IDENTIFICATION OF URINE BIOMARKERS FOR THE DIAGNOSIS OF TUBERCULOSIS IN ELEPHANTS

RANJINI MANUEL

(18–MVP–19)

THESIS

Submitted in partial fulfilment of the requirement for the degree of

MASTER OF VETERINARY SCIENCE

Veterinary Epidemiology and Preventive Medicine

2021

Faculty of Veterinary and Animal Sciences

Kerala Veterinary and Animal Sciences University

DEPARTMENT OF VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

POOKODE, WAYANAD- 673 576

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DECLARATION

I hereby declare that the thesis entitled **"Identification of urine biomarkers for the diagnosis of tuberculosis in elephants"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis, entitled **"Identification of urine biomarkers for the diagnosis of tuberculosis in elephants"** is a record of research work done independently by **RANJINI MANUEL (18-MVP-19)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to him.

Pookode **Dr. Deepa P.M**

Date: Chairman

Advisory committee

CERTIFICATE

We, the undersigned members of the advisory committee of **Ranjini Manuel (18-MVP-19)**, a candidate for the degree of Masters of Veterinary Science in Veterinary Epidemiology and Preventive Medicine, agree that this thesis entitled **"Identification of urine biomarkers for the diagnosis of tuberculosis in elephants"** may be submitted by **Ranjini Manuel (18-MVP-19** in partial fulfilment of the requirement for the degree.

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EXTERNAL EXAMINER

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Ranjini Manuel

Top

To Amma and Dada,

Who always picked me up when in distress and encouraged me to go on every adventure, especially this one

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

TB	Tuberculosis
PTB	Pulmonary tuberculosis
EPTB	Extrapulmonary tuberculosis
MOTT	Mycobacterium organism Other Than Tuberculosis
IUCN	International Union for Conservation of Nature
MoEFCC	Ministry of Environment, Forest and Climate change
LAM	Lipoarabinomannan
LF-LAM	Lateral Flow- Lipoarabinomannan
DPP	Dual-Path Platform
EDTA	Ethylenediaminetetraacetic acid
MAPIA	Multi antigen Print Immunoassay
MALDI-MS	Matrix-Assisted Laser Desorption/Ionization mass spectrometry
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
DMSO	Dimethyl Sulfoxide
SSA	Sulfosalicylic acid
HR-LCMS	High-Resolution Liquid Chromatography-Mass Spectrometry
AFB	Acid Fast Bacilli
kDa	Kilo Dalton
DNA	Deoxyribo Nucleic Acid
TBE	Tris-borate EDTA buffer
DW	Distilled water
OD	Optical Density
PCR	Polymerase chain reaction

BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology and Information
TLC	Total Leukocyte Count
TEC	Total Erythrocyte Count
Hb	Haemoglobin
HCT	Haematocrit
MCV	Mean Corpuscular Volume
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
ESR	Erythrocyte Sedimentation Rate
BUN	Blood Urea Nitrogen
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
bp	Base pairs
ml	Millilitre
µl	Microliter

Introduction

1. INTRODUCTION

In Indian culture, elephants are regarded as an epitome of divinity and royalty. According to the mythological and cultural beliefs, elephants are considered as a sacred symbol of power, spirituality and strength. This belief continues to be a part of Indian culture even today. These majestic pachyderms still form a vital part of the temple and religious festivities. However, there is a decline in the number of Asian elephants in India. Asian elephants are listed as endangered species as per International Union for Conservation of Nature (IUCN). According to the latest elephant census released by the Union Ministry of Environment, Forest and Climate change (MoEFCC) in the year 2017, there are about 29,964 elephants in India which was 30,711 in the year 2012. The decline in the number of elephants is mainly due to diseases, habitat loss, and poaching (Baskaran *et al.*, 2011).

Tuberculosis (TB) is one of the ancient maladies known to both humankind and the animal kingdom. For the past 2000 years, the disease has proven its existence among elephants (Iyer, 1937). Tuberculosis had been reported worldwide in captive elephants (Narayanan, 1925; Bopayya, 1928; Seneviratna *et al.* 1966; Pinto *et al.* 1973; Chandrasekharan *et al.,* 1995; Mikota *et al.,* 2000, 2001; Ratanakorn, 2001; Gavier- Wieden *et al.,* 2002; Chakraborty, 2003; and Rahman, 2003). The main causative agent responsible for the infection is *Mycobacterium tuberculosis,* though other species of mycobacterium like *M.bovis, M.tuberculosis complex, M.avium* are also isolated from the elephants (Payeur *et al.,* 2002). Incidence of TB in Asian elephants (*Elephas maximus*) is observed to be more than in African elephants (*Loxodonta aftricana*) (Feldman *et al.*, 2013). In Asian countries, the occurrence of TB is high among captive wild elephants due to the co-existence and close association with humans and other domestic livestock (Michalak *et al.*, 1998). There is a high risk of transmission of the disease when the grazing land is shared with livestock, when wild elephants breed with captive elephants or when captive elephants lives in close proximity with infected humans (Mikota *et al.,* 2006a, 2006b). Infected animals tend to shed the organism in the preclinical period which risks the transmission of the infection to elephants, humans, and other mammals (Oh *et al.*, 2002; Lewerin *et al.*, 2005). TB infection is chronic and slow-progressing, it often remains unnoticed or undiagnosed due to the lack of specific clinical signs. Common clinical signs include exercise intolerance, weakness, coughing, difficulty in breathing, weight loss and abnormal respiratory discharges from the trunk (Mikota *et al.,* 2001). Infected elephants may or may not exhibit clinical signs in the initial stages of the infection. Some infected elephants may display clinical signs only at terminal stages (Mikota *et al.,* 2000; Mikota, 2008). This makes the early diagnosis strenuous.

Definitive antemortem diagnosis of TB in elephants can be challenging and has serious limitations. For the past two decades, the diagnosis of TB predominantly relied on trunk wash culture and acid-fast staining techniques. Trunk wash culture is considered as one of the gold standard test for active TB infection in elephants however, the test has numerous limitations (Greenwald *et al.*, 2009; Mikota and Maslow, 2011). The disadvantages of trunk wash culture technique are, it is labour intensive, the technique needs trained personnel, the sample should be collected three times a week, turn-around time required for the result is 8-12 weeks and relatively expensive (Lyashchenko *et al.*, 2012). Traditional acid-fast staining technique alone cannot be used for TB diagnosis as it has low and variable sensitivity and hence recommended in combinations with other diagnostic techniques (Urbanczik, 1985; Ong *et al.*, 2013). In many species including elephants, intradermal tuberculin test was not validated as there were poor correlations on comparison with other diagnostic tools (Mann *et al.*, 1981; Mikota *et al.*, 2001). Intradermal tuberculin tests were known to be validated and used for screening only in cattle, bison and other cervids (Fowler, 1992). The radiographic investigation has limited value in case of the diagnosis of TB in elephants due to the anatomy and size of the animal, although radiographic evaluation can be used to some extent in the case of elephant calves.

Valuable and reliable diagnosis of tuberculosis could be achieved only by employing a combination of the diagnostic techniques (Angkawanish *et al.,* 2010). The antibody immunoassays like Elephant TB Stat-Pak, MAPIA and DPP Vet TB have shown 100 per cent sensitivity and 95 per cent of specificity which entitles it as a new generation serologic rapid point of care test for elephants. These tests are found to be more useful and superior to the existing techniques (Greenwald *et al.*, 2009; Lyashchenko *et al.*, 2012). Nucleic acid amplification techniques like Polymerase Chain Reaction (PCR) has also gained importance in the past few years in the TB diagnosis. Utilization of serological and molecular assays helps to comprehend active and latent TB status in elephants (Ong *et al.*, 2013).

The present study focusses on the utilization of serological, molecular, and proteomic diagnostic assays for TB diagnosis. An integral part of the study is the usage of urine as a biological sample. Urine was selected as a primary sample because it is least invasive, requires less labour, and easily available from all the elephants. A component of the bacterial cell wall, Lipoarabinomannan (LAM), released from the metabolically active or degrading bacteria known to have an immunogenic virulence factor (Hunter et al., 1986; Chan et al., 1991). No known glycosidase can degrade LAM, therefore it is expected to be excreted through urine. Hence LAM antigen can be detected in the urine using an immunochromatographic test which was considered as a point of care test (POCT) (Dheda *et al.,* 2010).

