sample
1.	Asiatic lion	03
2.	Barking deer	01
3.	Bengal tiger	06
4.	Black buck	06
5.	Chital (Spotted deer)	04
6.	Chimpanzee	01
7.	Elephant	02
8.	Giraffe	06
9.	Himalayan Black bear	02
10.	Hippopotamus	03
11.	Hog deer	01
12.	Hyena	04
13.	Indian Gaur	02
14.	Langur (Baboon)	02
15.	Leopard	05
16.	Nilgai - Blue Bull	01
17.	One-horned Rhinoceros	09
18.	Rhesus monkey	03
19.	Sambar deer	04
20.	Sangai (Manipuri) deer	05
21.	Sloth bear	05
22.	Swamp deer (Barahsigha)	02
23.	White tiger	01
24.	Wolf	04
25.	Zebra	02
Total		**84**

Table 6: Species wise distribution of faecal sample collected from captive birds

Sl. no.	Birds	Total no. of sample
1.	Budgerigar	01
2.	Cassowary	01
3.	Common Emerald dove	01
4.	Crane	02
5.	Crane Sarus	01
6.	Brahminy duck (Ruddy Shelduck)	01
7.	Emu	01
8.	Zebra finch	02
9.	Golden pheasant	02
10.	Hill myna	01
11.	Hornbill	01
12.	Illiger's Macaw	01
13.	Kite-Pariah/Black Kite	01
14.	Lady Amherst's pheasant	01
15.	Medium sulphur crested cockatoo	01
16.	Ostrich	02
17.	Parrot	01
18.	Peafowl	03
19.	Red & Green Macaw	01
20.	Scarlet Macaw	01
21.	Silver pheasant	03
22.	Vulture	01
Total		**30**

3.3.2 Plastics ware and glassware:

All the plastic wares used in this study were procured from national and international firms i.e. HiMedia laboratories Pvt. Ltd., Mumbai, Axiva (India), Greiner (India) and Axygen(USA) and glasswares were obtained from, Borosil (India) and Schott Duran (Germany) micro centrifuge tubes (Tarsons), tips (Eppendorf).

3.3.3 Instruments and equipment used for study:

The following instruments and equipment listed down were used during the present study:

i. BACTEC MGIT 960 (BD)
ii. Electronic balance (Denver, USA)
iii. Centrifuge machine (REMI, India/Thermo Scientific)
iv. Deep fridge -20°C (Blue Star, India)
v. Gel documentation system (Bio-Rad, USA)
vi. PCR machine (Himedia, XP- Thermal cycler)
vii. pH meter (LABMAN)
viii. Micropipette of various volumes(0.5µl-1000µl) (Eppendorf, Germany)
ix. Horizontal gel electrophoresis apparatus (Thermo Scientific, China)
x. Gel Doc (XR System Bio-Rad)
xi. Water bath (YSI, India)
xii. Vortex mixture (Tarson, India)
xiii. Autoclave (Instrumentation, India)
xiv. Incubator (Sonar, India)
xv. Water distillation apparatus (Millipore India Pvt. Ltd., New Delhi)
xvi. Laminar airflow bench (Klenzaids, India)
xvii. Microwave oven (LG, India)
xviii. Compound light microscope (Olympus)
xix. Refrigerator (LG)

xx. Centrifuge (Thermo scientific)

xxi. Vortex mixer (Cyclo Mixer)

xxii. SPINWIN Micro Centrifuge (Tarsons)

xxiii. Hot water bath (Nuon Lab)

xxiv. Micropipettes (Eppendorf)

xxv. Deep freezer (Elanpro)

3.3.4 Media, buffers reagents and kits used for study:

The media and reagents used in this study were procured commercially from HiMedia Mumbai and DSS Takara, India. The detail of media and their preparation, buffers and reagents that were used in this study.

3.3.4.1 Chemicals used in molecular studies:

i. NALC powder
ii. Sodium hydroxide (NaOH)
iii. Sodium citrate x 2 H_2O or anhydrous sodium citrate.
iv. N-acetyl-L-cysteine (NALC)
v. Na_2HPO_4
vi. KH_2PO_4
vii. PCR Master Mix (Takara)
viii. 100bp Gene ruler (Thermo Scientific, USA)
ix. Ethidium bromide (Sigma, USA)
x. Agarose (HiMedia)
xi. Glycerol (HiMedia)
xii. Diluent for DNA extraction (HiMedia)
xiii. Primers (Eurofins)
xiv. SD BIOLINE TB Ag MPT64 Rapid kit (Standard Diagnostics, Inc., Korea).

3.4 Methods

3.4.1 Sample collection:

The study included fresh faecal samples of 114 healthy captive wild animals (25 species of mammals and 22 species of birds).

In this investigation, each animal was only sampled once. Fresh samples were collected considering it as pollutant free less from contaminants, such as surface dust specks, sunlight, etc.

Approximately 10 g of freshly voided faecal matter were collected from a clinically healthy animal using gloves in leak-proof sterile vial from various captive animal and bird species. The three replicate samples were collected and transferred in glycerol, absolute alcohol (99.9%) and phosphate buffered saline (pH 7.4) and labeled accordingly including the details of animals and birds. After collection of the samples, they were transported to the laboratory in a chilled box, and transported immediately to Bihar Veterinary College (BVC), Patna and kept at -20^{o}C till further use.

Figure 2: Collection of faecal samples at Sanjay Gandhi Biological Park (Patna zoo)

3.4.2 Processing of samples:

The faecal samples were processed in a Biological Safety Cabinet. Samples and reagents were brought to room temperature prior to processing.

The processing of faecal samples was performed as per Egbe *et al*., (2016). Approximately, 2-3 grams of specimen were homogenized using a mortar and pestle with 5 ml of sterile normal saline (0.85%) and filtered with three-layered sterile gauge pieces. Equal volume of fresh NALC-NaOH-sodium citrate working solution was added to each tube, agitated on a vortex mixer for 30 seconds and incubated for 10–15 minutes at room temperature to decontaminate the specimen. Immediately after the incubation, the mixture was neutralized with phosphate buffer (pH 6.8). Tubes were centrifuged at 3200 g for 20 minutes at 4°C. The supernatant was discarded, and pellets were re-suspended in 1 ml PBS for inoculation of the media as well as DNA extraction. Processing was completed within 20 – 25 minutes to avoid the damage of mycobacteria by sodium hydroxide present in solution. The samples were split after processing for smear microscopy, LJ culture and MGIT culture.

Figure 3: Processing of faecal sample from captive wild animals

3.4.3 Detection of MTBC by multiplex –PCR

All the 114 processed faecal samples of captive wild animals were subjected to m-PCR reaction.

3.4.3.1 Extraction of DNA by QIAamp Fast DNA Stool Mini DNA kit:

The DNA was extracted from the processed faecal samples using QIAamp Fast DNA Stool Mini DNA kit (Cat no. 51604) as per manufacturer's instructions.

i. About 500 µl of decontaminated (processed) faecal sample was heat lysed in hot water bath (80 ºC) for 30 min.

ii. 1 ml of Inhibit EX Buffer was added to each stool sample and vortexed continuously for 1 min. or until the stool sample homogenize thoroughly.

iii. Suspension was heated at 70 ºC for 10 min. and vortexed for 15 s.

iv. The sample was centrifuged at 14000 RPM for 1 min. at 20 ºC.

v. 15 µl proteinase K was added in a new 1.5 ml micro centrifuge tube(MCT).

vi. 200 µl supernatant was added in the 1.5 ml micro centrifuge tube containing proteinase K.

vii. 200 µl Buffer AL was added and vortexed for 15 s.

viii. Incubated at 70 ºC for 10 min.

ix. 200 µl of absolute ethanol (96–100%) was added to the lysate and mixed by vortexing.

x. 600 µl lysate was applied to the QIAamp spin column centrifuge at 14000 RPM speed for 1 min.

xi. 500 µl Buffer AW1 was added, centrifuged at 14000 RPM for 1 min. and the filtrate was discarded.

xii. 500 µl Buffer AW2 was added and centrifuged at 14000 RPM for 3 min.

xiii. Collection tube containing the filtrate was discarded.

xiv. QIAamp spin column was placed in a new collection tube.

xv. 200 µl Buffer ATE was added directly onto the QIAamp membrane and incubated for 1 min. at room temperature.

xvi. Centrifuged at 14000 RPM for 1 min. to elute the DNA.

xvii. The DNA was stored at -20 ºC till further processing.

3.4.3.1. Optimisation of Uniplex PCR assay for *hsp*-65 and *esat*-6 gene:

To identify primer pairing, forward and reverse primers for *hsp*-65 and *esat*-6 gene individually amplified by uniplex PCR assay. While determining a successful primer pairing, strong versus weak bands generated determined whether primers were working efficiently and could later be combined into a multiplex reaction. The successful amplification was determined on visualization of band size on gel.

3.4.3.2 Optimisation of Multiplex PCR assay for *hsp*-65 and *esat*-6 gene for identification of Mycobacterium species:

Following optimisation of singleplex PCR, the DNA extracted from mycobacterial strains was used to standardise the multiplex reactions for detection and identification of the *Mycobacterium tuberculosis* complex. Multiplex PCR was optimized and performed using a pair of primers containing an *hsp*-65 gene (441 *bp*) fragment to identify mycobacterial genus and *esat*-6 gene (320 bp) fragment (Table-7) for identification of *Mycobacterium tuberculosis* complex (MTBC) species.

The standard sample used as positive control of PCR amplification was DNA extracted from the H37Rv lab strain of MTBC (Borrell *et al*., 2019). The PCR master mix (Takara RR330A) containing *Taq* DNA polymerase as well as green dye and 10 pM of each primer were used. To 20 µl of this total master mix, 5 µl of template DNA was added. The reaction was carried out in Prima Duo (HiMedia) cycler at the amplifying conditions of initial denaturation at 95°C for 10 min and 30 cycles of 95°C for 1 min, 59°C for 1 min and 72°C for 1 min and a final extension of 72°C for 10 min. After completion of PCR the resulting PCR products were electrophoresed on 1.8% agarose gel and stained with EtBr (Gopinath and Singh, 2009).

MTBC was considered positive in the specimens that had both fragments of 441 *bp* and 320 *bp* amplified the corresponding targets precisely. Each set of specimens was subjected to a negative reagent blank and a positive control reaction in parallel. The results which gave the correct negative and positive control (H37Rv) in each set were used for analysis.

Table 7: Details of Oligonucleotide primers

Target gene/Locus	Primer Sequences (5'-3')	Expected product size	Reference
***hsp*-65** (Heat shock Protein 65)	F- **ACCAACGATGGTGTGTCCAT** (20 Mer) R- **CTTGTCGAACCGCATACCCT** (20 Mer)	**441** *bp*	Telenti *et al.*, 1993.
***esat*-6** (Early secretary antigenic target)	F- **GCGGATCCCATGACAGAGCAGCAGT GGA** (28Mer) R-**CCCAAGCTTCCTATGCGAACATCCCA GTGACG** (32 Mer)	**320** *bp*	Kumar *et al.*, 2014.

F: Forward primer, **R:** Reverse primer **custom synthesized from Eurofins (India).**

3.4.3.3 Agarose gel electrophoresis:

For constitution of 1.8% of agarose gel, appropriate weight of agarose powder was weighed and added to the appropriate volume of 1x TAE buffer and left to hydrate for 15 minutes. The agarose-TAE mixture was then heated in a microwave until boiling. The mixture was taken out and mixed well. The agarose was left to cool for 5 minutes at room temperature and for per 100 ml of gel 2-3µl EtBr was added. Agarose was poured into the gel tray and left to set for 30 minutes at room temperature. The gel casting tray was then transferred into the gel tank. In gel tank, 1x TAE buffer was added as running buffer. After that comb was removed from the gel. DNA ladder of 100 *bp* ladder was added to first well of the gel and each amplicon was added to the corresponding wells. The gel tank lid was placed and connected to a power supply unit. After the run, an image was taken using gel doc system.

The PCR positive samples were carried to AIIMS, Bhopal by maintaining cold chain. The samples were further processed for culture of *Mycobacterium* in BSL-3 laboratory of AIIMS, Bhopal for confirmation of *Mycobacterium* by culture and identification of isolates.

3.4.4 Direct microscopy for screening of mycobacteria:

After the processing of specimens, smears were prepared from each specimen and were were subjected to Ziehl-Neelsen (ZN) staining and examined with a light microscope for the presence of acid-fast bacteria (AFB).

Ziehl-Neelsen Staining (ZN-staining):

For ZN-staining, new, clean and unscratched microscope slides were labelled with identification mark and date. The specimen was smeared over an area approximately 1x2 cm with the bacteriological loop. Air-dried smears were heat fixed by putting on hot plate set at 65-75^{o}C for at least 15 minutes. The slides were flooded with boiled Carbol Fuchsin and left for 5 minutes. The slides were washed with tap water and flooded with 3% acid-alcohol and destained for 1-2 minutes. The destained smears were washed again with water and allowed to dry. The slides were counterstained with methylene blue for 30-60 seconds. The stained slides were rinsed with water, drained and air-dried. The slides were examined under oil immersion objective lens (100x) of the microscope and pink colour bacilli were identified as acid-fast Bacilli. (Pfyffer G., 2007; Kalyan, 2018). A total of 100 fields were examined on each slide. For morphological determinations, AFBs were assessed in terms of size, colour, shape, pattern, distribution, and uniformity to differentiate them from debris. The presence or absence of AFBs was recorded.

3.4.5 Culture of *Mycobacterium* on MGIT and LJ medium:

The processed faecal sample confirmed by ZN microscopy were inoculated (0.1-0.2ml) onto LJ media slants (HiMedia) and in MGIT tubes according to manufacturer's instructions (Siddiqi *et al*., 2012). Solid culture in LJ was incubated at 37^{o} C for 8 weeks and MGIT tubes were incubated in MGIT 960 instrument till flagged positive or negative up to 6 weeks.

3.4.5.1 Culture of *Mycobacterium* in MGIT 960 system:

The BACTEC MGIT 960 culture tubes contain 7mL of Middlebrook 7H9 base, to which was added an enrichment supplement containing oleic acid, albumin, dextrose, and catalase (BBL OADC) and an antibiotic mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (BBL MGIT PANTA) as described in the manufacturer's instructions (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD). After inoculation of each tube with 0.5 ml of the processed specimen, the contents were mixed by inverting the tube 3-4 times, barcode was scanned, and tube inserted into the BACTEC MGIT 960 instrument and were incubated at 37^{o}C. The growth of mycobacterium was monitored every 60 minutes through an alarm signal for increase of fluorescence. The cultures were tested until positive or negative for 6 weeks. When either liquid medium was

signalled as positive, a sample of the broth was withdrawn aseptically. Using a sterile pipette, a small amount of sediment from the positive MGIT tube was placed to a slide. Smear was prepared, then ZN staining was performed. The sample flagging positive and AFB detected on ZN staining was considered as a true positive.

A specimen found positive in an MGIT tube, but ZN negative, additional investigation was carried out for turbidity or indications of microbial growth in the MGIT tube. A repeat smear and ZN staining were also performed on a concentrated sample. If no AFBs were detected on ZN staining, the MGIT tube was re-incubated for 3 days to allow mycobacterium to grow further, and a repeat ZN smear was prepared. Where this continued negative, the sample was declared as negative.

To detect false negative tubes, smears were prepared from all negative tubes after six weeks of incubation and stained with ZN staining technique.

Multiplex-PCR was also performed with AFB positive samples for further confirmation of *Mycobacterium tuberculosis* complex organism.