Proteomics analysis helps to identify the proteins which could be used as a biomarker for the diagnosis of TB. This was a comprehensive methodology to identify the proteins which could be used as a biomarker for the diagnosis of TB (Wheelock *et al.,* 2013). The possibilities of utilization of urine as a potential biological sample and identification of ideal biomarkers are unexplored till date in the field of elephant disease diagnosis. Thus, the present study envisages the identification of urine biomarkers for the early diagnosis of tuberculosis in elephants. Objectives of the study include:

1. Screening of elephants for tuberculosis (TB) using Chembio DPP VetTB assay
2. Proteomics study of urine of healthy and diseased elephants
3. Identification of biomarkers for the diagnosis of TB in elephants

Review of literature

2. REVIEW OF LITERATURE

2.1 SEROPREVALANCE

In a survey conducted on 387 captive temple elephants and Government as well as privately owned Asian elephants of India, Abraham *et al.* (2008) observed that 15 per cent of Asian elephants were seroreactive. There was higher rate of seroprevalence (25 per cent) among the group of elephants who were in close contact with the humans.

Tuberculosis (TB) seroprevalence study of 236 captive Asian and African elephants from various locations in the US and Europe established that 15.3 per cent of the elephants were TB antibody positive. Another important observation was the statistical significant increase (21.9 per cent) in seroprevalence among Asian elephants in comparison to African elephants (Greenwald *et al.*, 2009).

Mikota and Maslow (2011) observed that seroprevalence for TB was between 2 per cent - 4.4 per cent among 204 African elephants in the US.

Rosen *et al.* (2018) on her study on TB surveillance in Zimbabwe noted that there were six of 35 African elephants (17.1per cent) were positive for TB antibodies.

Kerr *et al.* (2019) screened African elephants in Kruger National Park (KNP) for TB antibodies in the retrospective cohort study of 222 free- ranging African elephant from 2004 to 2018. The estimated TB seroprevalence was found to be 6 – 9 per cent based on STAT- PAK and DPP Vet TB assay. According to the study, males and adult elephants had higher seroprevalence compared to females and younger elephants.

2.2 COLLECTION AND UTILITY OF CLINICAL SAMPLES

2.2.1 Blood sample

Ong *et al.* (2013) for a cross-sectional study conducted in peninsular Malaysia, collected blood samples from elephants for serological diagnosis. Blood was collected from auricular vein using 21- gauge butterfly catheter. Blood was drawn into a 10 ml syringe into a plain tube. Blood was allowed to clot at room temperature and was transferred to the laboratory in ice chest. Further clotted blood was centrifuged at 600g for 10 minutes at room temperature and used for the test.

Bansiddhi *et al.* (2015) for a blood compatibility test in Asian elephants, collected venous blood of 10ml volume from marginal ear vein. The blood was centrifuged at 2000 g for 15 min after clotting to extract serum.

2.2.2 Trunk wash sample

The practical difficulty of trunk wash sample collection was emphasised by Isaza *et al.* (1999). Study stated the draw backs of the trunk wash sample collection procedure, the main difficulties were to train the elephants prior for the collection procedure and restraining of trunks of uncooperative elephants. Since the procedure could be very dangerous to the handlers, safety precautions were mandatory during the collection. Other important point was the risk involved in the collection because of the zoonotic nature of the disease.

Lyashchenko *et al.* (2006) while working on the antibody responses in elephant tuberculosis opined that trunk wash method could only identify diseased animals at late course of disease with extensive shedding of organism and hence had poor sensitivity.

Kay *et al.* (2011) did DNA extraction techniques for detection of *Mycobacterium tuberculosis* complex organism in Asian elephant trunk wash samples. The trunk wash samples were collected by flushing with 60ml of sterile saline into the nostril using a catheter- tipped syringe. Elephant was made to leave the trunk elevated for few seconds and to exhale the saline. The contents were pooled in a sterile bag and stored in -80°C.

In a cross-sectional study conducted in peninsular Malaysia, Ong *et al.* (2013) collected trunk wash samples for PCR and culture. They reported that trunk wash sample was a valuable and useful sample for detection of mycobacterium organisms in TB diagnosis. Elephants were restrained carefully and 60ml of saline was instilled into the trunk. Trunk of the elephant was lifted up and then lowered after few minutes of instillation. The trunk wash sample was then collected in a sterile bag. The contents was split into 25 ml aliquots, one aliquots was taken for culture the other was taken for PCR.

Fagen *et al.,* 2014 opined that all elephants could not be trained to perform trunk wash and training an elephant was a protracted process

2.2.3 Urine sample

Thongboonkerd *et al.* (2002) constructed proteome map for normal human urine to further differentiate normal physiology and pathology using urine proteins as biomarkers.

Torrea *et al.* (2005) while performing nested PCR for diagnosing PTB in human patients using urine specimen confirmed that there were 64.3 per cent sensitivity in culture negative pulmonary tuberculosis (PTB) cases and 46.3 per cent sensitivity in extra pulmonary tuberculosis (EPTB) cases.

Rapid, non-invasive, point of care diagnosis of infectious diseases like pneumococcal pneumonia, histoplasmosis, legionella and tuberculosis were enabled by urine tests in humans (Peter *et al.,* 2006).

Gopinath *et al.* (2009) ran trial on urine as an adjunct specimen for diagnosing mycobacterial infection in humans and reported that *M. tuberculosis* was detected in 26.1 per cent of the culture and/or smear positive cases and further indicated that mycobacterial DNA was detected by PCR in 54.3 per cent of urine samples.

Interferon inducible protein (IP-10) was found elevated in urine specimens of active TB cases and showed potential in tracking disease progression and treatment response (Gowda *et al.* 2013 and Petrone *et al.* 2016) further indicating the importance of urine as an optimal specimen for biomarker identification.

Illera *et al.* (2014) separated elephant cows in individual boxes for the collection of urine from individual elephants. Urine was collected from the ground of the elephant barn in the next morning using a 5ml syringe, it was then placed in 5ml cryotubes. Urine samples were then centrifuged at 500 g at 20°C for 10min. Sample was collected for the assessment of ovarian cycles in African elephants. Further indicated that the urine collected from ground of barn could also be used for such studies with minimum interference with the results.

2.3 SCREENING USING LATERAL FLOW ASSAYS

2.3.1 Screening using DPP® (Dual Path Platform) VetTB - Elephant assay kits by Chembio Diagnostic Systems, INC.

Greenwald *et al.* (2009) described the efficiency of serological lateral flow assays. On comparison of Elephant TB Stat-Pak, Multiantigen Print Immunoassay (MAPIA) and Dual Path Platform (DPP) VetTB assay, all elephants included in the TB group were found to be positive for CFP10/ESAT-6 antibody and no positive results were obtained from non TB group. Therefore, the study indicated a 100 per cent sensitivity and 100 per cent specificity for all three assays. However four elephant sera that was reactive for MPB83 antigen were found to be positive for Mycobacterium organism other than Tuberculosis (MOTT).

Angkawanish *et al.* (2010) in their study conducted on four Asian elephants observed that three elephants developed antibodies for CFP10/ESAT-6 antigens using Elephant TB Stat-Pak assay. Their post-mortem findings confirmed TB lesions on lung, spleen, and mediastinal lymph nodes.

Lyashchenko *et al.* (2012) after their study of serodiagnosis to identify elephants with TB prior to culture confirmation reported that, in elephants

serological diagnostic tests were easy to perform and provided reliable results even months before the appearance of clinical signs. All 14 elephants included in the study showed positive antibody responses to *M. tuberculosis*. CFP10/ESAT-6 was identified as the key antigens. Elephant TB Stat-Pak, Multiantigen Print Immunoassay (MAPIA) and Dual Path Platform (DPP) VetTB assay showed 100 per cent, 95-100 per cent sensitivity and specificity respectively. Out of 14 elephants, 7 elephants responded to MPB83 antigen only, which signifies the presence of MOTT or mixed infections. Findings of the study favoured high test accuracy for serological diagnosis and DPP VetTB assay could be identified as a potential test for monitoring the treatment process in elephants.

2.3.2 Screening using Alere Determine™ TB LAM Ag assay

Kadival *et al.* (1986), Sada *et al.* (1983) and Watt *et al.* (1988) in their studies identified the utility of secreted or circulating mycobacterium antigens in the diagnosis of active TB. Detection of antigens in the urine of infected individuals could be a milestone in the TB diagnosis since it is less invasive and easily obtainable.

Lipoarabinomannan (LAM) is a heat stable 17.5kD glycolipid, found on the cell wall of *Mycobaterium tuberculosis*. It possesses 15 per cent of the total bacterial weight and has a virulence factor that is released by active or degrading organism (Hunter *et al.*, 1986).

Patel *et al.* (2009) in their study noted that LAM antigen which was not metabolically degraded in the body was filtered through kidney and excreted in urine in an intact form. It could be identified and detected in urine and it has potential to be used as a point of care test in TB diagnosis.

Dheda *et al.* (2010) in a case control study observed that irrespective of the anatomical location of the infection, LAM was excreted in the urine. Hence it could be used to identify both pulmonary and extra pulmonary TB. Detection of LAM showed promising diagnostic performance.

Minion *et al.* (2011) in a systemic review and meta-analysis of LAM detection assays noted that the results from Alere Determine TB Antigen immunochromatographic test showed a sensitivity of 40 per cent and specificity of 98 per cent which was enhanced compared to other LAM detection methods.

A study conducted by Elsawy and Redwan (2012) concluded that sensitivity of LAM was more than sputum smear microscopy. In the study 72 patients were included and 52 patients were confirmed with TB by sputum smear microscopy and culture. Sensitivity and specificity of LAM was found to be 94.3 per cent and 100 per cent, whereas sputum smear microscopy was 80.8 per cent and 100 per cent

respectively.

2.4 CLINICAL PATHOLOGY

2.4.1 Haematological evaluation

Giri *et al.* (2011) assessed the relationship between blood parameters and mycobacterium culture in captive elephants in Nepal. Study concluded that there was no significant influence of mycobacterium infection on serum biochemistry and haematological parameters. On haematological and serum biochemistry evaluation all values obtained were within the normal range.

Shafee *et al.* (2014) evaluated blood parameters of 600 (Male: 238 and Female: 362) patients with clinical signs of pulmonary tuberculosis and reported a marked change in Erythrocytic Sedimentation Rate (ESR), Haemoglobin (Hb) and lymphocytes in affected animals of both sexes. Haemoglobin was recorded lower than normal value in 55 per cent of males and 53 per cent of females. Total leukocyte count was also lower than normal values in 8 per cent and 6 per cent of male and female respectively. Neutropaenia was observed in 5 per cent and 8 per cent cases, while neutrophilia was recorded as 60 per cent and 64 per cent in male and female patients respectively. Lymphocytopaenia was also observed in 59 per cent and 43per cent patients in male and female respectively.

Allwin *et al.* (2015) reported that for healthy male Indian elephant (n=32) the packed cell volume (%), haemoglobin (gm/dl), total erythrocyte count (10^6/µl), mean corpuscular volume (fL), mean corpuscular hemoglobin (pg), mean corpuscular haemoglobin concentration (gm/dl), total leukocyte count (10^3/µl), neutrophil count, eosinophil count, lymphocyte count, monocyte count were (41.11±0.42), (11.55±0.47), (3.47±0.15), (112.22±5.50), (37.24±1.57), (28.26±0.10), (12.00±0.29), (5057.44±336.78), (397.78±88.01), (6319.84±319.83), (228.89±33.91) respectively. For healthy female Indian elephants (n=36) the values are (40.50±0.64), (11.56±0.38), (3.16±0.18), (109.05±4.23), (35.58±1.49), (28.07±0.74), (11.19±0.23), (3800.63±198.51), (323.75±50.22), (4886.25±153.53), (183.13±20.47) respectively.

Abay *et al.* (2018) evaluated blood parameters of 100 TB human patients and reported that the mean values of haemoglobin, platelet count, and neutrophils counts were significantly lower for pulmonary tuberculosis (PTB) subjects. Of the PTB infected patients 46 per cent were anaemic, 6 per cent were leukopaenic, 22 per cent were neutropaenic, 8 per cent were lymphopaenic, and 8 per cent were thrombocytopaenic. Erythrocyte sedimentation rate (ESR) value was increased in all patients.

2.4.2 Serum biochemical evaluation

Brown *et al.* (1980) reported that for apparently healthy Indian elephant (*Elephas maximus*) the blood urea nitrogen (mg/dl), creatinine level (mg/dl), glucose level (mg/dl), serum total protein level (g/dl), cholesterol level (mg/dl), aspartate transaminase level (IU/l), alanine aminotransferase level (IU/l), alkaline phosphatase level (IU/l) ranges were (03.60 to 05.80), (99.00 to 170.00), (02.40 to 03.70), (62.00 to 107.00), (01.41 to 02.99), (17.00 to 23.00), (08.00 to 11.00), (25.00 to 125.00) respectively.

Janyamethakul *et al.* (2017) reported that for healthy male Thailand elephants (*Elephas maximus*) (n=41) the blood urea nitrogen (mg/dl), creatinine level (mg/dl), total protein level (g/dl), aspartate aminotransferase level (IU/l), alanine aminotransferase level (IU/l), alkaline phosphatase level (IU/l) ranges were (03.10 to 27.20), (00.70 to 02.20), (06.50 to 08.90), (04.80 to 56.30), (00.00 to 04.90), (00.00 to 281.50) respectively. For healthy female elephants (n=108) values were (04.20 to 19.70), (00.90 to 01.80), (06.60 to 09.30), (10.10 to 39.60), (00.00 to 05.60), (00.00 to 225.40) respectively.

Akram *et al.* (2017) investigated the biochemical parameters in tuberculosis (TB) human patients in comparison with healthy individual and found a significant increase in the level of alanine aminotransferase, a non-significant increase in the level of aspartate aminotransferase, a significant increase in alkaline phosphatase and significant variation in the concentration of serum proteins.

2.4.3 Urine evaluation

2.4.3.1 Colour of urine

Urine colour analysed for healthy female Asian elephants (*Elephas maximus*) (n=22) showed that colour of urine in 54.54 per cent animals were pale yellow and in 45.45 per cent animals it was medium to dark yellow coloured (Weidner *et al.,* 2009).

2.4.3.2 Appearance of urine

Urine colour analysed for healthy female Asian elephants (*Elephas maximus*) (n=22) showed that colour of urine in 54.54 per cent animals were pale yellow and in 45.45 per cent animals it was medium to dark yellow coloured (Weidner *et al.,* 2009).

Urine appearance analysed for healthy female Asian elephants (*Elephas maximus*) (n=22) showed that the appearance of the urine in 36 per cent of females were clear and the urine of 64 per cent of females were cloudy (Weidner *et al.,* 2009).

2.4.3.3 Detection of urine protein

Weidner *et al.* (2009) reported that urine protein detected using sulfosalicylic acid test for healthy female Asian elephants (Elephas maximus) (n=22) were 100 per cent negative.

2.4.3.4 Evaluation of urine using Dipstick

Wiedner *et al.* (2009) collected urine samples from 22 healthy female adult Asian elephants (Elephas maximus) and evaluated using dipstick method. It was reported that in elephants urine was less concentrated compared to other mammals, had predominantly alkaline pH and crystalluria of varying types in all samples with minimal cellularity. They reported that colour and clarity were highly variable and ranged from clear and straw-coloured to deep yellow and opaque. Glucose and urobilinogen were not detected in samples whereas trace ketones, trace blood, trace protein, trace bilirubin, and bacteria were detected in few samples. Urine specific gravity (SG) ranged from 1.007–1.025. No correlation between clarity and S.G. Urine osmolality ranged from 244–802 mOsm/kg. pH measurements ranged from 6.0–8.5. No statistical correlations were found between age and either S.G or osmolality.

Altiparmak *et al.* (2015) studied 79 cases of renal tuberculosis and reported that the most common observations in urine of animals with tuberculosis were haematuria (79.7 per cent), pyuria (67.1 per cent) and dysuria (51.9 per cent).

2.5 MOLECULAR DIAGNOSIS USING POLYMERASE CHAIN REACTION

Clarridge *et al.* (1993) figured that PCR with *IS*6110 insertion sequence was an effective molecular tool for the identification of mcobacterium organisms. Out of the 218 respiratory samples confirmed for *Mycobacterium tuberculosis* by culture, 85 per cent (181) were PCR positive. Polymerase chain reaction in comparison with culture showed the positive predictive value, sensitivity, and specificity of 94.2 per cent, 83.5 per cent and 99 per cent respectively.

Mikota *et al.* (2000) and Lemaitre *et al.* (2004) in their studies proposed that PCR had the potential to replace trunk wash culture method and could be used for TB screening in elephants. Extraction of nucleic acids followed by PCR analysis could be the sensitive and rapid diagnostic technique for identification of mycobacterium from respiratory discharges, tissues and soil.

Torrea *et al.* (2005) evaluated PCR based analysis of human urine for diagnosing pulmonary tuberculosis (PTB) and extra-pulmonary tuberculosis (EPTB). The sensitivity of the test for the microbiologically positive PTB, microbiological negative PTB and EPTB was found to be 40.5 per cent, 66.7 per

cent and 57.1 per cent respectively and the specificity was 98.2 per cent.

Ong *et al.* (2013) in a cross sectional study conducted in Malaysia on TB in captive Asian elephants reported that there was a poor correlation between trunk wash PCR, trunk wash culture and serology. The ten elephants with seropositive results and twelve elephants with seronegative results were subjected to TB PCR. Only three of twenty two samples were positive for serology and PCR. Eight elephants showed negative results for both tests. Seven elephants was found to be seropositive and PCR negative. Hence the study pointed that negative serological finding with positive culture and PCR, were indicative of active TB infection whereas positive serological finding with no clinical signs and PCR negative were indicative of latent TB infection.