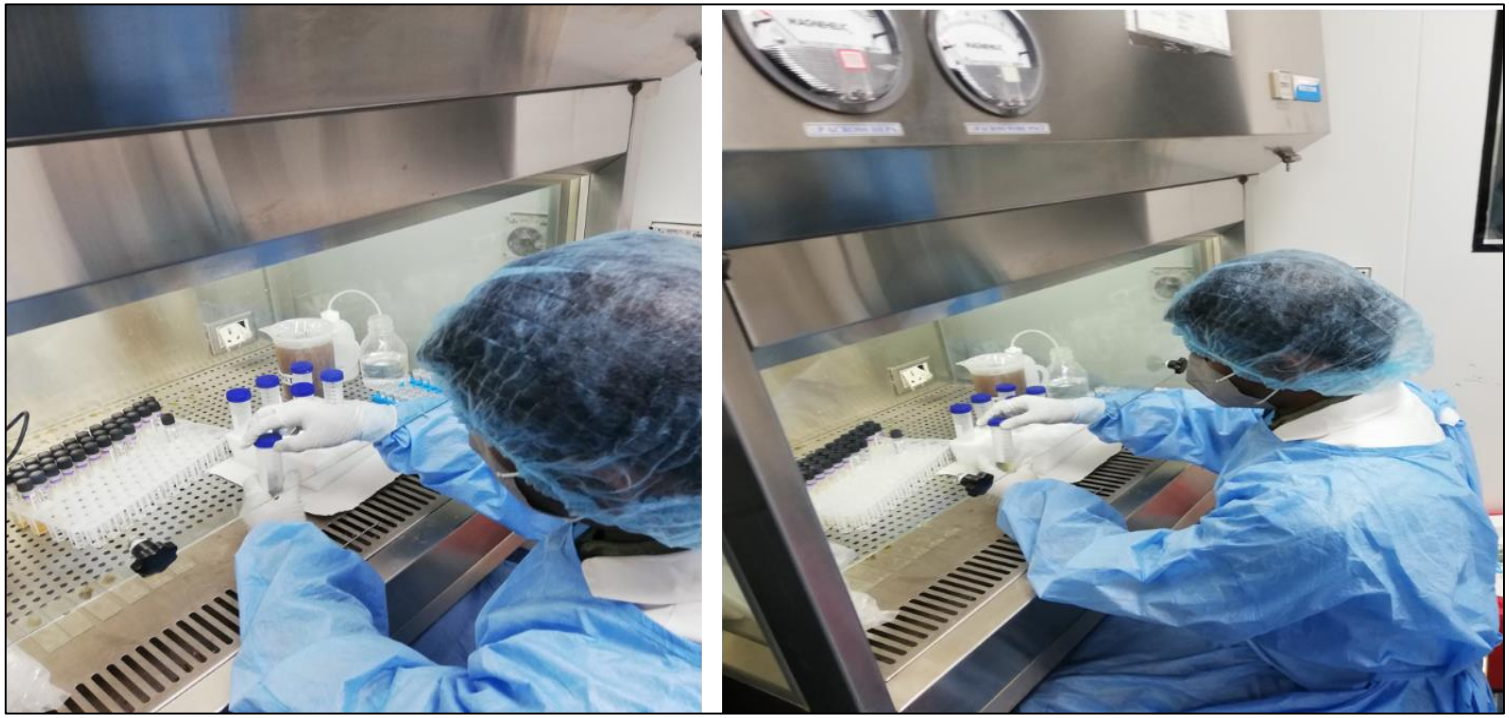


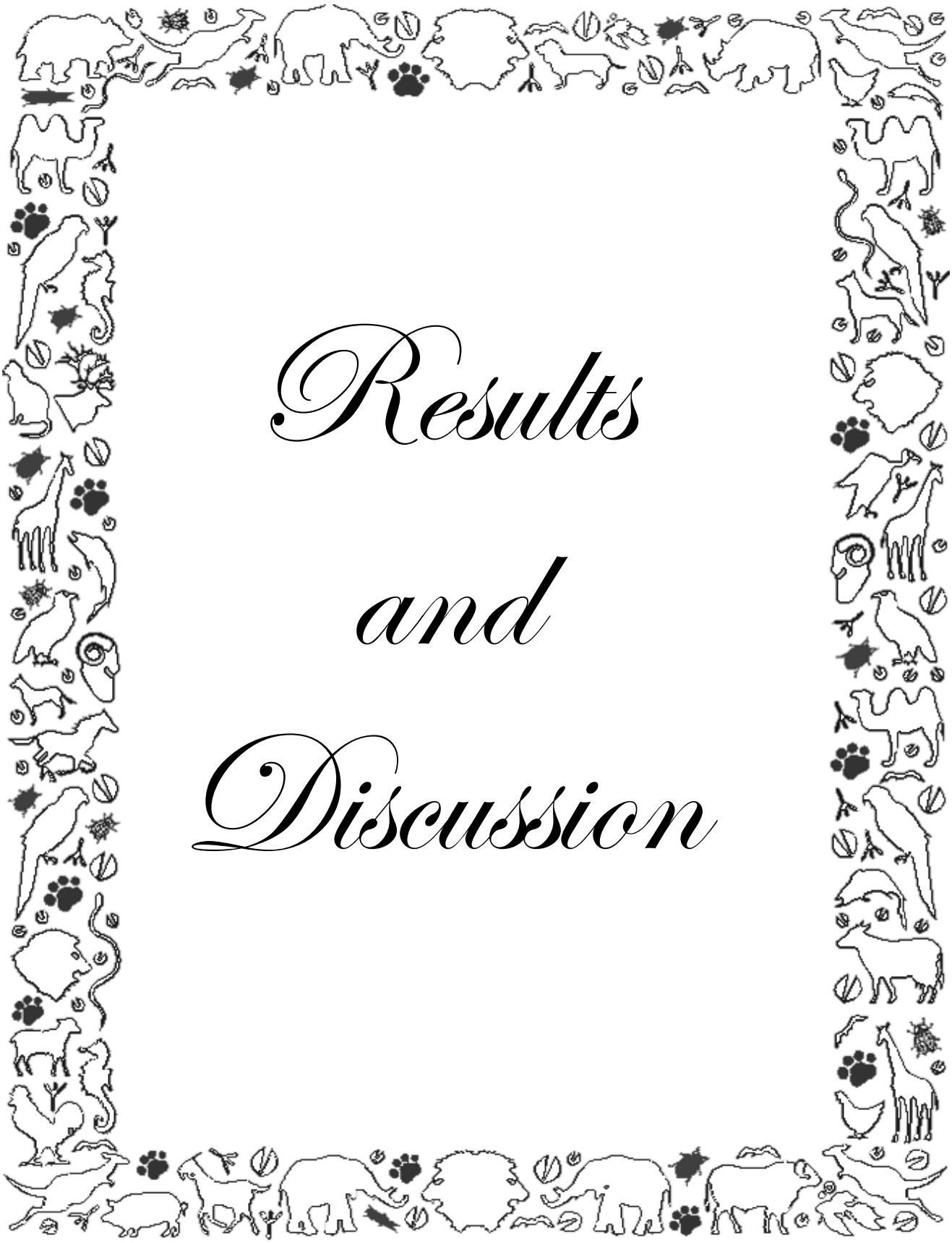
Figure 4: Inoculation of MTBC in MGIT tube

3.4.5.2 Culture of *Mycobacteria* on Lowentein-Jensen (LJ) medium:

For inoculation onto conventional LJ solid media (Himedia, Mumbai). The 0.2 ml of the suspension was used for each specimen. All processed faecal samples were inoculated on LJ medium and were incubated at 37°C. The inoculated LJ media were incubated at 37^{o}C and periodic visual scanning of LJ slopes was done weekly to observe growth on solid media for mycobacterial colonies. On detection of visible colonies on LJ slopes suggestive of *M. tuberculosis* complex were further processed by ZN staining to determine whether true positive or contaminated. Cultures were deemed a true positive for *M. tuberculosis* complex (MTBC) when it demonstrated growth on the LJ slope and revealing typical colony morphology, AFB detected on ZN staining microscopic appearance, MPT64 protein specific detection immune-chromatographic test (SD Bioline Kit, Standard Diagnostics, Inc., Korea). ZN positive culture samples were confirmed as *M. tuberculosis* complex by a m-PCR. Positive results demonstrated the presence of *Mycobacterium tuberculosis* complex organism A culture deemed to be negative if no mycobacterial growth were recorded by 8-week. LJ culture results were classified as positive, negative, or contaminated.

3.4.5.3 Detection of MTBC by MPT-64 antigen card test:

The study was performed directly on 09 samples flagged as positive by the MGIT 960 instrument. The MPT64 protein detection-based immunochomatographic test (Gaillard *et al*., 2011) (SD Bioline Kit, Standard Diagnostics Inc., Korea) antigen assay was performed on acid fast bacilli - positive MGIT cultures, as described by the manufacturer. Liquid culture (0.1 ml) was loaded onto the sample well. Solid culture isolates are prepared by scraping 2-4 colonies and suspending them in 200 liters of extraction buffer provided in the kit. The extracted solution was applied to the sample well in the same way as liquid culture. The test cassettes strips were incubated for 15-30 minutes at room temperature (RT). The pink band in the ‘C’ region confirmed the test validity. An additional pink band in the ‘T’ region was interpreted as positive for the MPT64 Ag. Only the pink band in the ‘C’ region and no band in the ‘T’ region was considered negative for the MPT 64 antigen. No band in ‘C’ region was interpreted as an invalid test (Arora *et. al.,* 2015). H37Rv was taken as a positive control for each new kit.



Results and Discussion

Results and Discussion

The present investigation aimed to study the occurrence of *Mycobacterium tuberculosis* complex (MTBC) in the faecal samples of captive zoo mammals and birds. The 114 faecal samples collected were distributed into two groups, including 84 faecal samples from captive wild mammals and 30 samples from captive wild birds.

The samples were subjected to conventional bacterial identification by Ziehl-Neelsen staining, molecular method for detection of DNA of *Mycobacterium tuberculosis* complex by multiplex-polymerase chain reaction (m-PCR) using primers of *hsp*-65 and *esat*-6 gene and subsequent culture examination using MGIT medium, Lowenstein-Jensen medium.

4.1 GENERAL OPERATING AND SOURCES PARAMETER

4.1.1 Number and information on the Zoo:

The Sanjay Gandhi Biological Park (Patna Zoo), Patna, Bihar was selected for the study and periodically visited. Patna zoo is maintained as per the guidelines of the Central Zoo Authority of India (CZA). A permission has been obtained from the competent authority to collect samples at the zoo premises.

4.1.2 Visit intervals and testing frequencies:

The Patna Zoo was visited following the curriculum. The visits were conducted with logistics planning and the presentation of the objective at the zoo.

4.1.3 Description of captive mammal and bird:

To accomplish the aim of study, 47 species of captive wild animals including 25 species of mammals and 22 species of birds that were housed at the Sanjay Gandhi Biological Park, Patna. The species and distribution of captive animals (mammals and birds) into various categories, selected during the period of study, has been presented in Tables 1, 2, 3 and 4. The details of the faecal sample collected from each species of mammal and bird have been depicted in Tables 5 and 6, respectively.

The present study encompassed 25 species of mammals across five different taxonomic orders (*Carnivora, Proboscidea, Perissodactyla, Artiodactyla,* and *Primate*)

and 22 species of birds covering ten different taxonomic orders (*Accipitriformes Anseriformes Bucerotiformes, Casuariiformes, Ciconiiformes, Columbiformes, Galliformes, Gruiformes, Passeriformes, Psittaciformes, Strigiformes* and *Struthioniformes*) enabling to examine a range of host factor such as diet type for harbouring MTBC. The captive wild animals were also studied for presence of MTBC as per their feeding habits viz. carnivorous, herbivorous and omnivorous.

4.1.4. Sampling from Captive mammal and bird:

A total of 114 faecal samples were collected from captive wild mammals and birds (Figure- 01) by non-invasive method without capturing, manipulating or putting them at health risk. The behavioural response of individual animals was also considered to prevent animal's susceptibility to human disturbance while collecting samples. One faecal specimen was collected from each animal. All the samples were transported to the Department of Veterinary Microbiology, Bihar Veterinary College, Patna, where further works were done.

The non-invasive approach to sampling is a strategy widely used by researchers in field studies for collection of biological samples and has become a major component of wildlife research (Garshelis, 2006; MacKay *et al*., 2008; Taberlet *et al*., 1999). The advantages of faecal sample-based wildlife research include easy collection, access to large sample sizes and at a reasonable cost and effort. (Biswas *et al*., 2019). Therefore, the analysis of non-invasive samples becomes a viable alternative, which offers a great deal of benefits to monitoring. It has been advocated that faecal DNA analysis can provide information about the host's gastrointestinal pathogen burden (Eriksson *et al*., 2017).

4.1.5. Processing of faecal sample:

In the present study N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method was used to digest and process faecal specimen for the recovery of acid-fast bacilli (AFB). To detect *Mycobacterium* in specimens via culture, an appropriate pre-treatment method of fluidification and decontamination is essential to eliminate competing microorganisms (Cadmus *et al*., 2011). This step is very crucial since commensal flora can prevent the growth as well as the detection of the presence of mycobacteria (Cadmus *et al*., 2011; Rivas *et al*., 2010; McClean *et al*., 2011; Kaufmann, 2003).

The N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method had been employed during the isolation of mycobacteria on solid media, liquid media and molecular detection from faecal specimens as per Kubica *et al*., (1963). Several workers have also used this method for identification of *Mycobacterium* (Sommers and Good, 1985; Vestal, 1975; Zingue *et al*., 2013). It has also been recommended as one of the most satisfactory digestion-decontamination procedures due to its rapidness and efficacy in reducing the number of contaminants and is also the most widely recommended to eliminate competing microorganisms (Cadmus *et al*., 2011)

4.1.6. Tentative identification of Mycobacterium by Ziehl-Neelsen (ZN) acid fast staining:

A microscopy (acid-fast staining) or culture are conventional methods of detecting Mycobacterium tuberculosis in the laboratory. In the present study the Ziehl-Neelsen (ZN) staining technique was used for primary screening of 114 faecal specimens for presence of acid-fast bacilli. The distributions of acid-fast bacilli (AFB) among 114 faecal samples of captive wild animals were shown in Table- 08 and 09.

Among these 114 faecal samples, 18 samples were tentatively identified AFB positive by ZN method, including 17 (14.91%) samples from captive wild mammal (Table 8; Figure 6), and 01 (0.87%) from captive wild bird (Table- 09; Figure- 07).

The AFB was identified as a dense cluster of red rod-shaped colonies, slightly curved, emerged in pairs or clumps (Figure 5). The captive wild mammals whose faecal sample were positive for AFB included bear, giraffe, lion, hippopotamus, elephant, black buck, spotted deer, zebra, rhesus monkey, Indian gaur, and sambhar deer. The Medium-sulphur - crested cockatoo only demonstrated AFB bacilli in its faecal sample. The ubiquitous Ziehl-Neelsen (ZN) stain is a primary diagnostic strategy for detection of tuberculosis worldwide that is recommended by the World Health Organization (WHO). Staining of specimen with the Ziehl-Neelsen (ZN) method may provide indications of acid-fast bacteria.

The microscopic examination is the fastest and cheapest method of visualization of acid-fast bacilli (Backues, 2008; Mikota, 2008; Miller, 2008; Lécu & Ball, 2011; Arun *et al*., 2014), and is thus the fastest, easiest, and least expensive technique (Mikota, 2008; Rishikesava *et al*., 2008). However, it does not differentiate between *Mycobacterium* species and other organisms with the same acid-fast staining characteristic (Eisenstadt and Hall, 1995).

Furthermore, the method lacks sensitivity (Wards *et al.*,1995), with a variable detection rate of 20-70% (Behr *et al*., 1999) and only detects the presence of AFB at concentrations exceeding 10^4 bacteria per millilitre (Rodriguez *et al*., 2004).

Table 8: Results of microscopic examination of faecal samples for AFB in captive wild mammals (n=84)

Sl. no.	Species	Total no. of samples	Smear positive for AFB (n)	Smear positive for AFB (%)	Smear negative for AFB	Smear negative for AFB (%)
1.	Asiatic lion	3	1	33.33	2	66.67
2.	Barking deer	1	0	0.00	1	100.00
3.	Bengal tiger	6	0	0.00	6	100.00
4.	Black buck	6	3	50.00	3	50.00
5.	Chital (Spotted deer)	4	1	25.00	3	75.00
6.	Chimpanzee	1	0	0.00	1	100.00
7.	Elephant	2	2	100.00	0	0.00
8.	Giraffe	6	1	16.67	5	83.33
9.	Himalayan Black bear	2	1	50.00	1	50.00
10.	Hippopotamus	3	2	66.67	1	33.33
11.	Hog deer	1	0	0.00	1	100.00
12.	Hyena	4	0	0.00	4	100.00
13.	Indian Gaur	2	2	100.00	0	0.00
14.	Langur (Baboon)	2	0	0.00	2	100.00
15.	Leopard	5	0	0.00	5	100.00
16.	Nilgai - Blue Bull	1	0	0.00	1	100.00
17.	One-horned Rhinoceros	9	0	0.00	9	100.00
18.	Rhesus monkey	3	1	33.33	2	66.67
19.	Sambar deer	4	1	25.00	3	75.00
20.	Sangai (Manipuri) deer	5	0	0.00	5	100.00
21.	Sloth bear	5	1	20.00	4	80.00
22.	Swamp deer (Barahsigha)	2	0	0.00	2	100.00
23.	White tiger	1	0	0.00	1	100.00
24.	Wolf	4	0	0.00	4	100.00
25.	Zebra	2	1	50.00	1	50.00
Total		**84**	**17**	**20.24**	**67**	**79.76**

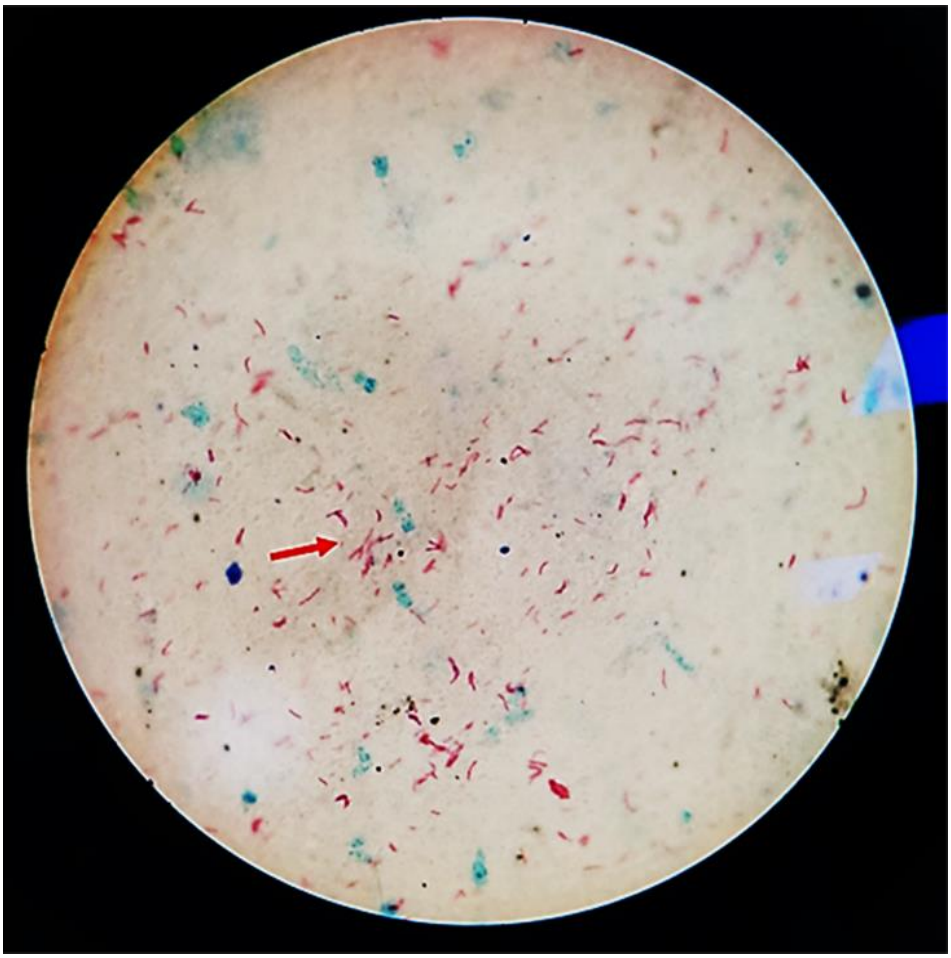


Figure 5: Microphotograph of *Mycobacterium* on an oil-immersion smear slide stained with Ziehl-Neelsen staining of a faecal sample. Long and rod-shaped bacilli indicated by a red arrow; magnification(x1000)

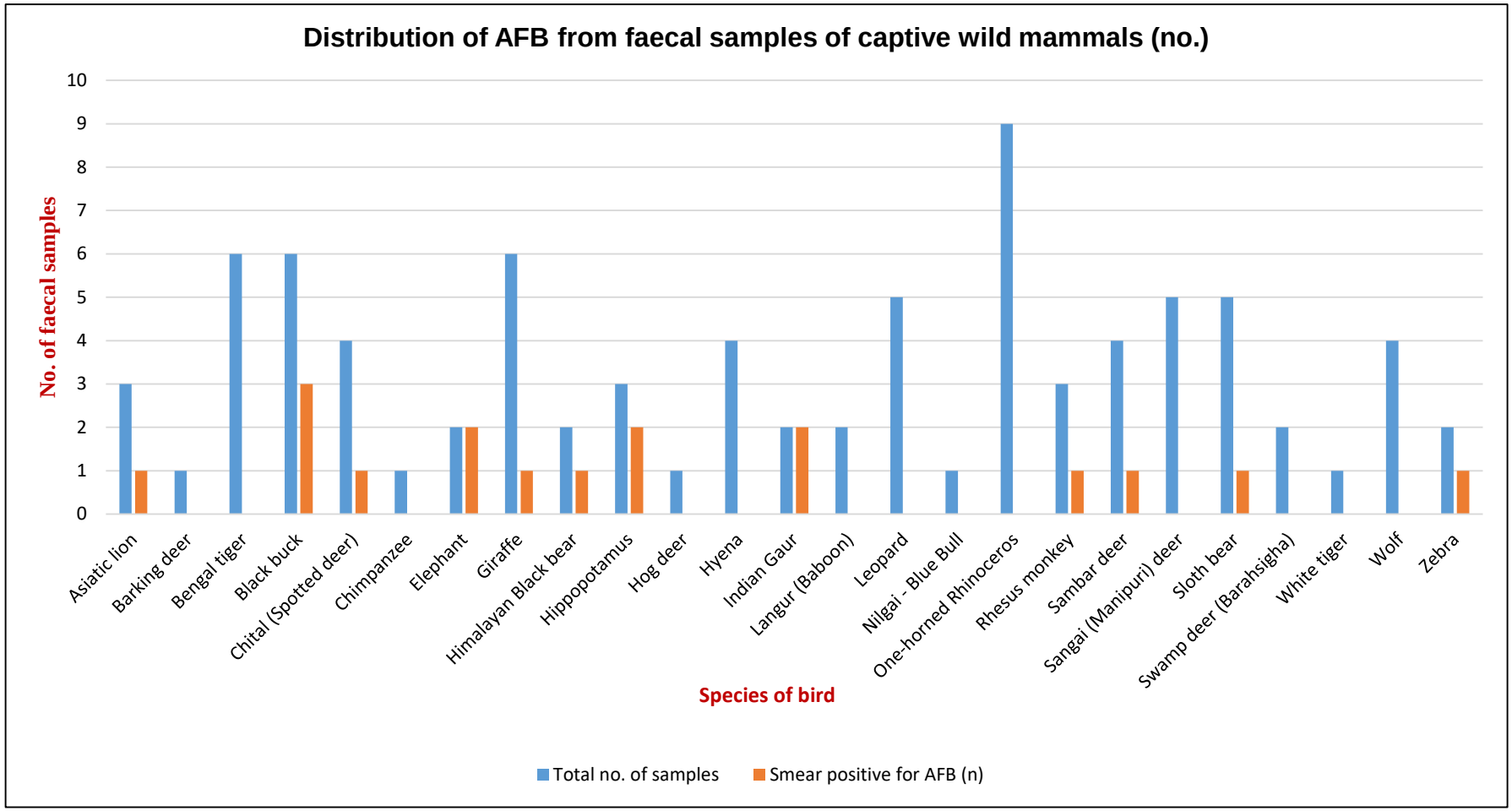


Figure 6: Bar diagram showing species wise distribution of AFB (ZN staining) in faecal sample (no.) of captive wild mammals

Table 9: Results of microscopic examination of faecal samples for AFB in captive wild birds (n=30)

Sl. no.	Species	Total no. of samples	Smear positive for AFB (n)	Smear positive for AFB (%)	Smear negative for AFB (n)	Smear negative for AFB (%)
1.	Budgerigar	1	0	0.00	1	100.00
2.	Cassowary	1	0	0.00	1	100.00
3.	Common Emerald dove	1	0	0.00	1	100.00
4.	Crane common	2	0	0.00	2	100.00
5.	Sarus crane	1	0	0.00	1	100.00
6.	Brahminy duck	1	0	0.00	1	100.00
7.	Emu	1	0	0.00	1	100.00
8.	Zebra Finch	2	0	0.00	2	100.00
9.	Golden pheasant	2	0	0.00	2	100.00
10.	Hill myna	1	0	0.00	1	100.00
11.	Hornbill	1	0	0.00	1	100.00
12.	Illiger's Macaw	1	0	0.00	1	100.00
13.	Medium sulphur crested cockatoo	1	1	100.00	0	0.00
14.	Ostrich	2	0	0.00	2	100.00
15.	Parrot	1	0	0.00	1	100.00
16.	Peafowl	3	0	0.00	3	100.00
17.	Red & Green Macaw	1	0	0.00	1	100.00
18.	Scarlet macaw	1	0	0.00	1	100.00
19.	Silver pheasant	3	0	0.00	3	100.00
20.	Kite-Pariah/Black Kite	1	0	0.00	1	100.00
21.	Lady Amherst's pheasant	1	0	0.00	1	100.00
22.	Vulture	1	0	0.00	1	100.00
Total		**30**	**1**	**3.33**	**29**	**96.67**

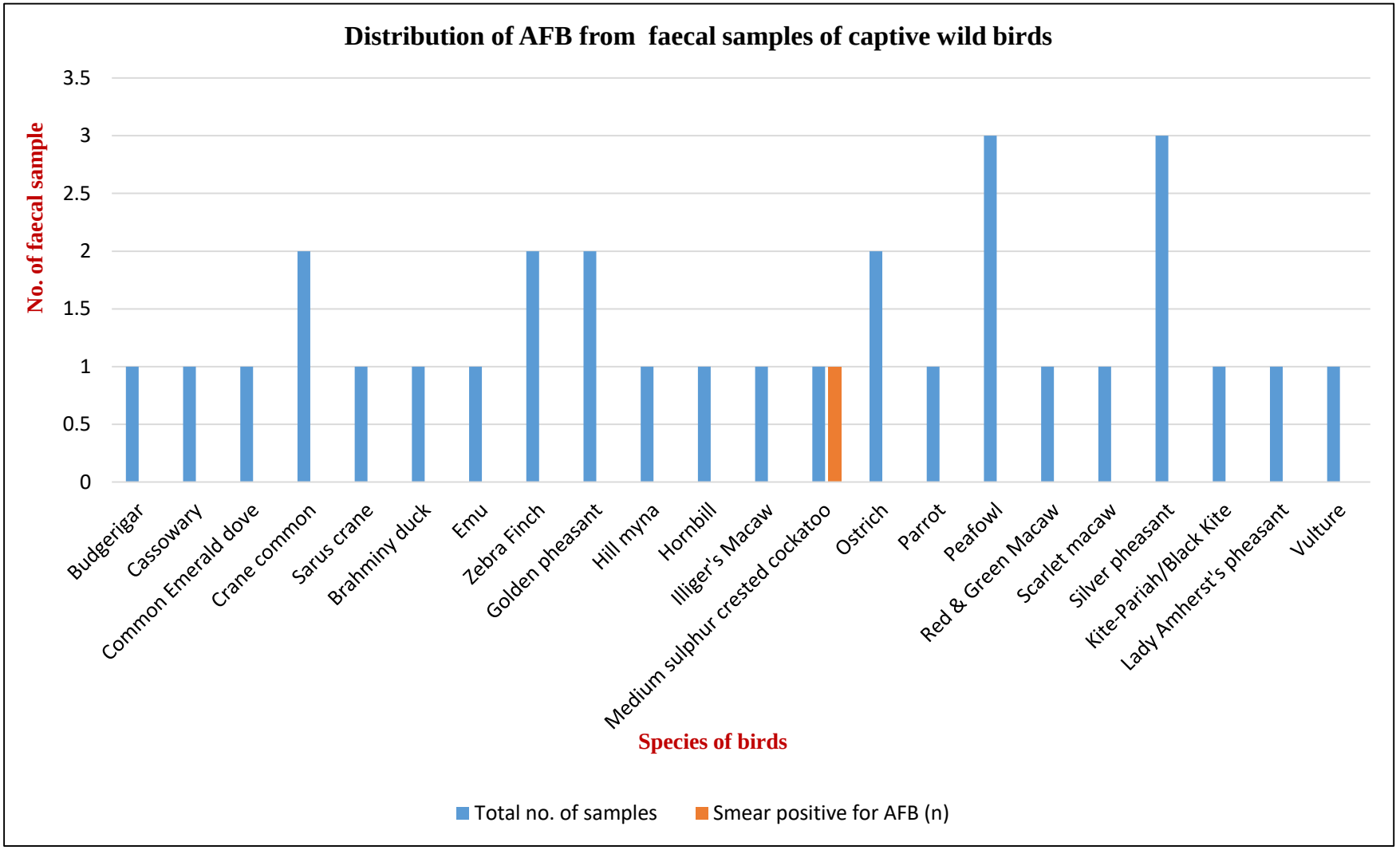


Figure 7: Bar diagram showing species wise distribution of AFB (ZN staining) in faecal sample from captive wild birds

4.1.7. Confirmatory identification of Mycobacterium tuberculosis complex by molecular method

4.1.7.1. Genomic DNA extraction:

In this study a commercial Qiagen QIAamp DNA Stool mini kit was used for extraction of DNA from faecal samples of captive wild animals. Earlier researchers have used Qiagen QIAamp DNA Stool mini kit for extraction of DNA from feacal samples and found it as relevant (Schuurman *et al*., 2007; Persson *et al*., 2011; Mirsepasi *et al*., 2014).

4.1.7.2. Optimisation m-PCR assay and detection of *Mycobacterium tuberculosis* complex species:

The purpose of PCR optimization was to produce a multiplex PCR reaction that was properly balanced and produced an equal quantity of PCR product for each set of gene.

4.1.7.2.1. Uniplex PCR:

To identify primer pairing, forward and reverse primers for *hsp*-65 and *esat*-6 gene individually amplified in Uniplex PCR assay. While determining a successful primer pairing, strong versus weak bands generated determined whether primers were working efficiently and could later be combined into a multiplex reaction. The successful amplification was determined on visualization of band size on gel. Both the gene segments *hsp*-65 (441 *bp*) and *esat*-6 gene (320 *bp*) amplified by PCR (Figure 8).

4.1.7.2.2. Multiplex PCR:

Following optimisation, the DNA extracted from mycobacterial strains was used to standardise the multiplex reactions for detection and identification of the *Mycobacterium tuberculosis* complex. Multiplex PCR was optimized and performed using a pair of primers containing an *hsp*-65 gene (441*bp*) fragment to identify mycobacterial genus and *esat*-6 gene (320*bp*) fragment (Table-7) for identification of MTBC species. MTBC was considered positive in the specimens that had both fragments of 441 *bp* and 320 *bp* amplified the corresponding targets precisely (Figure 9,10 and 11). Each set of specimens was subjected to a negative reagent blank and a positive control reaction in parallel. The results which gave the correct negative and positive control (H37Rv) in each set were used for analysis.

The selection of genetic markers for detecting MTBC included two gene sequences for genus identification and common gene possessed by different species of MTBC. The *hsp*65 gene, which codes for a 65 KDa protein, is found in all mycobacterial species and has epitopes that are both unique and shared by different mycobacterial species. (Deepa *et al*., 2005; Plikaytis *et al*., 1992; Wu *et al*., 2008; Shinnick *et al*., 1988; Aravindhan *et al*., 2007). In addition, *esat*-6 gene clusters are found in the genomes of mycobacteria (Gey Van Pittius *et al*., 2001).

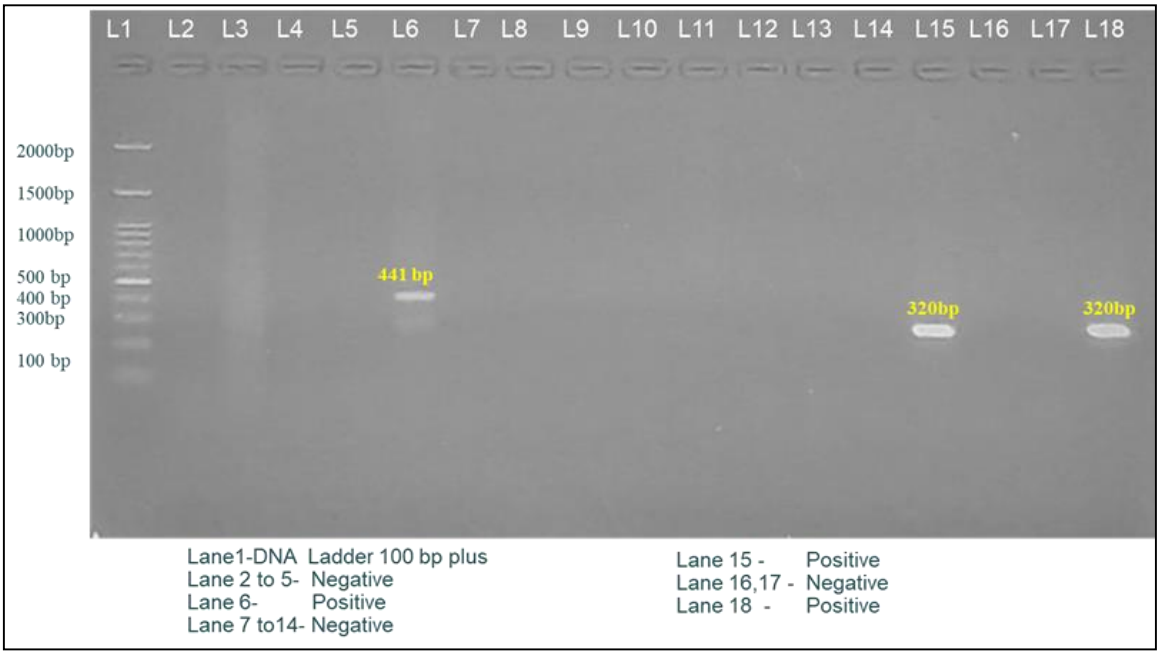


Figure 8: Optimisation of Uniplex PCR assay for *hsp*-65 and *esat*-6 gene

Multiplex PCR assay of DNA from MTBC species:

The inspection of Table 10 and 11 revealed that direct m-PCR on 114 faecal specimen-derived DNA yielded positive results in 10 (8.77%) samples, displaying simultaneous amplification of the two targets *hsp*-65 (441*bp*), and *esat*-6 (320*bp*) (Figure 12 and 13). None of the 30 faecal specimens from captive wild birds has been found positive for m-PCR assay. Mycobacterium tuberculosis complex (MTBC) species are, however, frequently found in mammals (Nicolet, 1985; Carter and Wise, 2004), which corroborates the present observation. *Mycobacterium tuberculosis* complex detection in faecal samples could be improved by nucleic acid amplification. These assays use *Mycobacterium tuberculosis* complex-specific primers that are unaffected by non-*Mycobacterium tuberculosis* flora present in faeces.