Heydari *et al.* (2014) investigated the diagnostic value of tuberculosis-polymerase chain reaction of human urine in the diagnosis of pulmonary tuberculosis and reported a sensitivity of 56.2 per cent and a specificity of 100 per cent for urine PCR when compared to positive sputum smear test.

2.6 PROTEOMIC PROFILING OF URINE FOR IDENTIFICATION OF BIOMARKERS

Lazar *et al.* (2004) identified the urinary proteins that bound with the pheromones and found that the major protein in elephant urine was albumin. Albumin occurred in urine as monomer (66kDa) and dimer (132kDa). The molecular mass of albumin was determined by Matrix-Assisted Laser Desorption/Ionization mass spectrometry (MALDI-MS) together with SDS-PAGE.

Young *et al.* (2013) on identification of tuberculosis biomarkers in human urine sample discovered that there were proteins in the urine which could be clearly discriminate TB infected individual with that of healthy individual. Study included 63 TB patients, out of which 21 and 24 individuals were categorized as active and latent TB patients. Urine protein were separated by SDS- PAGE, digested using trypsin, and run in high performance liquid chromatography- mass spectrometry (HPLC-MS) for protein profiling. Study inferred that urine protein biomarkers could be efficiently used for the diagnosis of TB because 10 mycobacterial proteins were exclusive for active TB patients and six mycobacterial proteins were exclusive for latent TB patients.

Bathala *et al.* (2015) was successful to characterize bovine urinary proteome which became a database for future biomarker studies in bovines. The study identified more than 1500 proteins from the urine of Karan fries cows. Proteins were analysed by two dimensional electrophoresis (2DE) and LC/MS. Proteins were categorised on the basis of cellular localization, and found that 29% of proteins

were cytoplasmic and 20 per cent extracellular. Large proportion of these proteins had catalytic (32 per cent), binding (30 per cent), metabolism (25 per cent) and cellular process (20 per cent) activities.

Pastushkova *et al.* (2016), Guo *et al.* (2015) and He *et al.* (2012) proposed that urine proteome of healthy individuals showed wide variability depending upon the age, sex, diet, physical activity, drug consumption, and environmental conditions. Identification of various proteins in different conditions could be utilized for diagnostic purposes.

On comparison of urine proteomic profiles of pulmonary TB infected and healthy humans using two dimensional electrophoresis and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF- MS), 19 proteins were identified exclusive to TB infected individuals and could be used to differentiate between TB infected and healthy subjects. These were confirmed with western blotting and qRT-PCR. Mannose-binding lectin 2 and a 35-kDa fragment of inter-α-trypsin inhibitor H4 were the two proteins that exhibited the highest differential expression. These biomarkers has the diagnostic sensitivity and specificity of 85.87 per cent and 87.50 per cent respectively (Wang *et al.,* 2018).

In the study for identification of urinary reference values and urine proteome in giraffes (*Giraffa camelopardalis*), one dimension electrophoresis (SDS-PAGE together with mass spectrometry was used in the identification and separation of 15 highly represented urinary proteins. Important proteins identified were uromodulin, albumin, alpha-1B-glycoprotein, and haptoglobin. Other main proteins detected in the urine of giraffes were also previously reported in camels, dogs, cats and cows. Study suggested that there were common sets of proteins present in urine of healthy mammals (Zhu *et al.*, 2020).

Materials and methods

3. MATERIALS AND METHODS

The present study was conducted in the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Pookode, Wayanad during the period of September 2019 to December 2020. A total of 86 captive Asian elephants from Kerala formed the subject of the study.

3.1 SELECTION OF ANIMALS

Apparently healthy adult animals were randomly selected irrespective of sex, bodyweight and parity. Neonates, pregnant animals and animals with clinical illness were excluded from the study.

3.2 COLLECTION OF CLINICAL SAMPLES

The clinical samples collected for the study included blood, serum, urine and trunk wash. All the samples were transported to the laboratory maintaining cold chain and then transferred to -20°C immediately. Samples were stored at -20°C until further processing.

3.2.1 Collection of blood samples

Animal was placed in lateral recumbency. A total of 4ml blood was drawn from superficial marginal vein located at the external aspect of the ear using a clean, sterile 16-gauge disposable hypodermic needle. A quantity of 1ml and 3 ml blood was transferred into an EDTA vacutainer tube and serum vacutainer tube respectively for haematological and serum biochemical evaluation. Serum was separated and stored at -20°C until further evaluation.

3.2.2 Collection of trunk wash sample

Trunk wash samples were collected as per the guidelines for control of tuberculosis in elephants by United States Department of Agriculture (USDA) (Ramiro and Cornelia, 2010). Sterile saline (60 ml) was flushed into one nostril of the elephant's trunk using a catheter-tipped syringe. The elephant was then instructed to raise the trunk and leave it elevated for 30-60 seconds, after which it lowered the trunk and exhaled. Trunk wash contents were collected in a sterile plastic bag and then aseptically transferred into a sterile, leakproof, screw top container and stored at -20°C until further evaluation.

3.2.3 Collection of urine samples

Urine samples were collected from elephants at dawn in the presence of mahout under sterile conditions. Mid-stream urine (200 ml) was collected in sterile urine collection. A quantity of 100ml urine was transferred into centrifugation vials

of 50ml capacity and protease inhibitor cocktail (Freeze dried Protease inhibitor developed by Promega Corporation was reconstituted with 1ml DMSO (Dimethyl Sulfoxide) to achieve a protease inhibitor cocktail (50X)) was added at the rate of 50µl/100ml urine. Mixed the samples thoroughly to attain uniform distribution of protease inhibitor. Urine samples were stored at -20°C until further evaluation.

3.3 SCREENING USING LATERAL FLOW ASSAYS

All the elephants included in the study were screened for tuberculosis using DPP VetTB assay and Alere Determine TB LAM Ag assay. The samples used for the screening included serum and urine.

3.3.1 Screening using DPP® (Dual Path Platform) VetTB - Elephant assay kits by Chembio Diagnostic Systems, INC.

The sera samples were screened using rapid lateral-flow immunochromatographic test by DPP® (Dual Path Platform) VetTB - Elephant assay kits by Chembio Diagnostic Systems, INC, USA. Test was manually performed by adding 5 µl of serum to the sample plus buffer well followed by 2 drops of buffer. After 5 min, 4 drops of buffer were added into the buffer well. The kit was left undisturbed for 15 min and results were read positive or negative based on the colour appearance of control and test lines as per guidelines of the kit.

3.3.2 Screening using Alere Determine™ TB LAM Ag assay

Urine samples were screened using rapid lateral-flow kit, Alere Determine™ TB LAM Ag test (LF-LAM, Abbott, USA) for Lipoarabinomannan detection as per guidelines of the kit. The test was performed manually by applying 60 µl of urine to the Alere Determine™ TB LAM Ag test strip and incubated at room temperature for 25 min. Strips were then read according to the appearance of coloured line in patient and control windows.

3.3.3 Screening using acid fast staining technique

Trunk wash samples were centrifuged at 5000rpm for five min. Supernatant was discarded and a drop from sediment was made into a smear on a clean, grease free microscopic slide. The smear was heat fixed. Carbol fuchsin was applied over the fixed smear and heat was applied below the slide for one min. After one min, the slide was rinsed under running tap water. Decolorizing agent (1 per cent acid alcohol) was poured into the slide until the smear was decolorized. The smear was then counterstained with methylene blue for two min and was rinsed under running tap water. Slide was air dried and observed under oil immersion 100X of compound microscope as per standard method (Murray *et al.,* 1980).

3.4 CLINICAL PATHOLOGY

3.4.1 Haematological evaluation

The whole blood samples were subjected to complete blood count using automated analyzer (Orphee mythic 18vet, Switzerland) available at College of Veterinary and Animal Sciences, Mannuthy on the same day of sample collection. Results thus obtained were recorded. The parameters checked were:

a) Total red blood cell count (10^6/µl)

b) Total leucocyte count (10^3/µl)

c) Haemoglobin (g/dl)

d) Granulocytes (%)

e) Lymphocytes (%)

f) Monocyte (%)

g) Platelet Count (x10^3/µl)

h) Packed Cell Volume (%)

i) Mean Corpuscular Volume (fL)

j) Mean Corpuscular Haemoglobin (pg)

k) Mean Corpuscular Haemoglobin Concentration (g/dl)

3.4.2 Serum biochemical evaluation

The sera samples were subjected to biochemical analysis using a semi-automated biochemical analyser (Master T, Hospitex diagnostics Italy) available at College of Veterinary and Animal Sciences, Mannuthy on the same day of sample collection. The results thus obtained were recorded. The parameters estimated were:

a) Alanine amino transferase (U/l)

b) Aspartate amino transferase (U/l)

c) Total protein (g/dl)

d) Albumin (g/dl)

e) Globulin (g/dl)

f) A:G ratio

g) Serum cholesterol (mg/dl)

3.4.3 Urine evaluation

Urine was evaluated for its colour, appearance, osmolality, mucous threads, pH and protein. Dipstick (Mission urine reagent strip from ACON LABS INC. USA) was used to assess leukocytes, nitrite, urobilinogen, blood, specific gravity, ketone, bilirubin and glucose.