Additionally, due to its efficiency and accuracy, the m-PCR-based method employed in the current study to identify MTBC offers an advantage over traditional bacteriological examination. Furthermore, conventional technique suffers from significant limitations, such as the low sensitivity of acid-fast bacillus detection by microscopy and the slow growth of tubercle bacilli in culture media (Lewinsohn *et al*., 2017). Furthermore, it is crucial to investigate other diagnoses when initial acid-fast-bacillus (AFB) smears and cultures result in negative results and to perform essential investigations.

Nucleic acid screening tests have gained acceptance in the last years, lowering turnaround times and enhancing sensitivity to overcome the drawbacks of traditional techniques. (Piersimoni and Scarparo, 2003; Alcaide and Coll., 2011; Walzl *et al*., 2018).

The findings of this study demonstrated that the m-PCR technique is quick and sensitive in amplifying MTBC specific sequences. Moreover**,** nucleic acid amplification employed in current study is a culture-independent technique for the rapid detection and quantification of MTBC in faecal sample based upon unique genetic markers. It is therefore possible to significantly reduce the delay associated with bacterial culture by using m-PCR to detect MTBC, specifically as an "early warning" regarding the risk of MTBC.

The low number of MTBC diagnosed in this study by m-PCR might be due to the less template DNA in faecal samples or likely to be contaminated by presence of presence of non-target DNA. (Taberlet *et al*., 1996; Henry *et al*., 2011). Moreover, it is speculated that inhibitory substances present in faecal samples might have hindered the extraction of DNA. According to reports, it is nonetheless difficult to isolate DNA from faecal samples because PCR-inhibiting organic compounds hinder the recovery of DNA (Scarparo *et al*., 2000; Pontiroli *et al*., 2011, Gioffre *et al*., 2004) and thus significantly affect the sensitivity of PCR. The current investigation is substantiated by these findings. Furthermore, the few numbers of positive samples obtained in this investigation could be attributable to low viability of *Mycobacterium tuberculosis* complex bacteria during transition in the gut, which require further investigation. Additionally, the low number of MTBC in faecal specimens may also be due to the decontamination process by NaLC/NaOH that might have denatured the DNA. These findings underscore the importance of proper sample preparation (Mtafya *et al*., 2019).

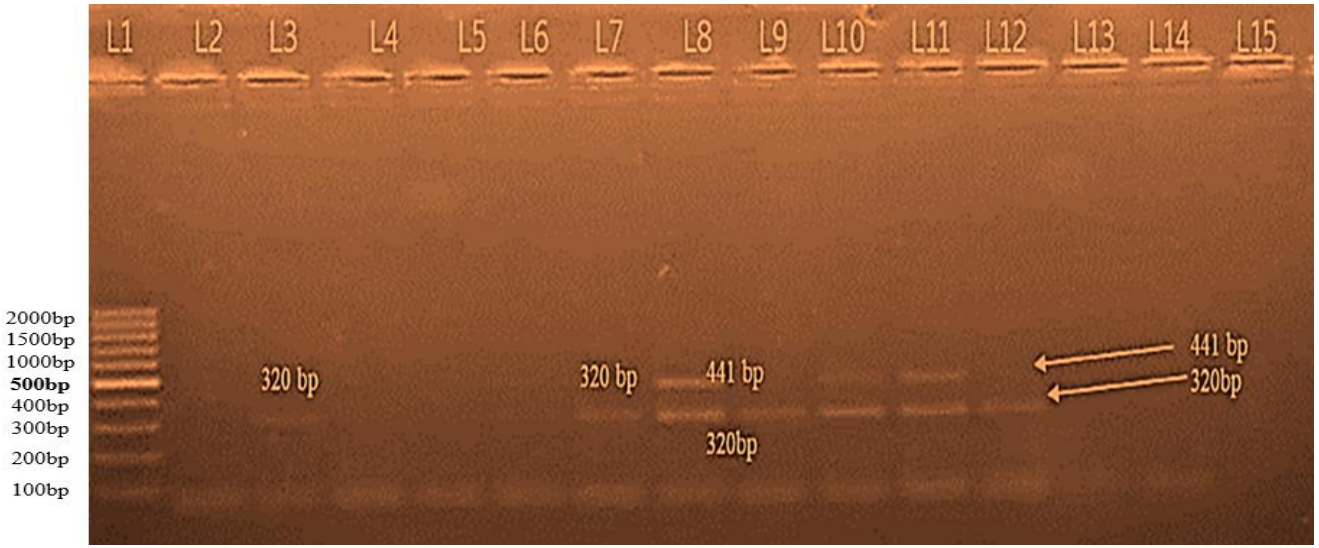


Figure 9: The results of standardization of '*Mycobacterium tuberculosis* complex' (MTBC) in faecal samples of captive wild animals by Uniplex and Multiplex PCR. Band sizes are indicated next to the marker in base pairs

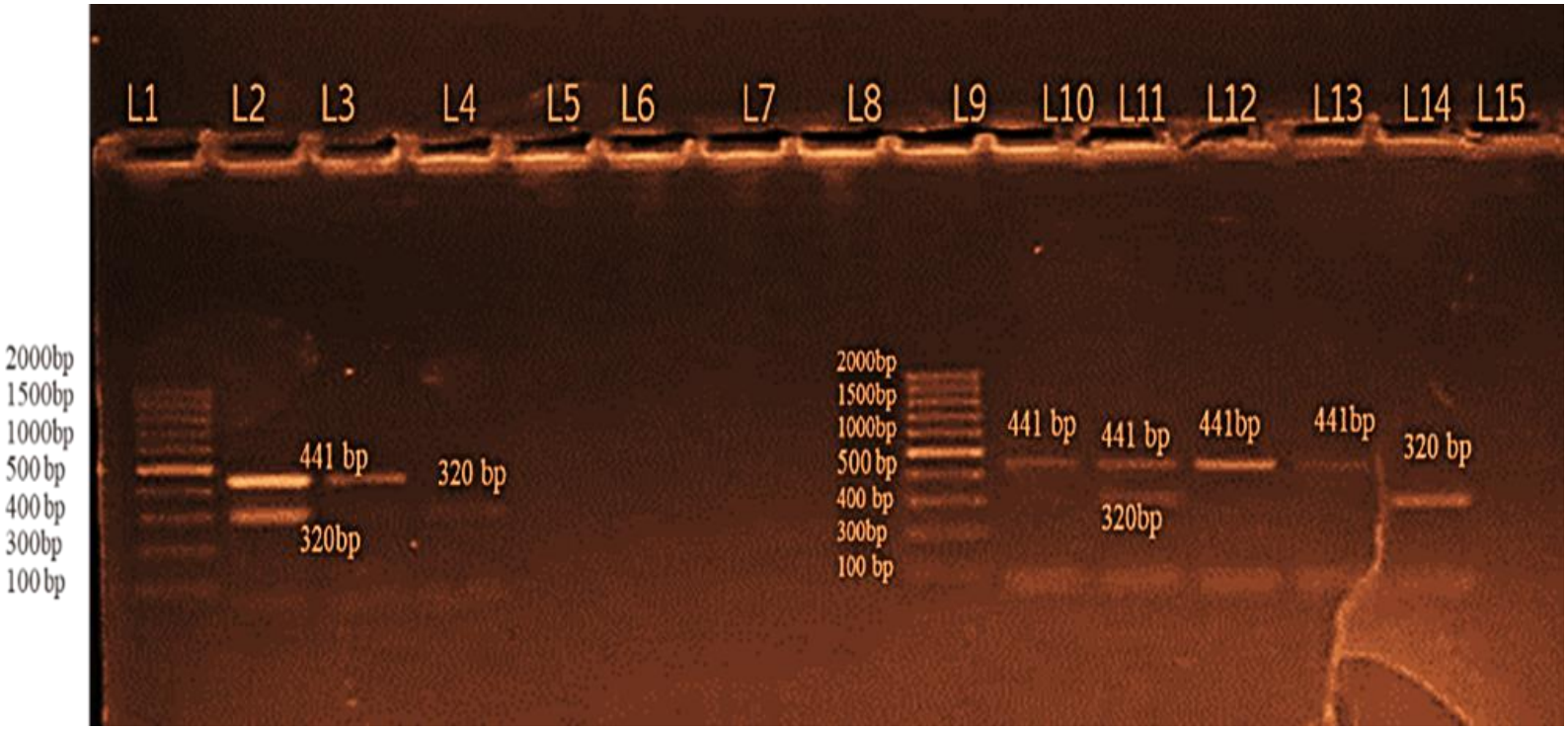


Figure 10: The results of Uniplex and Multiplex PCR assay applied for standardization of hsp-65 gene and esat-6 gene primers from DNA of faecal samples of captive wild animals. Base pair is indicated next to each band size.
Lane1- DNA Ladder (100 *bp* plus).

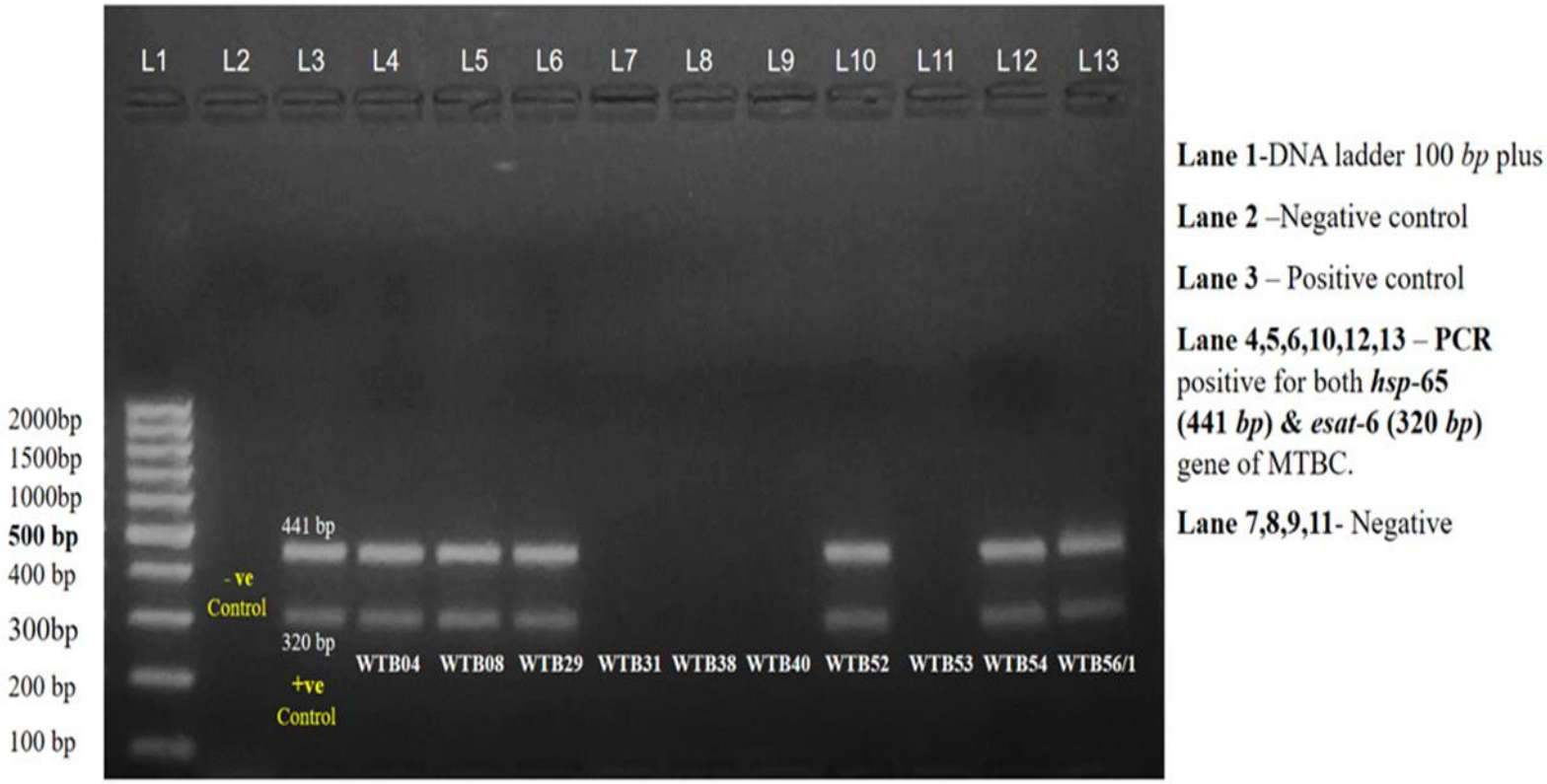


Figure 11.1: The results of Multiplex PCR assay for identification of *hsp*-65 gene and *esat*-6 gene of DNA of faecal samples from captive wild animals

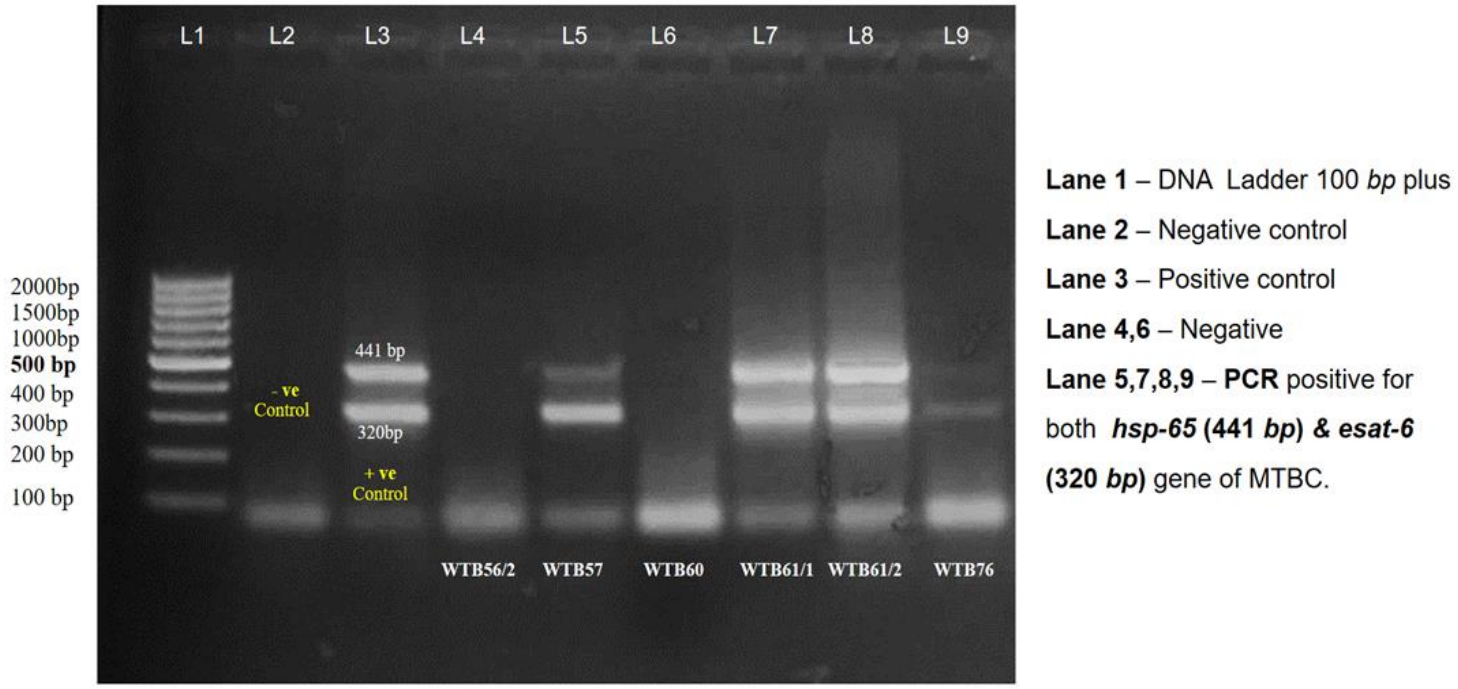


Figure 11.2: The results of Multiplex PCR assay for identification of *hsp*-65 gene and *esat*-6 gene of DNA of faecal samples from captive wild animals