3.4.3.1 Colour of urine

Urine sample was evaluated visually based on colour of the urine and graded straw yellow, amber and dark brown colour.

3.4.3.2 Appearance of urine

Urine sample was evaluated visually based on appearance of the urine and graded clear, cloudy and turbid.

3.4.3.3 Detection of protein

Presence of Protein in urine was checked using sulfosalicylic acid (SSA) urine test. 10 ml of clear mid-stream urine was taken in a test tube, added 10 ml of sulfosalicylic acid to it and checked for turbidity.

3.4.3.4 Evaluation of urine using Dipstick

Urine samples collected were evaluated using Mission urine reagent strip from Acon labs inc. USA. Dipstick was immersed in urine sample in centrifuge vial of 50ml capacity. The parameters evaluated were:

a) Leukocytes
b) Nitrite
c) Urobilinogen
d) Protein
e) pH
f) Blood
g) Specific Gravity
h) Ketone bodies
i) Bilirubin
j) Glucose

3.5 MOLECULAR DIAGNOSIS USING POLYMERASE CHAIN REACTION

Confirmation of *Mycobacterium tuberculosis* was accomplished by Polymerase Chain Reaction (PCR) technique. It amplified the targeted bacterial genome IS*6110*. Trunk wash and urine were the samples subjected to Polymerase Chain Reaction.

3.5.1 Glassware and reagents

Borosil® glassware, analytical or guaranteed grade reagent and chemicals were used for the study.

3.5.1.1 Preparation of glassware

Borosil® glassware such as conical flasks, beakers, measuring cylinders and glass bottles were kept submerged in 0.1% hydrochloric acid overnight. They were washed in running tap water and immersed in detergent solution for a day. The glassware was washed in running tap water followed by distilled water. Following this, glassware was allowed to air dry. Conical flasks and measuring cylinders were plugged with non-absorbent cotton. Glass bottles and beakers were packed securely and sterilisation was done by dry heat using hot air oven at 160°C for one hour.

3.5.2 Isolation of bacterial genomic DNA

Bacterial DNA was isolated using DNeasy Blood and Tissue Kit for 50 reactions, produced by M/s Qiagen inc Germany. (Catalog no. 69504) As per the protocol of the kit.

3.5.2.1 Preparation of Buffer AW1 and Buffer AW2

Buffer AW1 and buffer AW2 were supplied as concentrates. Before using for the first time, an appropriate volume of ethanol (96 to100 per cent) was added as indicated on the bottle and mixed thoroughly.

3.5.2.2 Procedure for isolation of bacterial genomic DNA

The trunk wash sample collected in sterile bag was first thawed and transferred into centrifuge vial of 50ml capacity. Trunk wash sample was centrifuged at 15000rpm for 30 min at 4ºC. The supernatant was discarded and the sediment, resuspended in 100 ul PBS. To this, 180 µl of buffer ATL and 20 µl of proteinase K solution was added and incubated at 56°C in a dry heat bath until fully lysed. Exactly 200 µl buffer AL was added to this and thoroughly mixed by vortexing. To this, 200 µl of absolute ethanol was added and vortexed thoroughly. The solution was transferred to a DNeasy Mini spin column placed in a two ml collection tube. It was then centrifuged at 8000 rpm for one min. The collection tube was discarded and the spin column was placed in a new two ml collection tube. Buffer AW2 (500 µl) was added to this and centrifuged for one min at 8000 rpm. The collection tube was again discarded and the spin column was placed in a new two ml collection tube. To this, 500 µl of buffer AW2 was added and centrifuged for three min at 14,000 rpm. The collection tube was discarded and the spin column was transferred to a new 1.5 ml microcentrifuge tube. The DNA was eluted by adding 200 µl buffer AE to the centre of the spin column membrane. This was kept for incubation at room

temperature (20ºC) and the centrifuged for one min at 8000 rpm.

3.5.3 DNA confirmation by Agarose gel electrophoresis

The purity and quality of the extracted genomic DNA were evaluated by agarose gel electrophoresis (1.5 per cent).

3.5.3.1 Reagents required

a. Agarose
b. TBE buffer (Tris-Borate EDTA buffer) (0.5X, pH 8.2)
   The solution was sterilized by autoclaving and diluted 20 times to get working strength of 0.5X TBE.
   A stock solution of 10X TBE was prepared as given below.

- Tris base 10.80 gm
- Boric acid 5.50 gm
- EDTA (0.5M, pH 8) 4 ml
- DW up to 100 ml

c. Gel loading dye (6X) (Thermo-scientific Cat. No. R0611)
d. Ethidium bromide (10mg /ml)
   Ethidium bromide 100 mg
   Distilled Water 10 ml
   The suspension was stirred to ensure that the dye had dissolved. The container then wrapped in aluminium foil and stored at room temperature.

3.5.3.2 Procedure for agarose gel electrophoresis

The purity of the DNA sample was checked by electrophoresis on 1.5 per cent of agarose gel as described below.

a. Required quantity of 1.5g agarose was weighed and dissolved in 100ml of 1.0 per cent of TBE and melted in a microwave oven for 1min.
b. The gel tray was placed on levelled gel caster. The ends of the tray were sealed by adjusting the cam lever of the gel caster. Level was once again checked using levelling bubble provided by the manufacturer and required adjustments were done. The comb was placed in proper position and the melted agarose was poured into gel tray carefully avoiding air bubbles and allowed to solidify.
c. On solidification, comb was removed carefully and the tray was released from the gel caster by loosening the cam lever.
d. The gel tray was held in electrophoresis tank and 1X TBE buffer was poured to submerge the gel in the tank.

e. The DNA samples were mixed with 1/6th volume of 6X loading dye and carefully loaded into the wells using micropipette.
f. The electrophoresis was carried out at 70V at room temperature till the bromophenol blue dye had just reached the end of the gel.
g. Following the electrophoresis, DNA bands were visualized using gel documentation unit.

3.5.4 Polymerase Chain Reaction (PCR)

3.5.4.1 Reagents required

a. Emerald Amp GT PCR Master Mix (Takara, Japan)

b. Primers (Sigma Aldrich, USA)

c. Nuclease free water

3.5.4.2 Primers

The following primer sequence were used for the detection of *M. tuberculosis* complex

Gene	Primer sequence 5'-3'	Product size (bp)	Reference
IS6110	INS1- CGTGAGGGCATCGAGGTGGC	245	Kulkarni *et al*., 2012
	INS2- GCGTAGGCGTCGGTGACAAA		

3.5.4.3 Reconstitution of the primer

The lyophilized primers were reconstituted in required volume of nuclease free water to make a stock solution of 100 pmol/µl concentration. The reconstituted primers were stored at -20ºC. Working solution with the concentration of 10 pmol/µl was prepared using sterile nuclease free water. Reconstituted primer (stock and working) solutions were stored at -20ºC for further use.

3.5.4.4 Procedure

Polymerase chain reaction was standardized using *M. tuberculosis* DNA isolated from culture H37Rv as positive control (A kind donation from Dr. Ajay

Kumar, scientist G, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram) and nuclease free water as negative control. The PCR reaction mixture and cycling conditions are given below

Components of PCR reaction mixture.

Sl. No.	Name of the reagent	Quantity
1	Template DNA	3.00µl
2	Emerald Amp GT PCR Master Mix	12.5µl
3	Forward primer	1.00µl
4	Reverse primer	1.00µl
5	Nuclease free water	7.5µl
Total		**25.0µl**

Optimization of PCR for IS6110 gene (Kulkarni *et al*., 2012)

Initial	94°C	3 min	
Denaturation	94°C	1 min	35 cycles
Annealing	65°C	1 min	
Extension	72°C	1 min	
Final Extension	72°C	10 min	

3.5.4.5 Detection of PCR products by electrophoresis

After completion of PCR reaction, amplified products were subjected to electrophoresis in 1.5 per cent agarose gel with 6X gel loading dye. A 100 bp DNA ladder was used as molecular weight marker.

3.5.5 Sequencing of PCR products and phylogenetic analysis

Amplicon obtained from PCR was further confirmed by sequencing the

product. DNA sequence of the amplicon was obtained by outsourcing the product to M/s Agrigenome, Kochi. Amplicons of two positive sample were sequenced. The sequence was subjected to BLAST analysis and phylogenetics of the sequence by neighbour joining method was worked out with Clustal W alignment and replications with 1000 bootstrap values as per Takamura-3 parameter model.

3.6 PROTEOMIC PROFILING OF URINE FOR IDENTIFICATION OF BIOMARKERS

3.6.1 Urine protein precipitation

Urine protein was precipitated by phenol extraction protocol.

3.6.1.1 Reagent required for urine protein precipitation

a. Extraction buffer (pH 8)

- 0.5M Tris buffer 60.55g
- 50mM EDTA 18.61g
- 700mM Sucrose 239.6g
- 100mM Potassium Chloride 7.4g
- DW up to 1000ml

b. Working extraction buffer (pH 8)

- 0.5M Tris buffer 60.55g
- 50mM EDTA 18.61g
- 700mM Sucrose 239.6g
- 100mM Potassium Chloride 7.4g
- 2% beta mercaptoethanol 20ml
- 1mM PMSF 10ml
- DW up to 1000ml

2 % mercaptoethanol and 1mM PMSF are added just before use.

c. Precipitation solution

- 0.1M Sodium acetate 8.2g
- Methanol up to 1000ml

Store at -20°C

d. Phenol tris equilibrated 100ml

e. Acetone 100ml

3.6.1.2 Procedure for urine protein precipitation

a. Urine sample (10 ml) was mixed with 20 ml of working extraction buffer.

b. Samples were vortexed. Phenol tris equilibrated (15 ml) was added, and solutions were incubated on a shaker for 10 min at room temperature.
c. To eliminate cell debris, samples were centrifuged at 5500rpm at 4 °C for 10 min.
d. The upper phenolic phase was transferred to a new tube, and back-extracted with 40 ml of extraction buffer (1:9 ratio).
e. Samples were incubated on a shaker for 10 min, vortexed and centrifuged at 5500rpm at 4 °C for 10 min.
f. The phenol phase was carefully transferred to a new tube and four volumes of chilled precipitation buffer was added. Tubes were mixed by inversion, and samples were incubated overnight at -20 °C.
g. Proteins were pulled down by centrifugation at 5500rpm at 4 °C for 10 min.
h. After centrifugation, the pellets were washed three times with ice-cold precipitation buffer and centrifuged for 10 min at 5500rpm at 4 °C.
i. The pellets were then washed with ice-cold acetone for 5 min at 5500rpm at 4 °C.
j. Pellets were dried under air at room temperature for 10 min.

3.6.2 Urine protein solubilisation

The protein precipitated from urine is dissolved in a solubilisation buffer.

3.6.2.1 Reagents required (solubilisation buffer)

The following reagents should be mixed together and stored at -20°C

a. 7M Urea 2.1 g
b. 2M Thiourea 760 mg
c. CHAPS 4%
(3- [(3- cholamidopropyl) dimethylammonio]-1-propanesulfonate)
d. 30mM Tris 180 mg
e. DW up to 5 ml

3.6.2.2 Procedure

Protein pellets were dissolved in 100µL of solubilisation buffer. Samples were incubated in solubilisation buffer for at least 1 hour at room temperature.

3.6.3 Quantification of urine protein

Protein concentration was determined by the Bradford protein quantification micro assay using a UV/Vis spectrophotometer UV-4 (Biorad, USA).

To determine the level of protein in urine, protein of known concentration was loaded in the spectrophotometer in serial dilutions and visible absorbance spectra were recorded at 595nm.

3.6.3.1 Reagents required (Bradford reagent)

The reagent was prepared freshly each time with following constituents.

a. Coomassie brilliant blue (R-250) 25.0 mg
b. Methanol (95 %) 12.5 ml
c. Ortho phosphoric acid 25.0 ml
d. DW up to 250.0 ml

3.6.3.2 Procedure for quantification of urine protein

250µl of Bradford reagent (Biorad, USA) was added to an aliquot of 50µl protein sample to be assayed, after ten min of adding the reagents; the absorbance was measured at 595 nm. Finally, the protein content was calculated by comparing the absorbance value with that of protein with known concentration in serial dilution from 10µg/µl to 0.625µg/µl.

3.6.4 Quality analysis of urine protein by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein quality was analysed by Electrophoresis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a mini vertical gel system (15 % T, 2.67 % C) with the buffer system of Laemmli (1970).

3.6.4.1 Reagents required

a. Solution A (Acrylamide and bisacrylamide stock)
- Acrylamide 29.2 g
- Bisacrylamide 0. 8 g
- DW up to 100.0 ml

b. Solution B (1.5 M Tris Cl, pH 8.8)
- Tris base 18.2 g
- Hydrochloric acid up to 2.0 ml
- DW up to 100.0 ml

c. Solution C (0.5 M Tris Cl, pH 6.8)
- Tris base 6.1 g
- Hydrochloric acid 4.2 ml
- DW up to 100.0 ml

d. Solution D (10 % Ammonium per sulphate)
- Ammonium per sulphate 0.1 g
- DW 1.0 ml

e. TEMED (N, N, N, N - Tetra methylene diamine)
f. Low range protein marker (SRL- BioLit BLM005)
g. Separating gel (12%)

- Solution A 4.0 ml
- Solution B 2.5 ml
- 10 percent SDS 100 µl
- Solution D 100 µl
- TEMED 10.00 µl
- DW 3.30 ml

h. Stacking gel (5%)

- Solution A 670 µl
- Solution C 1.25 ml
- 10 percent SDS 50.0 µl
- Solution D 50.0 µl
- TEMED 5.0 µl
- DW 2.9 ml

i. Running buffer (1X)

- Tris base 3.0 g
- Glycine 14.40 g
- SDS 1.0 g
- DW up to 1000ml

j. Sample buffer (2X)

- 10 percent SDS 4.0 ml
- 2-mercaptoethanol 0.50 ml
- Solution C 2.50 ml
- Glycerol 2.30 ml
- Bromophenol blue (0.05%) 50µl
- DW 70 ml

k. Staining solution

Coomassie Brilliant Blue (R 250)	200mg
Methanol	50ml
Glacial acetic acid	10ml
DW	up to 100ml

The stain was filtered through Whatman No.1 filter paper to remove any particulate matter.

l. Destaining solution

- Methanol 40ml
- Glacial Acetic acid 10ml
- DW up to 100ml

Prepared fresh, immediately before use

3.6.4.2 Gel Cassette Preparation

3.6.4.2.1 Glass cassette and casting stand assembly

The casting frame was placed upright with the pressure cams in the open position and facing forward on a flat surface. Spacer plate and short plate were placed on top of each other and oriented the spacer plate so that the labelling is "up". The two glass plates were slid into the casting frame, keeping the short plate facing the front of the frame. Once the glass plates were in place, the pressure cams were engaged to secure the glass cassette sandwich in the casting frame.

Casting frame was placed on the casting stand by positioning the casting frame (with the locked pressure cams facing out) onto the casting gasket while engaging the spring-loaded lever of the casting stand onto the spacer plate. After placing the casting frame, the assembly was tested for presence of leaks by pouring a small amount of isopropanol in between the spacer plate and short plate and was made sure that no leakage was found. A comb was placed completely into the assembled gel cassette and a mark was made 1 cm below the comb teeth indicating the level to which the resolving gel was to be poured and the comb was removed.

3.6.4.2.2 Gel preparation

The resolving gel monomer solution was prepared and poured up to the mark using a disposable plastic pipette. The solution was poured smoothly to prevent it from mixing with air. Immediately the monomer solution was overlaid with isopropanol and allowed the gel to polymerize for 45 min. The gel surface was rinsed with water. The top of the resolving gel was dried using a blotting paper and on to which stacking gel was added. Desired comb was inserted between the spacers starting at the top of the spacer plate, making sure that the tabs at the ends of each comb were guided between the spacers. The comb was seated in the gel cassette by aligning the comb ridge with the top of the short plate and the stacking gel was allowed to polymerize for 30 to 45 min. The comb was removed and was rinsed the wells thoroughly with running buffer.

Plates were removed from casting assembly and placed in electrophoresis apparatus. Running buffer was filled in the inner tank and up to indicated mark in the outer tank.

3.6.4.2.3 Sample preparation and loading

An aliquot of the sample corresponding to the required amount of protein (25mg) was mixed with $1/4^{th}$ volume of sample buffer and heated to $100^{o}C$ in a dry bath for five min. Each sample was then carefully charged into each of the 48 wells

of the stacking gel using a micropipette. Similarly, one of the wells was loaded with standard protein molecular weight marker (MW), after keeping in the water bath for 100ºC for five min. Electrophoresis was carried out at a constant current of 10 mA, 50 V for the stacking gel and 15 mA, 100 V for the resolving gel, until the dye front reached the lower end of the gel.

3.6.5 Staining and De-staining of the Gel

After the electrophoresis was over, the plates were removed and gel was taken out and immersed in staining buffer for one hour. This was followed by immersing in destaining solution. Destaining solution was changed every two hours and was replaced with double distilled water and observed under gel documentation system.