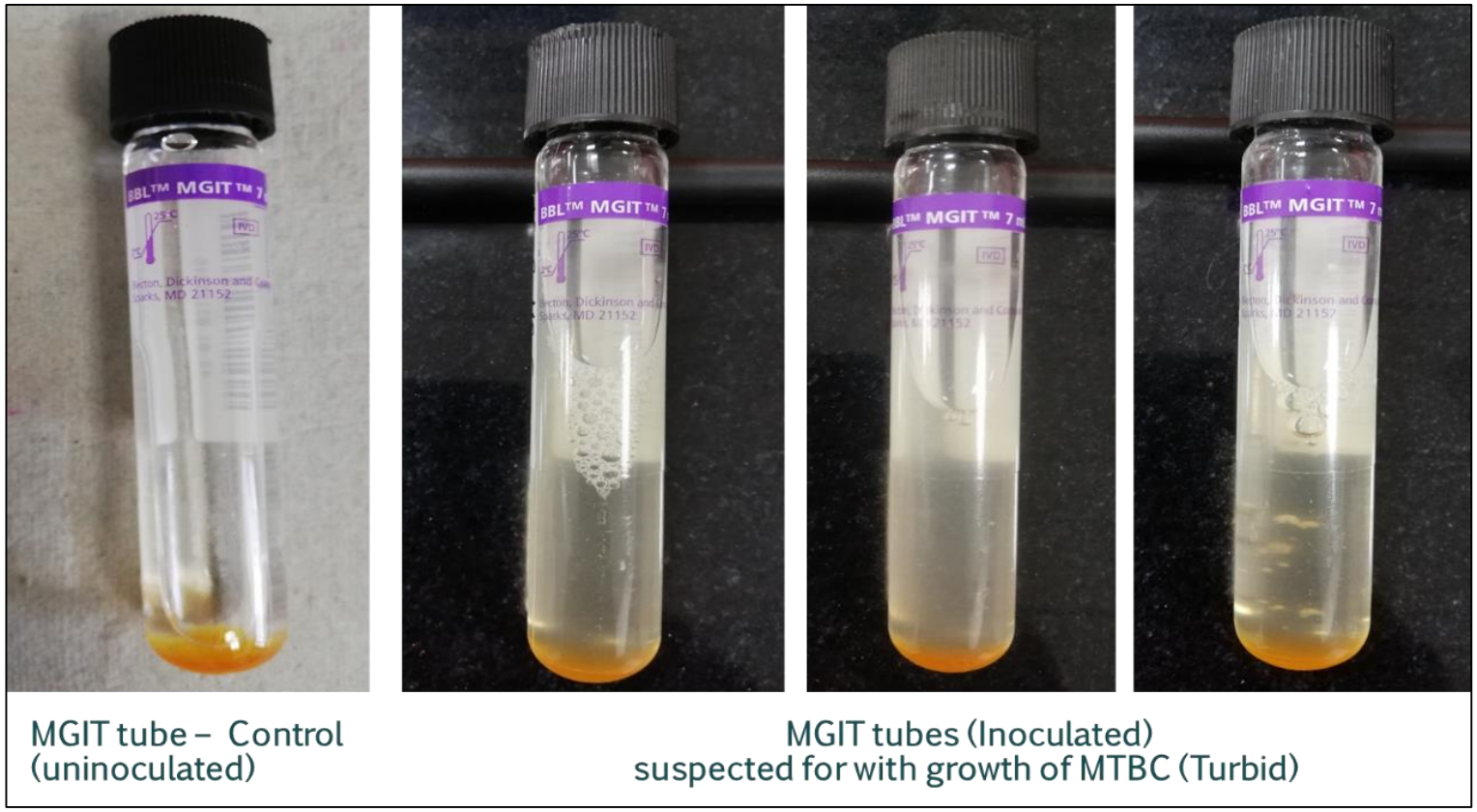


Figure 12: Suspected growth of MTBC in MGIT tubes

Figure 13: Inspection for growth of MTBC in MGIT tube

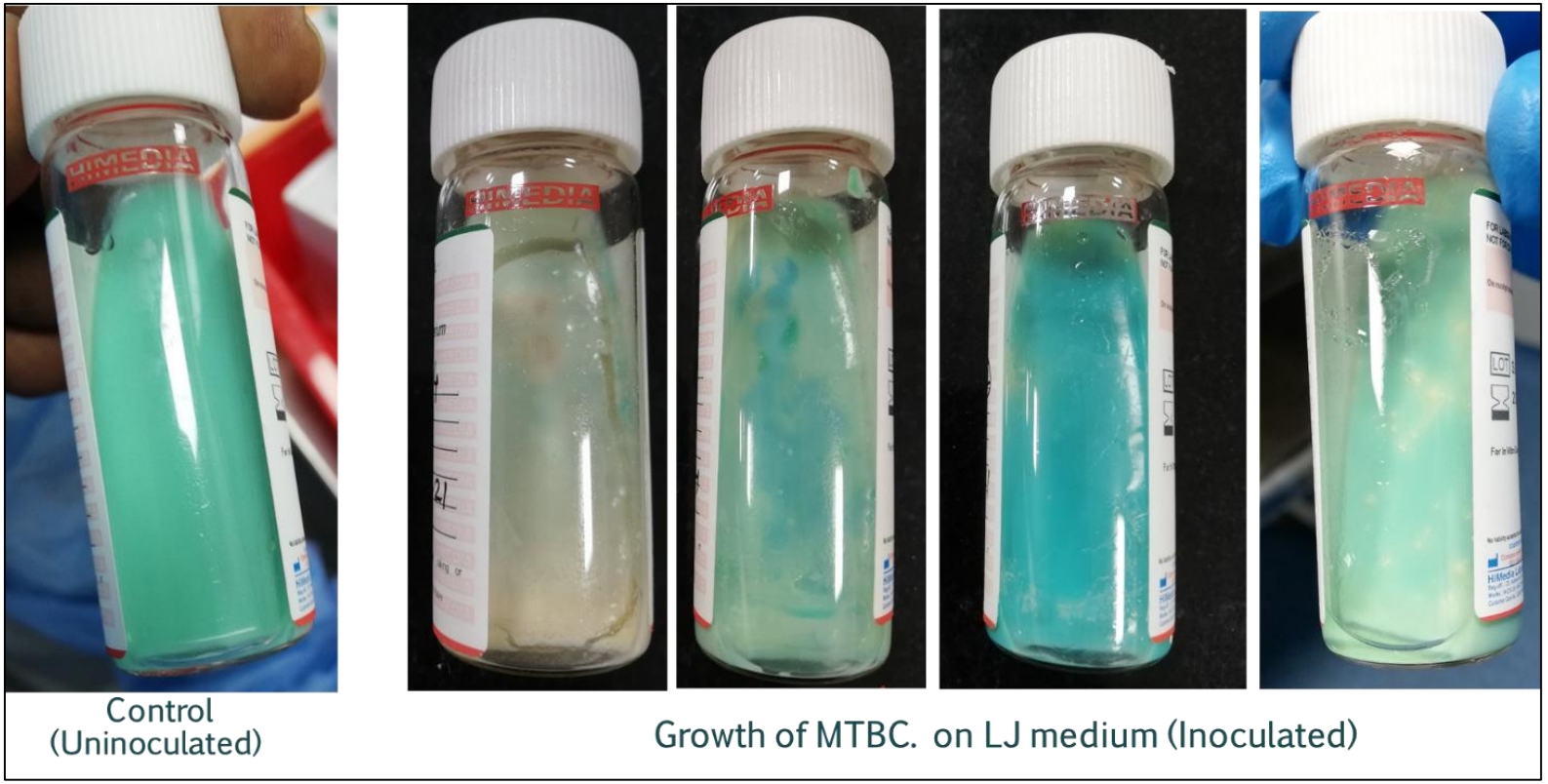


Figure 14: Growth of m-PCR positive MTBC on Lowenstein-Jensen (LJ) Medium

4.1.8. Comparison of m-PCR confirmed *Mycobacterium tuberculosis* complex species by conventional culture of faecal sample from captive wild animals:

To confirm the presence of *Mycobacterium tuberculosis* complex (MTBC) in faecal samples from captive wild animals that had been tested positive by m-PCR, two culture methods were used in the current investigation. The liquid system was carried out in BACTEC MGIT 960 system (Becton, Dickinson and Company) and Lowenstein -Jensen medium was used as conventional solid medium for growth of bacteria (Chien *et al*., 2000). A total of ten m-PCR positive samples were subjected to MGIT liquid culture and Lowenstein-Jensen (L-J) medium for cross confirmation. A positive culture was confirmed by the presence of acid-fast bacilli upon Ziehl-Neelsen (ZN) staining.

Table - 10 displays a summary of results for detection of MTBC detected in MGIT and LJ medium. At the end of 6 weeks of incubation, MTBC had been detected in broth from all specimens positive for m-PCR (Figure 14 and 15). Similar growth was observed in Lowentein-Jensen medium (LJ) after eight weeks of incubation (Figure 16). The result of this study is in agreement with those of other studies (Hanna *et al*., 1999; Idigoras *et al*., 2000; Cruciani *et al*., 2004).

The perusal of Table-10; Figure 18, showed that there was no difference in the m-PCR positive MTBC recovered from either the BACTEC system or a solid medium, LJ. Corresponding sensitivity and yield were observed when m-PCR and culture positive were compared. No samples from captive wild birds maintained in captivity had any positive m-PCR assay results (Table 11), hence no cultures were performed on those samples. The culture method is still regarded as the "Gold Standard" for mycobacterium detection (Hermes *et al*., 2018).

Since MTBC grows more quickly on liquid media, it is recommended to use liquid media for faster isolation (CLSI, 2008) and to meet turnaround time objectives for laboratories. Furthermore, MGIT 460 offers the advantages of being easy to use, having a faster detection time, higher capacity, and providing continuously monitored analysis of clinical samples for mycobacteria, which are all very important considerations when selecting a microbiological technique. According to several studies, the MGIT 960 system is a standard method for detecting mycobacteria in cultures (Alcaide *et al*., 2000, Cruciani *et al*., 2004, Hanna *et al*., 1999). Since its introduction, the MGIT 960 system has been widely deployed for the recovery of mycobacteria (Badak *et al*., 1996; Tortoli *et al*., 1999; Idigoras *et al*., 2000; Whyte *et al*., 2000; Somoskovi *et al*., 2000; Lu *et al*., 2002; Lee *et al*., 2003; Ogwang *et al*., 2015).

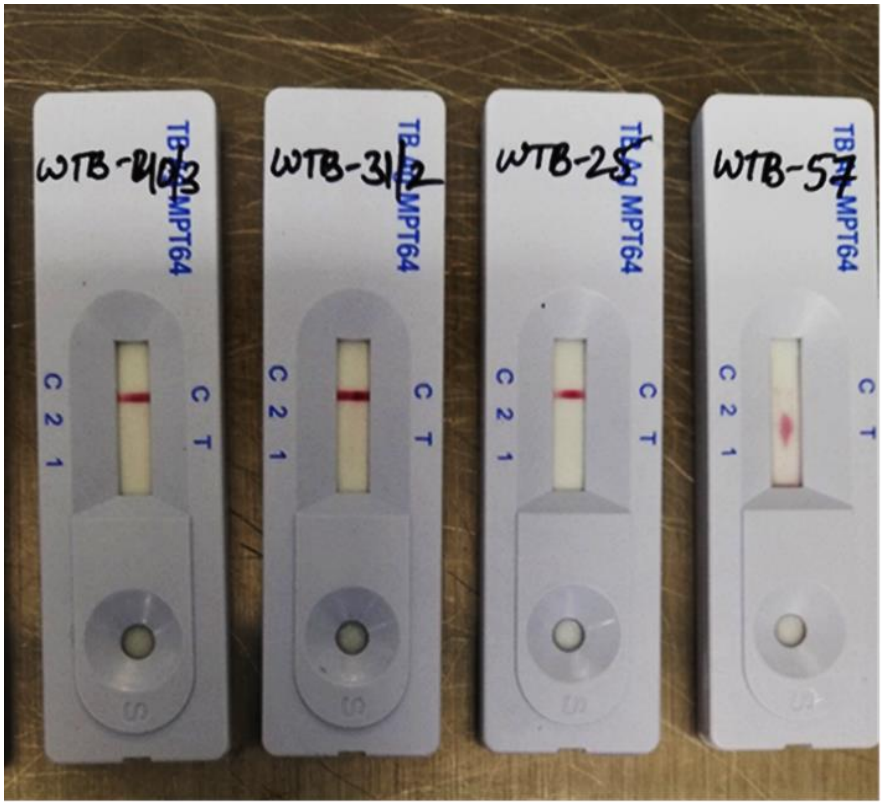
The findings of growth of MTBC in LJ medium is depicted in Table 10. All the ten m-PCR positive faecal samples have grown on LJ medium. Solid media are still important in the recovery of mycobacteria from extra pulmonary specimens, regardless of the advantages and higher sensitivity of the MGIT 960 cultivation system. Hillemann *et al*., (2006) reported that solid media are known to perform significantly better in the recovery of mycobacteria from extra pulmonary specimens (Hillemann *et al*., 2006).

4.1.9. Comparison of m-PCR confirmed *Mycobacterium tuberculosis* complex species of faecal sample from captive wild animals by MPT64 card test:

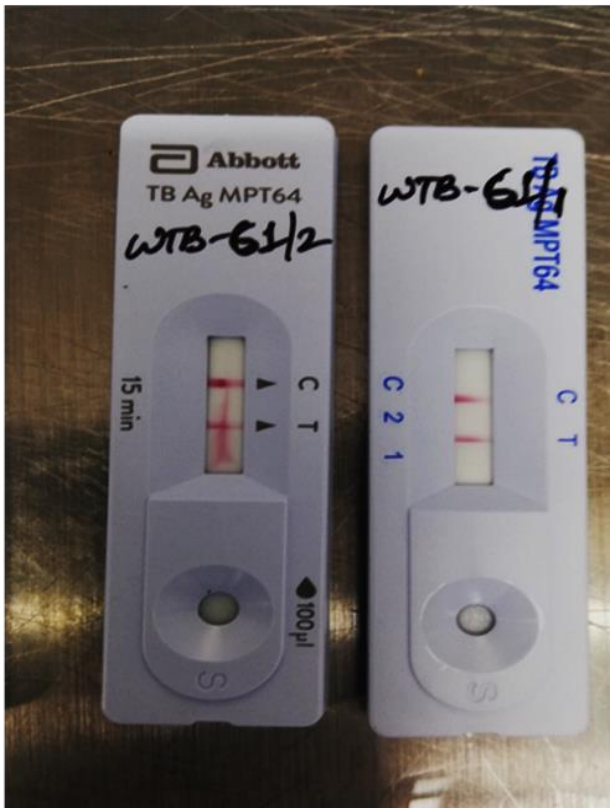
For MTBC detection, the MPT64 card test performance was evaluated. In the current study, a total of 10 acid-fast bacilli-positive liquid cultures that have been positive for m-PCR were evaluated. The control band was seen in all tested cultures, validating the test. The presence of the MPT 64 antigen was confirmed by the H37Rv control, which revealed the appearance of a pink band in the test region (T band). The result of test is depicted in Table-10. The faecal sample of Indian Gaur only displayed positive result for MTBC among all studied animals (Figure 17).

Only strains of the *M. tuberculosis* complex are known to have the MPT64 antigen, which has potential for use as a diagnostic tool. The MPT64 test is recommended as a reliable and efficient method for distinguishing between non-tuberculous Mycobacterium (NTM) and tuberculous bacteria since the MPT64 protein is specific to species of *Mycobacterium tuberculosis* complex (MTBC) and absent from non-tuberculous Mycobacterium (NTM) (Harboe *et al*., 1986; Mahairas *et al*., 1996; Elhay *et al*., 1998; Abe *et al.*, 1999).

The overall sensitivity of MPT-64 test performed in this investigation was 22.22% as compared to 67–100% in previous studies (Baba *et al*., 2008; Tadele *et al*., 2014; Jorstad *et al*., 2018). The MPT64 test performance was lower in the present study compared to previous studies. This could partly be explained by different procedures for culture used across the previous studies. Secondly, assuming that the cultured samples used for testing might have lower mycobacterial load and were therefore not detected by the MPT64 test.



Negative



Positive

Figure 15: MPT64 card test Identification of the *Mycobacterium tuberculosis* complex by the MPT64 kit. Left – negative, Right - strong positive

4.1.9.1. Taxonomic order wise occurrence of MTBC in faecal samples of captive wild mammals:

The data obtained by m-PCR and culture were further analysed for taxonomic order-wise occurrence of MTBC among captive wild mammals that are depicted in Table 11 and Figure 19. The perusal of table shows that faecal samples of order *Proboscidea* (50.00%) had the highest occurrence of MTBC in its faecal samples followed by *Artiodactyla* (20.00%) and *Carnivora* (3.33%). No MTBC has been detected among animals of order *Perissodactyla* and *Primate*. Elephant, the only members of order *Proboscidea* has been taken for investigation showed positive for MTBC in faecal sample. Several literatures evidence that elephant are reported positive for MTBC or its members. Yakubu (2015) has reported the prevalence of members of MTBC in captive Asian elephants. Recently members of the MTBC have been found in a captive Asian elephant (*Elephas maximus*) and free-ranging African savanna elephant (*Loxodonta africana*) (Miller *et al*., 2021; Suga *et al*., 2021). The members of MTBC have also been reported in elephants from India (Chandranaik *et al*., 2017). There are reports of members MTBC prevalence ranging from 0 to 23.33 percent among elephant populations (Rajbhandari *et al*., 2022), which is lesser than the value obtained in this study. It can be argued that the faecal samples were used as candidate in this study in contrast to other study which had been studied on tissue samples. Due to a variety of factors involved, the likelihood of identifying bacteria in faeces samples is lower than that of tissue samples. Additionally, due to the small number of samples included in this investigation, the overall significance of the results could not be established.