3.6.6 Preparation of protein and peptides for mass spectrometry analysis

For in solution digestion, 15µg of pooled protein sample of TB positive elephants confirmed by DPP® (Dual Path Platform) VetTB, Alere Determine^{TM} TB LF-LAM assay and PCR (n=6) and 15µg of pooled protein sample of healthy elephants (n=6) were subjected for enzymatic digestion and followed by high resolution liquid chromatography mass spectrometry.

3.6.6.1 Enzymatic digestion

Proteins of interest were digested with trypsin. It cleaves proteins into peptides with an average size of 700-1500 Daltons.

3.6.6.1.1 Reduction and alkylation

The precipitated protein (1µg/µl) of 15µl volume was transferred in to an eppendorf tube. For the reduction reaction 2µl of DDT (85mM) and 15µl of ammonium bicarbonate (50mM) were added into the precipitated protein. At 56˚C the mixture was incubated for 40 min. For the alkylation reaction to take place 7µl of indole-3- acetic acid (55mM) and 12µl of ammonium bicarbonate (50mM) were added. The mixture was incubated in a dark room at room temperature for 30 min. Precipitation of the mixture was prepared using 126µl of ice cold acetone and vortexed for uniform concentration. The precipitated mixture was incubated at -20˚C for overnight. The mixture is thawed and centrifuged at 15000 rpm for 10 min. The supernatant were separated and sediment was allowed to dry for 5 min. The formed pellet was resolublized with 27µl of Ammonium bicarbonate (50mM) solution.

3.6.6.1.2 Trypsin digestion

For the digestion process 1.65µl of trypsin and 1.35µl ammonium bicarbonate (50mM) were added to achieve 550ng/µl concentration of trypsin. To

this mixture 132µl of 100% acetonitrite (LC-MS grade) was also added. Incubation was carried out at 37˚C for 3 hours. The reaction was subsequently stopped with 5 per cent formic acid (LC-MS grade). Obtained peptides were vacuum dried and desalted by zip tip and stored at -80°C.

3.6.6.2 Clean up or desalting of the final peptide mixture

Interfering substances such as detergents, salts, lipids and nucleic acids were removed from the precipitated urinary protein preparations using 2D-Clean Up kit (GE Healthcare, USA). The pellet was rehydrated in the same 2D-DIGE lysis buffer and total protein concentration was estimated using 2D-quant kit (GE Healthcare, USA) as per the manufacturer's instruction

3.6.7 High Resolution Liquid Chromatography Mass Spectrometry (HR-LCMS)

The digested peptides were reconstituted in 0.1 per cent formic acid in LCMS grade water and subjected to nano-LC (Nano-Advance, Bruker, Germany) followed by identification by captive spray-Maxis-HD qTOF (Bruker, Germany) mass spectrometer (MS) with high mass accuracy and sensitivity. The peptides were enriched in nano-trap column (Bruker Magic C18AQ, particle size-5 µm, pore size-200 Å) and eluted on to analytical column (Bruker Magic C18AQ, 0.1 × 150 mm, 3 µm particle size and 200 Å pore size) using linear gradient of 5–45 per cent acetonitrile at 800 nl/min over 55 min. Positive ions were generated by electro spray and the QTOF operated in data dependent acquisition mode. A TOF MS survey scan was acquired (m/z range of 400–1400) and 6 most intense were sequentially selected by Q1 for MS-MS analysis. HR-LCMS was carried out in Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay.

Software analysis: Identification of peptide from MS was performed from global data base and instrument based software. Enumeration of biomolecules specific to particular phase and putative pathway analysis were evaluated by suitable software. Similarly for protein bioinformatics, calculator available at SIB based ExPASy bioinformatics resource portal (http://web.expasy.org/cgi-bin/compute_pi/pi_tool), and bioinformatics software like PANTHER were used. Molecular and biological function analysis of each identified proteins were assessed by predict protein tool. It ascribed some common functions to all the five differentially expressed proteins. Further functional analysis were assessed with gene ontology programme Blast2GO (B2G) which aided to identify their putative role in combined biological process, molecular function and sub-cellular localization.

3.7 STATISTICAL ANALYSIS

The statistical analyses were performed using IBM SPSS version 24. The

performance of LF- LAM assay and Acid fast staining tests were evaluated on the basis of indices such as sensitivity, specificity, positive predictive value and negative predictive value by taking DPP VetTB assay as standard. Cohen's kappa statistic (κ) was estimated to find out the agreement between the tests. Descriptive statistics of various study parameters were expressed in mean and standard error for continuous variable and in frequency and percentage for categorical variables. The statistical difference between haematological, serum biochemical parameters were assessed using independent t test and for urine test parameters Chi-square test of independence were adopted.

Plate 1. Blood sample collection

Plate 2. Trunk wash sample collection

Plate 3 urine sample collection

Plate 4 Orphee mythic 18vet - automated haematological analyser

Plate 5. Master T, Hospitex diagnostic Italy – semi automated biochemical analyser

Plate 6 urine dipstick

Plate 7. Separation or organic phase in phenol precipitation

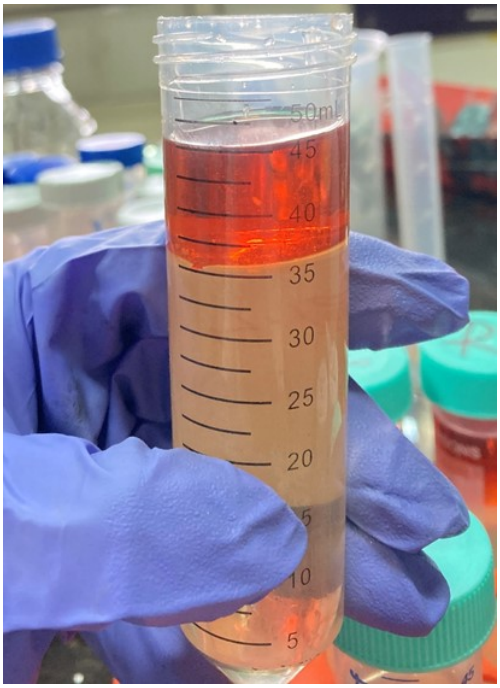


Plate 8 protein pellet formed after phenol precipitation

Plate 9 protein sample loaded for Bradford quantification method

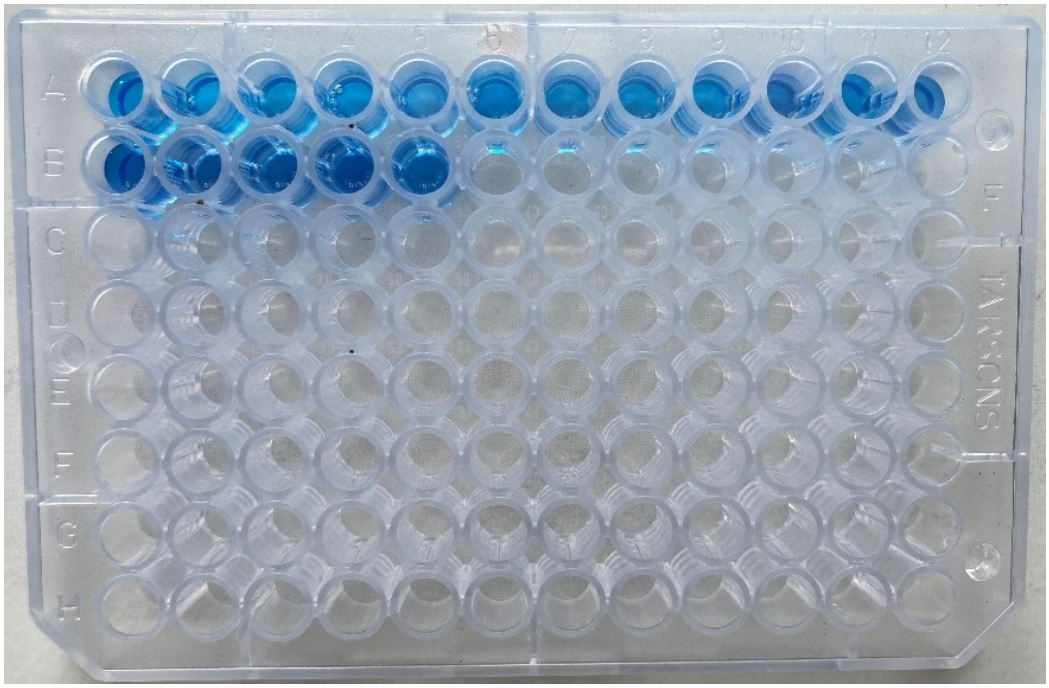
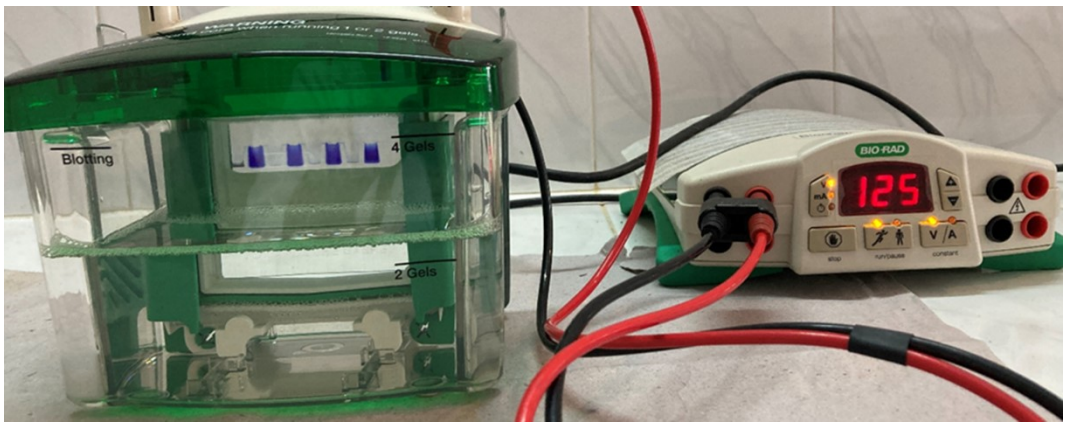