MTBC or its member has been reported in animals under *Artiodactyla*, in deer (Kaneene *et al*., 2002; Gowtage-Sequeira *et al*., 2009; Nugent *et al*., 2015; Hota *et al*., 2020), including Hippopotamus (Kerr *et al*., 2022; Kanyala *et al*., 2022), Giraffe (Krajewska-Wędzina *et al*., 2018; Hlokwe *et al*., 2019; Faraja *et al*., 2021). Although this study showed no MTBC in the faeces of *Perissodactyla*, including one-horned rhinoceros, other researchers have reported the presence of MTBC in rhinoceros. (Dwyer *et al*., 2022; Dwyer *et al*., 2020; Goosen *et al*., 2022). Notwithstanding, MTBC has been documented in prior investigations, the present investigation demonstrated that faecal samples of mammals in the taxonomic order *Primate* were negative (Wilbur *et al*., 2012; Holmberg *et al*., 1982; Matz-Rensing *et al*., 2015). This study showed that the order *Carnivora* did not contained MTBC in their faeces, as determined by m-PCR tests and cultural examinations.

Table 10: Comparison of the PCR and culture for detection of MTBC in faecal sample of various species of captive wild mammals (n=84)

Sl. no.	Carnivore	Species wise number of samples	No. of samples positive for AFB	No. of sample positive by PCR	No. of sample positive by MIGT	No. of sample positive by LJ medium	No. of sample positive by MPT64 card
1.	Bengal Tiger	6	0	0	0	0	0
2.	White Tiger	1	0	0	0	0	0
3.	Asiatic Lion	3	1	0	0	0	0
4.	Leopard	5	0	0	0	0	0
5.	Hyena	4	0	0	0	0	0
6.	Wolf	4	0	0	0	0	0
7.	Barking deer	1	0	0	0	0	0
8.	Black Buck	6	3	1	1	1	0
9.	Spotted deer	4	1	0	0	0	0
10.	Elephant	2	2	1	1	1	0
11.	Giraffe	6	1	1	1	1	0
12.	Hippopotamus	3	2	2	2	2	0
13.	Hog deer	1	0	0	0	0	0
14.	Indian Gaur	2	2	2	2	2	2
15.	Nilgai - Blue Bull	1	0	0	0	0	0
16.	One horned Rhinoceros	9	0	0	0	0	0
17.	Sambar deer	4	1	1	1	1	0
18.	Sangai (Manipuri) deer	5	0	0	0	0	0
19.	Swamp deer	2	0	0	0	0	0
20.	Zebra	2	1	0	0	0	0
21.	Chimpanzee	1	0	0	0	0	0
22.	Langur	2	0	0	0	0	0
23.	Rhesus Monkey	3	1	0	0	0	0
24.	Sloth bear	5	1	1	1	1	0
25.	Himalayan black bear	2	1	1	1	1	0
	Total	**84**	**17**	**10**	**10**	**10**	**2**

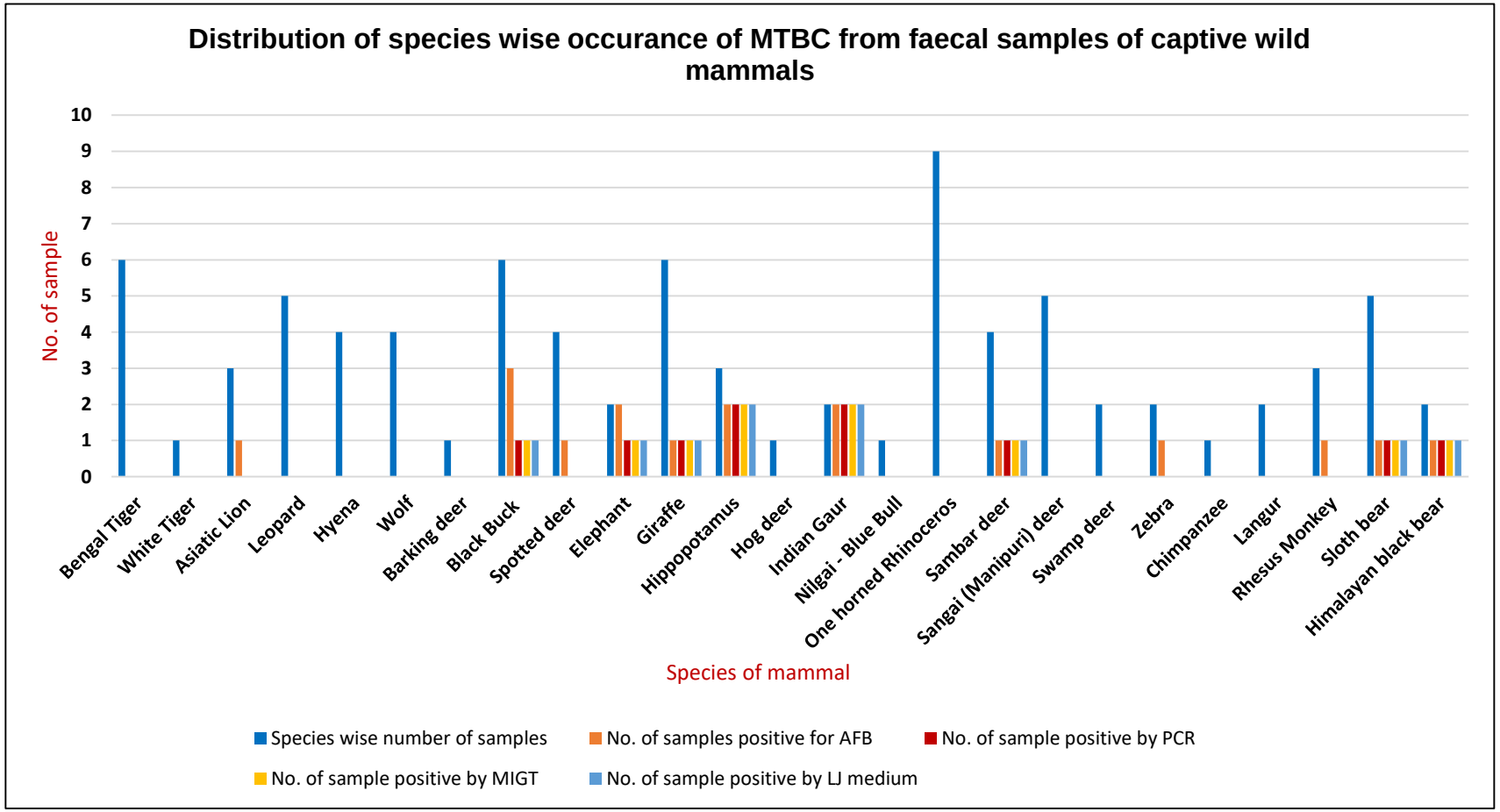


Figure 16: Bar diagram showing species wise occurrence of MTBC from faecal samples of captive wild mammal

Table 11: Results of PCR and culture of faecal sample of various order of captive wild mammals (n=84)

Sl. no.	Order of mammal	Species wise number of samples	No. of samples positive for AFB	No. of sample positive by PCR	No. of sample positive by MIGT	No. of sample positive by LJ medium
1.	*Carnivora*	30	2 (3.33%)	2 (3.33%)	2 (3.33%)	2 (3.33%)
2.	*Proboscidea*	2	2 (100.00%)	1 (50.00%)	1 (50.00%)	1 (50.00%)
3.	*Perissodactyla*	11	1 (9.09%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
4.	*Artiodactyla*	35	11 (31.43%)	7 (20.00%)	7 (20.00%)	7 (20.00%)
5.	*Primate*	6	1 (16.67%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
	Total	**84**	**17 (20.24%)**	**10 (11.90%)**	**10 (11.90%)**	**10 (11.90%)**

***Figure in parenthesis indicate percentage (%)**

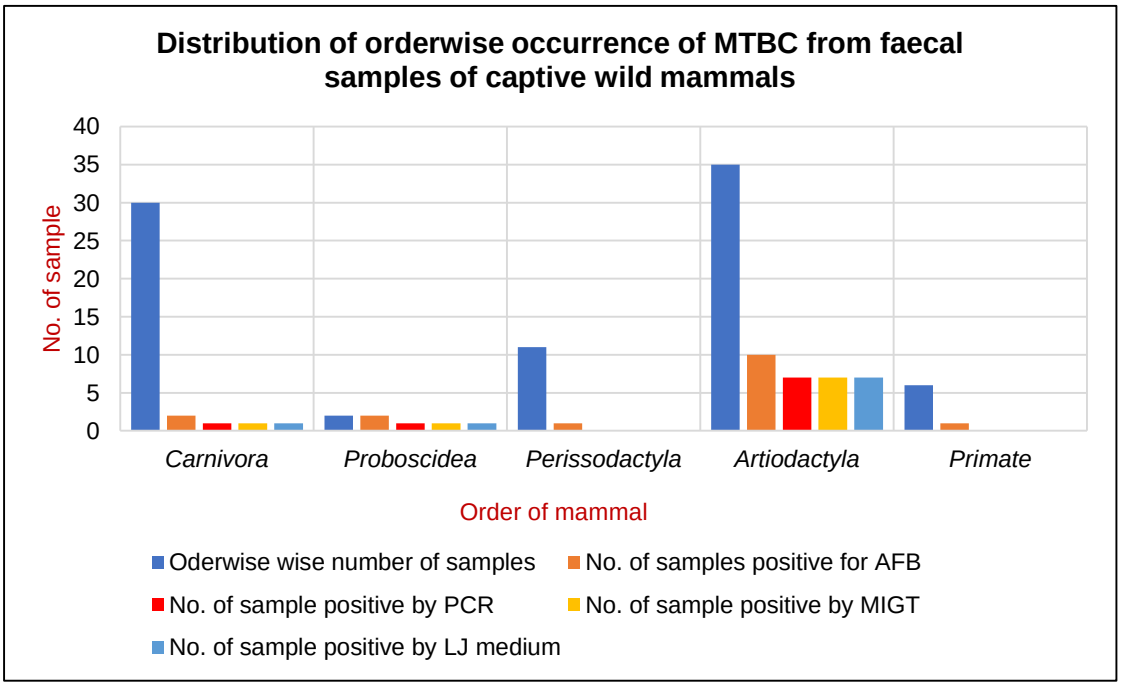


Figure 17: Bar diagram showing order wise occurrence of MTBC from faecal samples of captive wild mammals

4.1.9.2. Occurrence of MTBC in faecal samples of captive wild mammals based on feeding habits:

A further analysis of the species-specific data obtained for MTBC occurrence among captive wild mammals was conducted according to their feeding habits. The Tables 12, 13, and 14 shows the analysis of data linked to the occurrence of MTBC in captive wild mammals correlated with feeding habits.

All faecal samples from carnivore mammals were negative for MTBC bacteria (Table 12; Figure 20). Further perusal of data (Table 13; Figure 21) showed that 08 (16.67%) faecal samples from herbivorous mammal were detected positive for MTBC bacteria by m-PCR and 02 (15.38%) omnivores carried MTBC bacteria in their faeces (Table 14; Figure 22). The overall occurrence of MTBC bacteria among mammals was higher in herbivores followed by omnivores. Although, no literature has been found regarding occurrence of MTBC in faecal samples of wild mammals, however, the MTBC has been reported in herbivores (Asian elephant) in Malaysia (Ong *et al*., 2013; Yakubu *et al*., 2016). In the present study no faecal samples of carnivorous animals were detected for presence of MTBC (Table 12). It is documented that carnivores are resistant to Mycobacterial infection; however, it has been reported in wild carnivores like lion, tigers, leopards, caracals and bears (Das and Jayarao, 1986; Arora, 1994). Moreover, *Mycobacterium bovis*, a member of MTBC had been reported in leopards as well as other felids (Thorel *et al*., 1998), lions (Keet *et al*., 1996), tiger (*Panthera tigris*) (Lumeij *et al*., 1987). Moreover, some workers have recorded wild animal species encountered by MTBC bacteria include badgers, coyotes, raccoons, hares, rabbits, hedgehogs, capybaras, lions, elk, wild boars, foxes, primates, and grey seals (Matos *et al*., 2014; Gortazar *et al*., 2012; Barnett *et al*., 2013). All the reports are contradictory to findings of present study.

It has been documented by several workers that infected animals might excrete members of MTBC in faeces (Little *et al*., 1982; Phillips *et al*., 2003; Palmer *et al*., 2004; Payne, 2014). The MTBC detected in current study might have been excreted in faeces of animal under study.

In our study the MTBC has been confirmed by m-PCR, culture and MPT64 test in herbivorous mammals of Sanjay Gandhi Biological Park, Patna. It is speculated that herbivorous animals ingest relatively more vegetable feed, which might be contaminated with mycobacteria, or their surface is contaminated with soil containing mycobacteria and

the ingestion of water and feed contaminated with MTBC bacteria and successful survival in gastrointestinal tract of captive wild herbivores may be the reason for presence of MTBC bacteria as reported in present study. Several workers have documented similar results (Grange, 2001; Smith *et al*., 2004; Aboubaker *et al*., 2016). It has been hypothesised that the mucosal layer of the gastrointestinal (GI) tract may be infected with the *Mycobacterium* spp. (Debi *et al*., 2014; Rasheed *et al*., 2007). Most mycobacterial pathogens are excreted via faeces due to gastrointestinal infections (Torrea *et al*., 2005).

The perusal of Table 14 revealed that the faecal sample of Himalayan black bear (Omnivore) has found positive for MTBC. Veeraselvam *et al*., (2008) have detected *Mycobacterium bovis* in fresh faecal samples of sloth bear by PCR.

Table 12: Results of PCR and culture of faecal sample of captive wild carnivorous mammals (n=23)

Sl. no.	Carnivore	Species wise number of samples	No. of samples positive for AFB	No. of sample positive by PCR	No. of sample positive by MIGT	No. of sample positive by LJ medium
1.	Bengal Tiger	6	0	0	0	0
2.	White Tiger	1	0	0	0	0
3.	Asiatic Lion	3	1	0	0	0
4.	Leopard	5	0	0	0	0
5.	Hyena	4	0	0	0	0
6.	Wolf	4	0	0	0	0
	Total	**23**	**1**	**0**	**0**	**0**

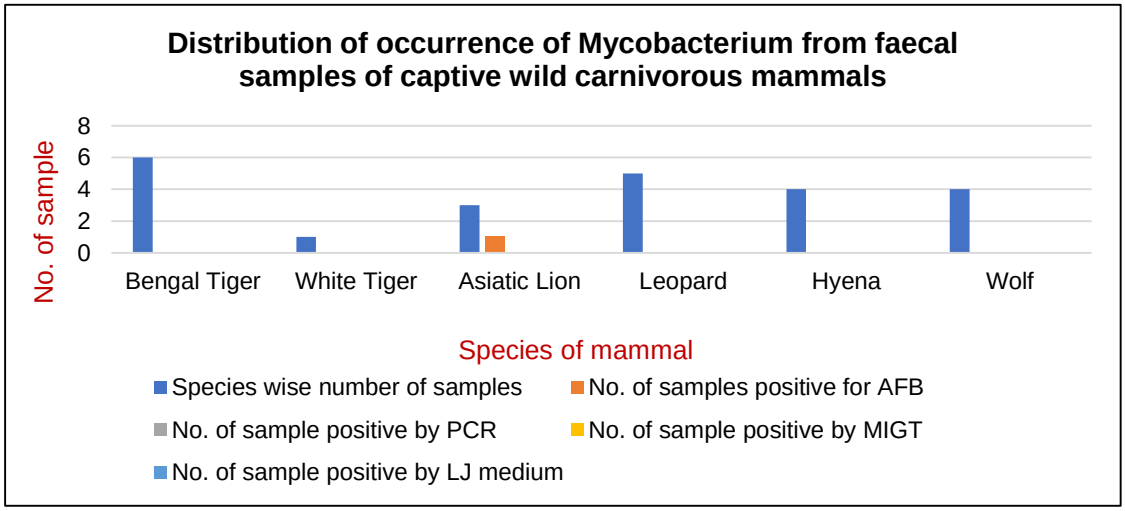


Figure 18: Occurrence of *Mycobacterium* from faecal samples of captive wild carnivorous mammals

Table 13: Results of PCR and culture of faecal sample of captive wild herbivorous animals (n=48)

Herbivore	Species wise number of samples	No. of sample positive for AFB	No. of sample positive by PCR	No. of sample positive by MIGT	No. of sample positive by LJ medium
Barking deer	1	0	0	0	0
Black Buck	6	3	1	1	1
Spotted deer	4	1	0	0	0
Elephant	2	2	1	1	1
Giraffe	6	1	1	1	1
Hippopotamus	3	2	2	2	2
Hog deer	1	0	0	0	0
Indian Gaur	2	2	2	2	2
Nilgai - Blue Bull	1	0	0	0	0
One horned Rhinoceros	9	0	0	0	0
Sambar deer	4	1	1	1	1
Sangai (Manipuri) deer	5	0	0	0	0
Swamp deer	2	0	0	0	0
Zebra	2	1	0	0	0
	48	**13 (27.08%)**	**8 (16.67%)**	**8 (16.67%)**	**8 (16.67%)**

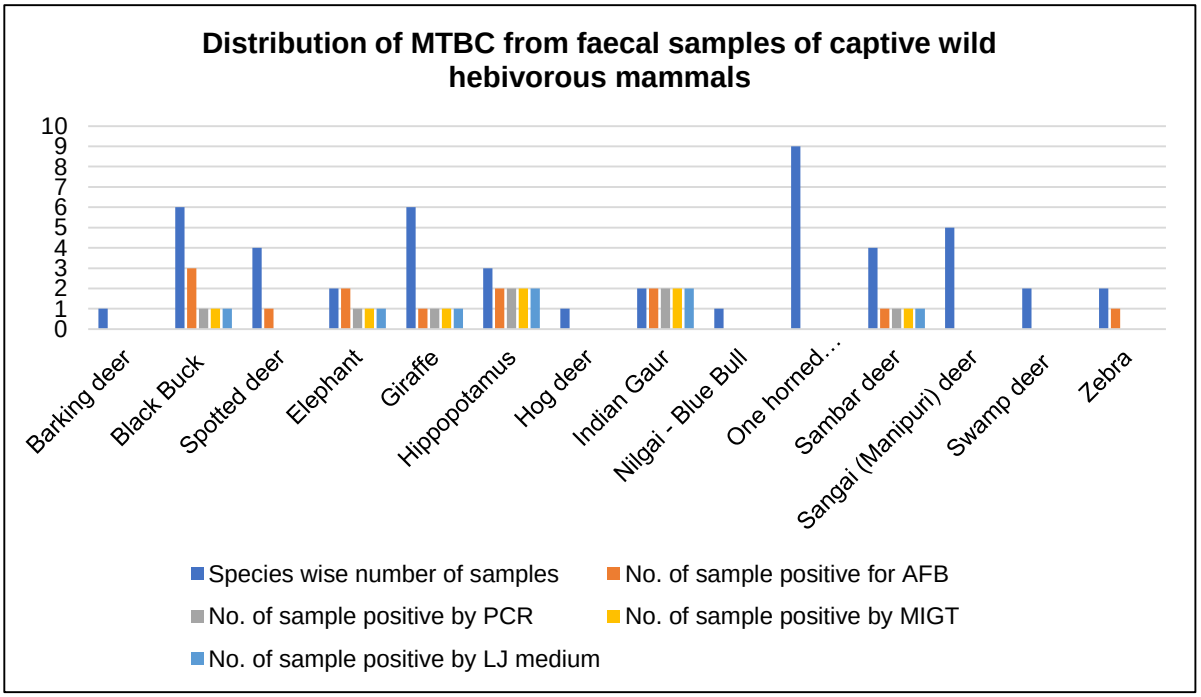


Figure 19: Occurrence of MTBC from faecal samples of captive wild herbivorous mammals

Table 14: Results of PCR and culture of faecal sample of captive wild omnivorous animals (n=13)

Omnivore	Species wise number of samples	No. of sample positive for AFB	No. of sample positive by PCR	No. of sample positive in MIGT culture	No. of sample positive in LJ medium
Chimpanzee	1	0	0	0	0
Langur	2	0	0	0	0
Rhesus Monkey	3	1	0	0	0
Sloth bear	5	1	1	1	1
Himalayan black bear	2	1	1	1	1
	13	**3 (23.07%)**	**2 (15.38%)**	**2 (15.38%)**	**2 (15.38%)**

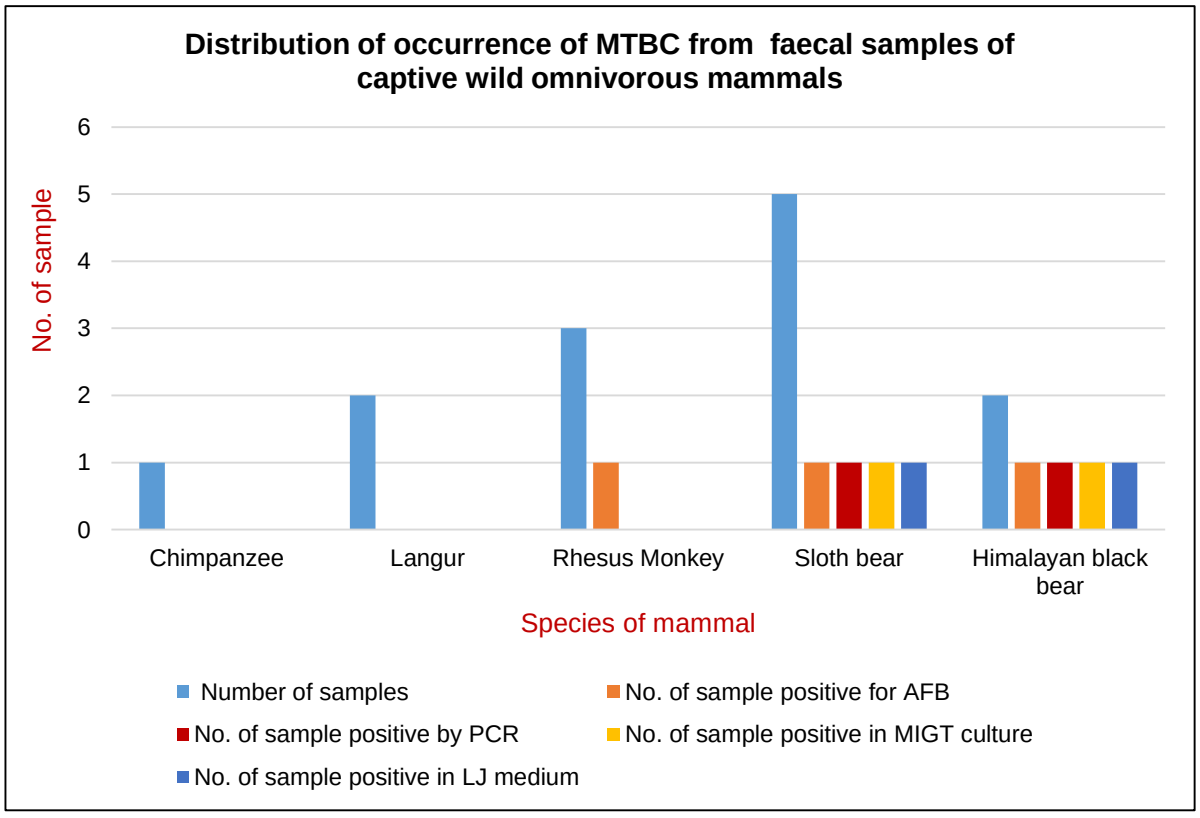


Figure 20: Bar diagram showing occurrence of MTBC from faecal samples of captive wild omnivorous mammals

Table 15: Results of PCR and culture of faecal sample of captive wild birds (n=30)

Sl. no.	Species	Total no. of samples	No. of sample positive by ZN staining	No. of sample positive by PCR	No. of sample positive by MIGT	No. of sample positive by LJ medium
1.	Budgerigar	1	0	0	0	0
2.	Cassowary	1	0	0	0	0
3.	Common Emerald dove	1	0	0	0	0
4.	Crane	2	0	0	0	0
5.	Sarus crane	1	0	0	0	0
6.	Brahminy duck	1	0	0	0	0
7.	Emu	1	0	0	0	0
8.	Finch Zebra	2	0	0	0	0
9.	Golden pheasant	2	0	0	0	0
10.	Hill myna	1	0	0	0	0
11.	Hornbill	1	0	0	0	0
12.	Illiger's Macaw	1	0	0	0	0
13.	Medium sulphur crested cockatoo	1	1	0	0	0
14.	Ostrich	2	0	0	0	0
15.	Parrot	1	0	0	0	0
16.	Peafowl	3	0	0	0	0
17.	Red & Green Macaw	1	0	0	0	0
18.	Scarlet macaw	1	0	0	0	0
19.	Silver pheasant	3	0	0	0	0
20.	Kite-Pariah/Black Kite	1	0	0	0	0
21.	Lady Amherst's pheasant	1	0	0	0	0
22.	Vulture	1	0	0	0	0
Total		**30**	**1**	**0**	**0**	**0**

4.1.10. Occurrence of *Mycobacterium tuberculosis* complex (MTBC) species by conventional culture of faecal sample from captive wild birds:

The results of the MTBC in faecal samples from captive wild birds tested by m-PCR, MGIT, and LJ medium are shown in Table 15. The analysis of the data reveals that all 30 faecal samples from captive wild birds tested negative for the m-PCR assay, despite the faecal sample of one species, the Medium Sulphur Crested Cockatoo, showing positive results for ZN staining. Further, none of the faecal samples grown by any of the two culture methods employed throughout this experiment. The MPT64 test was not performed since there was no positive culture.

The incidence of MTBC varies by species, age, living conditions, and whether or not they are captive and is less well understood in the wild and in captivity. Few researchers have recorded 0.5% to 14% of MTBC cases identified during bird necropsies (Gerhold and Fischer, 2005; Smit *et al*., 1987; Leite *et al*., 1998). However, there is no documentation regarding the presence of MTBC in wild birds. Nevertheless, it is believed that at least 1% of wild birds have mycobacterium incidence. According to various researchers, mycobacterial infections affect captive wild birds more severely than do those found in the wild (Friend and Franson, 1999). The results of the current investigation are consistent with previous observations.

4.1.10.1. Taxonomic order wise occurrence of MTBC in faecal samples of captive wild mammals:

Table-16 & Figure-17 shows the taxonomic orderwise distribution of MTBC in captive wild birds. Analyzing the table demonstrates that *Accipitriformes, Anseriformes, Bucerotiformes, Casuariiformes, Galliformes, Gruiformes, Passeriformes,* and *Struthioniformes* were among the 10 taxonomic orders of wild birds studied for occurrence of MTBC showed positive results in any of the methods except one *Passeriformes* member that was found positive for acid fast bacilli by ZN staining.

4.1.10.2. Occurrence of MTBC in faecal samples of captive wild birds based on feeding habits:

The results of MTBC in faecal samples from captive wild carnivorous and omnivorous birds are shown in Tables 17 and 18, respectively. Herbivorous birds were

excluded from the study. Tables were analyzed, and it was apparent that no captive wild bird faecal samples, regardless of dietary habits, contained MTBC.

Table 16: Results of PCR and culture of faecal sample of various order of captive wild birds (n=30)

Sl. no.	Order of birds	Species wise number of samples	No. of samples positive for AFB	No. of sample positive by PCR	No. of sample positive by MIGT	No. of sample positive by LJ medium
1.	*Accipitriformes*	2	1	0	0	0
2.	*Anseriformes*	1	0	0	0	0
3.	*Bucerotiformes*	1	0	0	0	0
4.	*Casuariiformes*	2	0	0	0	0
5.	*Columbiformes*	1	0	0	0	0
6.	*Galliformes*	9	0	0	0	0
7.	*Gruiformes*	3	0	0	0	0
8.	*Passeriformes*	3	0	0	0	0
9.	*Passeriformes*	6	1	0	0	0
10.	*Struthioniformes*	2	0	0	0	0
	Total	**30**	**2**	**0**	**0**	**0**

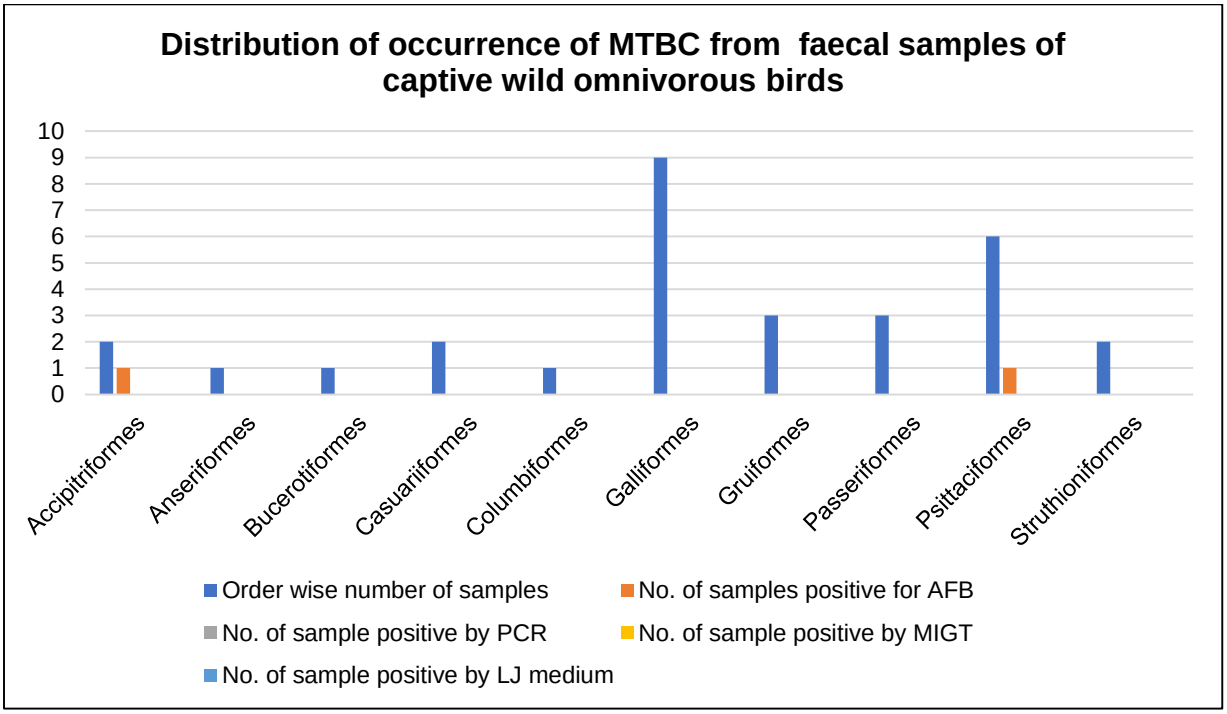


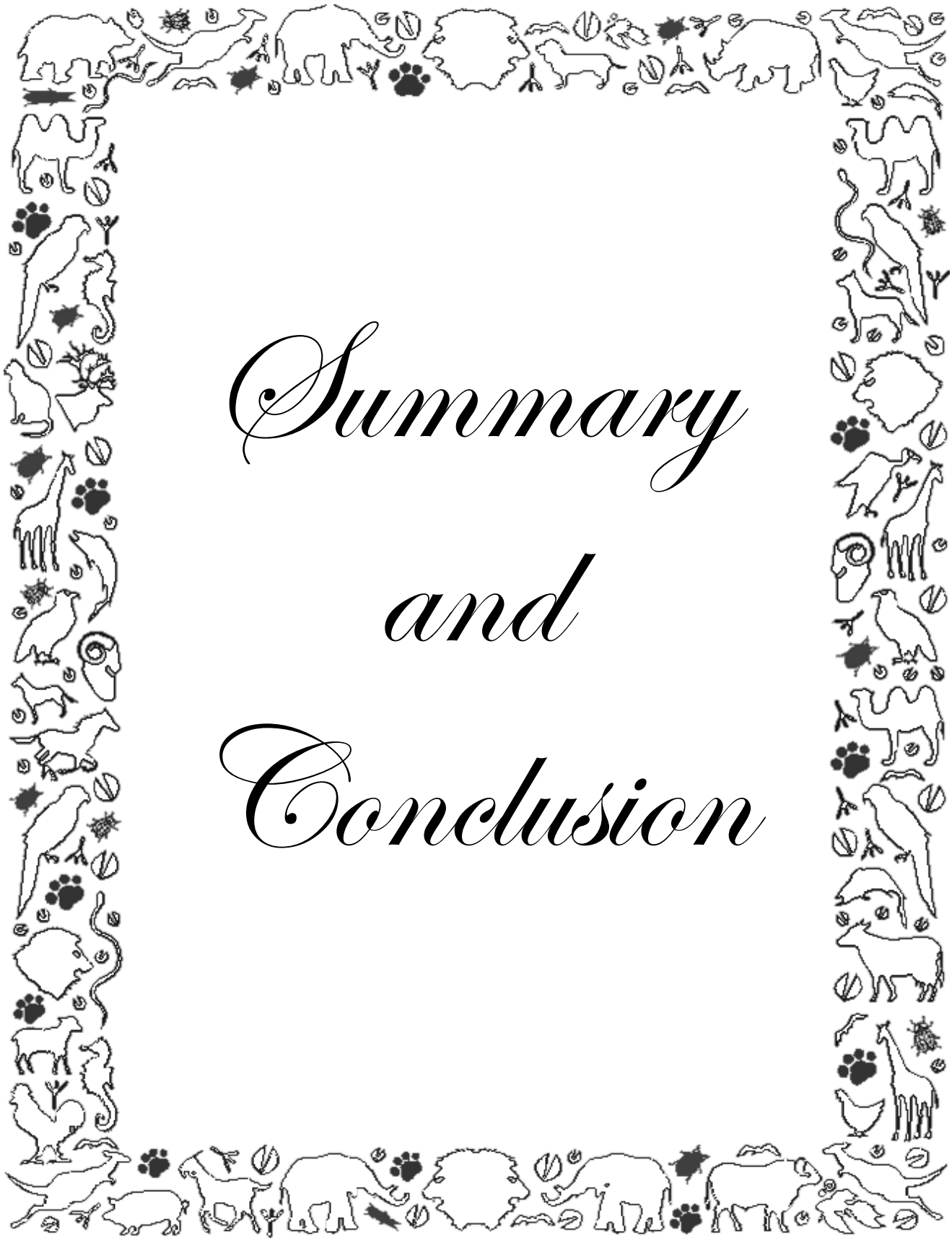
Figure 21: Order wise occurrence of MTBC from faecal samples of captive wild birds

Table 17: Results of PCR and culture of faecal sample of captive wild carnivorous birds (n=01)

Sl. no.	Species	Species wise number of samples	No. of samples positive for AFB	No. of sample positive by PCR	No. of sample positive by MIGT	No. of sample positive by LJ medium
1.	Kite-Pariah/Black Kite	1	1	0	0	0

Table 18: Results of PCR and culture of faecal sample of captive wild omnivorous birds (n=29)

SL. no.	Species	Species wise number of samples	No. of samples positive for AFB	No. of sample positive by PCR	No. of sample positive by MIGT	No. of sample positive by LJ medium
1.	Budgerigar	1	0	0	0	0
2.	Cassowary	1	0	0	0	0
3.	Common Emerald dove	1	0	0	0	0
4.	Crane	2	0	0	0	0
5.	Sarus crane	1	0	0	0	0
6.	Brahminy duck	1	0	0	0	0
7.	Emu	1	0	0	0	0
8.	Finch Zebra	2	0	0	0	0
9.	Golden pheasant	2	0	0	0	0
10.	Hill myna	1	0	0	0	0
11.	Hornbill	1	0	0	0	0
12.	Illiger's Macaw	1	0	0	0	0
13.	Medium sulphur crested cockatoo	1	1	0	0	0
14.	Ostrich	2	0	0	0	0
15.	Parrot	1	0	0	0	0
16.	Peafowl	3	0	0	0	0
17.	Red & Green Macaw	1	0	0	0	0
18.	Scarlet macaw	1	0	0	0	0
19.	Silver pheasant	3	0	0	0	0
20.	Lady Amherst's pheasant	1	0	0	0	0
21.	Vulture	1	0	0	0	0
Total		**29**	**1**	**0**	**0**	**0**



Summary and Conclusion

Chapter 5

Summary and conclusion

The current study aimed to determine the occurrence of *Mycobacterium tuberculosis* complex (MTBC) in faecal samples from captive zoo mammals and birds.

To accomplish the objective, 48 species of captive wild animals were selected, including 25 species of mammals and 23 species of birds housed at the Sanjay Gandhi Biological Park in Patna. A total of 114 faecal samples including 84 faecal samples from captive wild mammals and 30 samples from captive wild birds by non-invasive method without capturing or manipulating them. In order to examine the effects of host taxonomy on faecal MTBC occurrence, this study explicitly set out to investigate the MTBC of various species at large taxonomic scales (*i.e*., among all study species) and minor taxonomic scales (*i.e*., among taxonomic order related species) and host dietary guilds.

The N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method was used in the present study to process samples for the recovery of acid-fast bacilli (AFB), culture and extraction of bacterial DNA. The samples were subjected to conventional bacterial identification via Ziehl-Neelsen staining, culture examination through use of MGIT medium, Lowenstein-Jensen medium, and a molecular method for detecting DNA of *Mycobacterium tuberculosis* complex via multiplex polymerase chain reaction (m-PCR) using primers from the *hsp*-65 and *esat*-6 genes.

The AFB was identified as a dense cluster of red rod-shaped colonies that appeared in pairs or clumps, slightly curved and isolated against a blue background. Bear, Giraffe, Lion, Hippopotamus, Elephant, Black buck, Spotted deer, Zebra, Rhesus Monkey, Indian Gaur, Sambar deer, and Medium-sulphur - crested cockatoo were among the captive wild animals whose faecal samples tested positive for AFB.

Qiagen QIAamp DNA Stool mini kit was used in this study to extract DNA from faecal samples of captive wild animals.

The m-PCR assay was used to detect Mycobacterium tuberculosis complex species. Forward and reverse primers for the *hsp*-65 and *esat*-6 genes were individually amplified in a singleplex PCR assay to identify primer pairing. The DNA extracted

from mycobacterial strains was further standardised for the multiplex reactions with a pair of primers containing a *hsp*-65 gene (441*bp*) fragment to identify mycobacterial genus and an *esat*-6 gene (320*bp*) fragment to identify *Mycobacterium tuberculosis* complex (MTBC) species.

Direct m-PCR on 114 faecal specimen-derived DNA yielded positive results in 10 (8.77%) samples, demonstrating simultaneous amplification of the two targets *hsp*-65 (441*bp*) and *esat*-6 (320*bp*). None of the 30 faecal specimens collected from captive wild birds tested positive for the m-PCR assay.

The comparison of the findings of m-PCR, MGIT, and LJ medium revealed that the sensitivity and yield of m-PCR positive MTBC recovered from either the BACTEC system or a solid medium (LJ) were comparable and equivalent. After 6 weeks of incubation, MTBC had been observed in the broth of all specimens that had tested positive for m-PCR. In Lowentein-Jensen (LJ) media, identical growth had been seen after eight weeks of incubation.

For MTBC detection, the MPT64 card test performance was assessed. The current investigation studied on ten liquid cultures that had positive m-PCR results and were positive for acid-fast bacilli. The overall sensitivity of the MPT64 test was 22.22%.

To further comprehend the frequency of MTBC in faecal samples, the dataset of five mammalian orders (*Proboscidea*, *Artiodactyla*, *Carnivora*, *Perissodactyla*, and *Primates* was examined. The results of the m-PCR and culture assays showed that the frequency of MTBC was highest in taxonomic order *Proboscidea* (50.00%), followed by *Artiodactyla* (20.00%), and *Carnivora* (3.33%). *Perissodactyla* and *Primates* have not been found to have MTBC.

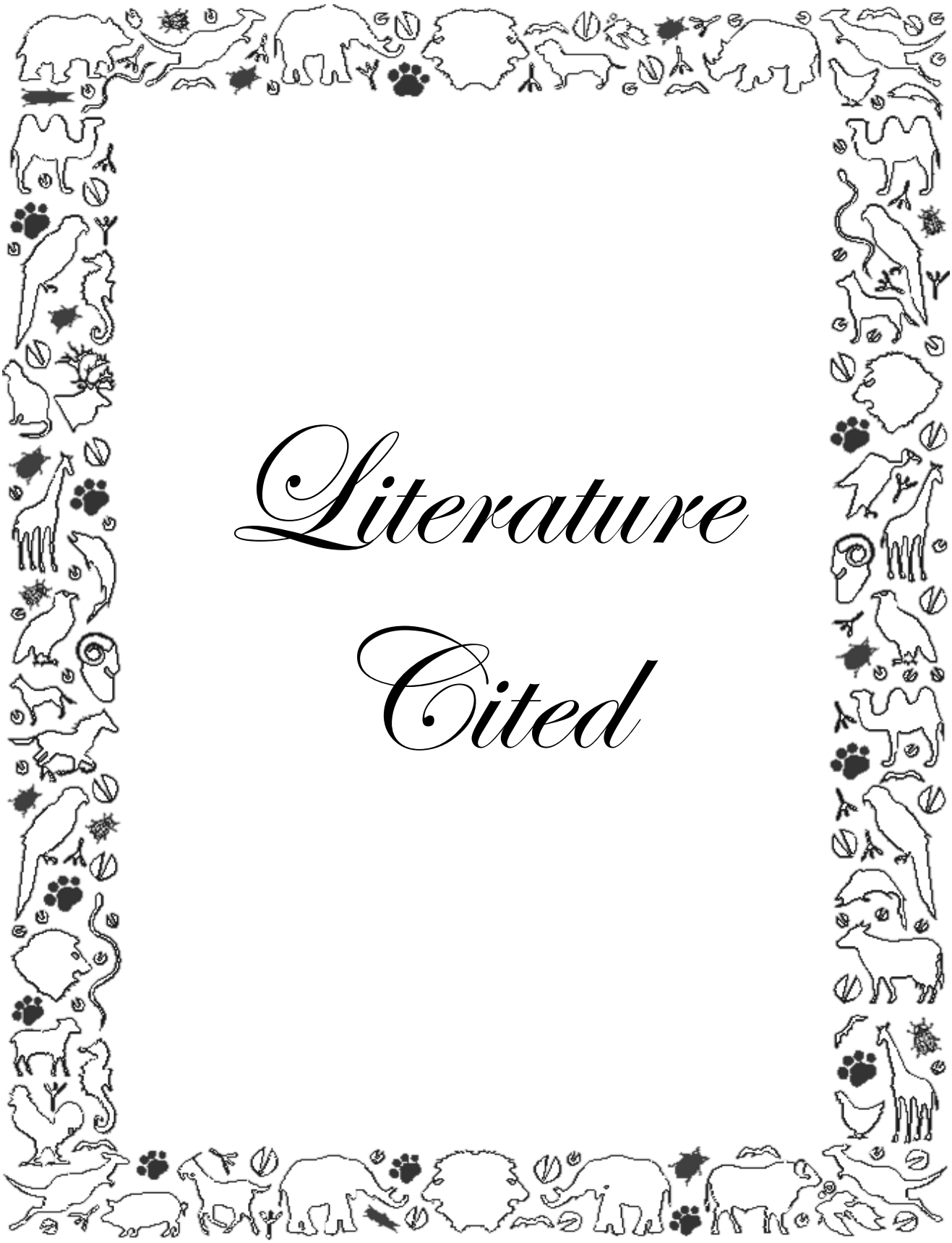
To expound upon the foregoing analyses, the second interpretation focused on a comprehensive examination of animal species that represented the three dietary guilds – carnivorous, herbivorous, and omnivorous animals – based on their feeding habits. In this study, no MTBC was found in any of the faecal samples from captive wild carnivores. Further examination of the data revealed that m-PCR detected positive for MTBC bacteria in 08 (16.67%) faecal samples from herbivorous mammals and 02

(15.38%) omnivores. Herbivores had the highest overall frequency of MTBC bacteria among mammals, followed by omnivores.

This research concluded that, 10 different species of captive wild mammals maintained at Sanjay Gandhi Biological Park, Patna had MTBC in their faeces that could be detected by m-PCR and cultured using MGIT and LJ medium. Two species of omnivores also showed abundances of their MTBC, and among the fourteen species of herbivores that reside in the zoo, the MTBC abundances were found among the six host species. These data collectively imply that, compared to contemporary omnivores, herbivores carried a considerably higher proportion of MTBC in their faeces. Future research employing molecular techniques, like m-PCR, will assist in revealing the presence of MTBC species and contribute to the development of novel techniques to accomplish preliminary screening of MTBC species for captive wild animals.

Future recommendations

Further research is required on a number of variables linked to the dissemination of *Mycobacterium* spp. According to the current exploratory study, captive wild mammals had MTBC. These findings should be validated by comparing them to those of upcoming investigations, and molecular methods requires to be employed to specify the MTBC species found during the study. The incidence of MTBC, as well as the gene abundance as a function of species and dietary habits, also needs to be established in conjunction with the metagenomics investigation of the microbiota in faeces.



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Appendix

Appendix

List of captive wild animals employed for study

Sl. no.	Sample ID	Species	Sl. no.	Sample ID	Species
1.	WTB-01	Sloth Bear	28.	WTB-28	Leopard
2.	WTB-02	Sloth Bear	**29.**	**WTB-29**	**Hippopotamus**
3.	WTB-03	Sloth Bear	30.	WTB-30	Langur(Baboon)
4.	**WTB-04**	**Himalayan Black Bear**	31.	WTB-31	Zebra
5.	WTB-05	Himalayan Black Bear	32.	WTB-32	Rhesus monkey
6.	WTB-06	Sloth Bear	33.	WTB-33	Langur(Baboon)
7.	WTB-07	Giraffe (Baby)	34.	WTB-34	Rhinoceros
8.	**WTB-08**	**Giraffe**	35.	WTB-35	Rhinoceros
9.	WTB-09	Giraffe	36.	WTB-36	Rhinoceros
10.	WTB-10	Giraffe	37.	WTB-37	Rhinoceros
11.	WTB-11	Giraffe	38.	WTB-38	Rhinoceros
12.	WTB-12	Asiatic Lion	39.	WTB-39	Black buck
13.	WTB-13	Asiatic Lion	40.	WTB-40	Sambar - Spotted deer
14.	WTB-14	Bengal tiger	41.	WTB-41	Rhinoceros
15.	WTB-15	Bengal tiger	42.	WTB-42	Rhinoceros
16.	WTB-16	Bengal tiger	43.	WTB-43	Rhinoceros
17.	WTB-17	Bengal tiger	44.	WTB-44	Rhinoceros
18.	WTB-18	White Tiger	45.	WTB-45	Swamp deer
19.	WTB-19	Leopard	46.	WTB-46	Nilgai-Bluebull
20.	WTB-20	Leopard	47.	WTB-47	Wolf
21.	WTB-21	Leopard	48.	WTB-48	Wolf
22.	WTB-22	Leopard	49.	WTB-49	Wolf
23.	WTB-23	Rhesus monkey	50.	WTB-50	Wolf
24.	WTB-24	Asiatic Lion	51.	WTB-51	Giraffe
25.	WTB-25	Bengal tiger	**52.**	**WTB-52**	**Sloth Bear**
26.	WTB-26	Bengal tiger	53.	WTB-53	Zebra
27.	WTB-27	Chimpanzee	**54.**	**WTB-54**	**Elephant**

Sl. no.	Sample ID	Species	Sl. no.	Sample ID	Species
55.	WTB-55	Elephant	83.	WTB-83	Finch Zebra
56.	**WTB-56/1**	**Hippopotamus**	84.	WTB-84	Illiger's Macaw
	WTB-56/2	Hippopotamus	85.	WTB-85	Medium sulphur crested cockatoo
57.	**WTB-57**	**Black buck**	86.	WTB-86	Ostrich
58.	WTB-58	Barking deer	87.	WTB-87	Crane common
59.	WTB-59	Hog deer	88.	WTB-88	Crane common
60.	WTB-60	Rhesus Monkey	89.	WTB-89	Sarus crane
61.	**WTB-61/1**	**Indian Gaur**	90.	WTB-90	Brahminy Duck (Ruddy Shelduck)
	WTB-61/2	**Indian Gaur**	91.	WTB-91	Vulture
62.	WTB-62	Budgerigar	92.	WTB-92	Peafowl
63.	WTB-63	Finch Zebra	93.	WTB-93	Hyena
64.	WTB-64	Hill myna	94.	WTB-94	Hyena
65.	WTB-65	Scarlet Macaw	95.	WTB-95	Hyena
66.	WTB-66	Red & Green Macaw	96.	WTB-96	Hyena
67.	WTB-67	Golden Pheasant	97.	WTB-97	Cassowary
68.	WTB-68	Silver Pheasant	98.	WTB-98	Emu
69.	WTB-69	Silver Pheasant	99.	WTB-99	Black buck
70.	WTB-70	Peafowl	100.	WTB-100	Black buck
71.	WTB-71	Peafowl	101.	WTB-101	Black buck
72.	WTB-72	Hornbill	102.	WTB-102	Black buck
73.	WTB-73	Golden Pheasant	103.	WTB-103	Chital (Spotted deer)
74.	WTB-74	Kite-Pariah / Black Kite	104.	WTB-104	Chital (Spotted deer)
75.	WTB-75	Sangai (Manipuri) deer	105.	WTB-105	Chital (Spotted deer)
76.	**WTB-76**	**Sambar deer**	106.	WTB-106	Sambar deer
77.	WTB-77	Swamp deer	107.	WTB-107	Sambar deer
78.	WTB-78	Ostrich	108.	WTB-108	Sambar deer
79.	WTB-79	Lady Amherst Pheasant	109.	WTB-109	Sangai (Manipuri) deer
80.	WTB-80	Common Emrald Dove	110.	WTB-110	Sangai (Manipuri) deer
81.	WTB-81	Parrot	111.	WTB-111	Sangai (Manipuri) deer
82.	WTB-82	Silver Pheasant	112.	WTB-112	Sangai (Manipuri) deer

Images of captive wild mammals and birds used for collection of faecal samples:

Figure: ***Captive wild animals employed during study to collect faeces***

Figure: ***During the experiment, rhinoceros were recruited to collect faeces samples***

Figure: ***Giraffes being employed in the investigation to collect samples***

Figure: ***Zebra used for collection of faecal sample***

Figure: ***Antelopes used for faecal sample collection***

Figure: ***Black buck used for faecal sample collection***

Figure: ***Elephant and Gorilla used for collection of faecal sample***

Figure: ***Captive wild birds used for study***


Resume

Brief Resume of the Student

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01	B.V.Sc. & A.H.	Bihar Veteriary College, Patna	Bihar Animal Sciences University, Patna	2019	70.05
02	M.V.Sc.	Bihar Veteriary College, Patna	Bihar Animal Sciences University, Patna	2023	81.50

Title of M.V.Sc. Thesis: **Molecular detection of '*Mycobacterium tuberculosis* Complex' in faecal samples of captive zoo animals**.