Plate 10 SDS-PAGE apparatus – Biorad



Results

4. RESULT

The present study was conducted in the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Pookode, Wayanad during the period of September 2019 to December 2020. A total of 86 captive Asian elephants from Kerala formed the subject of the study.

4.1 SCREENING USING LATERAL FLOW ASSAYS

4.1.1 Screening using DPP® (Dual Path Platform) VetTB - Elephant assay kits by Chembio Diagnostic Systems, INC.

The sera from 86 elephants were screened for tuberculosis. Sera of 32 (37.2 per cent) out of 86 elephants were seropositive and 54 (62.8 per cent) out of 86 elephants were seronegative for tuberculosis (Table 1) (Fig 1) (Plate 11 and 12).

4.1.2 Screening using Alere Determine™ TB LAM Ag assay (LF-LAM)

Urine samples from 86 elephants were screened for mycobacterium. Urine of 29 (33.7 per cent) out of 86 elephants were positive and 57 (66.3 per cent) out of 86 elephants were negative for Lipoarabinomannan (LAM) antigen of mycobacterium (Table 2) (Fig 2) (Plate 13 and 14).

On comparison with the standard DPP VetTB assay, LF- LAM assay has the sensitivity of 90 .63 per cent, specificity 100 per cent , positive predictive value 100 per cent, negative predictive value 94.7 per cent, accuracy 95.51 per cent and kappa statistic value 0.924 (p - value <0.001) (Table 3) (Fig 3).

On comparison with the Acid Fast Bacilli (AFB) staining method, LF- LAM assay has the sensitivity of 100 per cent, specificity 74.03 per cent , positive predictive value 31 per cent, negative predictive value 100 per cent, accuracy 76.74 per cent and kappa statistic value 0.374 (p - value <0.001) (Table 4) (Fig 4).

4.1.3 Screening using acid fast staining technique

Trunk wash smear from 86 elephants were screened for acid fast bacilli using acid fast staining method. Smear of 9 (10.46 per cent) elephants were found to be positive for acid fast bacilli and remaining 77 (89.54 per cent) elephants were negative for acid fast bacilli (Table 5) (Fig 5).

On comparison with DPP VetTB assay, acid fast staining shows sensitivity of 28.13 per cent, specificity 100 per cent, positive predictive value 100 per cent, negative predictive value 70.1 per cent, accuracy 73.26 per cent and kappa statistics value of 0.329 (p-value <0.001) (Table 6)(Fig 6).

4.2 CLINICAL PATHOLOGY

The 32 Elephants with positive seroreactivity for tuberculosis in DPP VetTB assay were considered as TB seropositive group and 32 elephants which were negative in DPP VetTB assay, LF-LAM, AFB staining were considered as control group.

4.2.1 Haematological evaluation

The mean value of leukocyte count (TLC) of TB seropositive group was found to be 23.15 ± 0.5 $\times 10^3/\mu l$. Out of 32 seropositive, five (15.62 per cent) infected elephants had TLC greater than 26 $\times 10^3 \mu l$, 22 (68.75 per cent) seropositive elephants had TLC ranging 21 × 10^3 to 26 $\times 10^3/\mu l$ and five (15.62 per cent) seropositive elephants had TLC below 20 $\times 10^3$ /µl. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean TLC of TB seropositive and control group (Table 7) (Fig 7a).

The mean value of absolute lymphocyte count of TB seropositive group was found to be 14.43 ± 0.5 $\times 10^3/\mu l$. Out of 32 seropositive three (9.3 per cent) seropositive elephants had lymphocyte count below 10 $\times 10^3/\mu l$, 19 (59.37 per cent) seropositive elephants had lymphocyte count ranging 10 $\times 10^3/\mu l$ to 16 $\times 10^3/\mu l$ and ten (31.25 per cent) seropositive elephants had lymphocyte count greater 16 $\times 10^3/\mu l$. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean absolute lymphocyte count of TB seropositive and control group (Table 7) (Fig 7a).

The mean value of absolute monocyte count of TB seropositive group was found to be 3.08 ± 0.15 $\times 10^3/\mu l$. Out of 32 seropositive, 17 (53.12 per cent) seropositive elephants had monocyte count below 3 $\times 10^3/\mu l$. Seven (21.87 per cent) seropositive elephants had monocyte count ranging 3 × $10^3/\mu l$ to 4 $\times 10^3/\mu l$ and eight (25 per cent) seropositive elephants had monocyte count greater 16 $\times 10^3/\mu l$. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean absolute monocyte count of TB seropositive and control group (Table 7) (Fig 7a).

The mean value of absolute granulocyte count of TB seropositive group was found to be 5.64 ± 0.33 $\times 10^3/\mu l$. Out of 32 seropositive, 15 (46.87 per cent) seropositive elephants had granulocyte count below 5 $\times 10^3/\mu l$. 11 (34.37 per cent) seropositive elephants had granulocyte count ranging 5 $\times 10^3/\mu l$ to 8 $\times 10^3/\mu l$ and six (18.75 per cent) seropositive elephants had granulocyte count greater 8 $\times 10^3/\mu l$. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean absolute granulocyte count of TB seropositive and control group (Table 7) (Fig 7a).

The mean value of erythrocyte count (TEC) of TB seropositive group was

found to be 3.34 ± 0.13 × 10^6/µl. Out of 32 seropositive, nine (28.12 per cent) seropositive elephants had TEC less than 2.3 ×10^6/µl. 15 (46.87 per cent) seropositive elephants had TEC ranging 3 × 10^6/µl to 4 ×10^6/µl and eight (25 per cent) seropositive elephants had TEC greater than 4 ×10^6/µl. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean TEC of TB seropositive and control group (Table 7) (Fig 7a).

The mean value of haemoglobin (Hb) of TB seropositive group was found to be 11.73 ± 0.59g/dl. Out of 32 seropositive, nine (28.12 per cent) seropositive elephants had Hb below 10g/dl. 16 (50 per cent) seropositive elephants had Hb ranging 11g/dL to 14 g/dl and seven (21.8 per cent) infected elephants had Hb higher than 15g/dl. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean Hb of TB seropositive and control group (Table 7) (Fig 7a).

The mean value of haematocrit (HCT) of TB seropositive group was found to be 41.97 ± 2.01 per cent. Out of 32 seropositive, nine (28.12 per cent) seropositive elephants had HCT below 11 per cent. 16 (50 per cent) seropositive elephants had HCT ranging 11 per cent to 14 per cent and seven (21.8 per cent) seropositive elephants had HCT higher than 15 per cent. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean HCT of TB seropositive and control group (Table 7) (Fig 7a).

The mean value of mean corpuscular volume (MCV) of TB seropositive group was found to be 124.25 ± 1.6 fL. Out of 32 seropositive, three (9.3 per cent) seropositive elephants had MCV below 110 fL, 21 (65.62 per cent) seropositive elephants had MCV ranging 110 to 130 fL and eight (25 per cent) seropositive elephants had MCV higher than 130 fL. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean MCV of TB seropositive and control group (Table 7) (Fig 7a).

The mean value of mean corpuscular haemoglobin (MCH) of TB seropositive group was found to be 34.59 ± 0.6 pg. Out of 32 seropositive, eight (25 per cent) seropositive elephants had MCH below 30 pg. Seven (21.87 per cent) seropositive elephants had MCH ranging 30 to 36 pg and 17 (53.12 per cent) seropositive elephants had MCH higher than 36 pg. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean MCH of TB seropositive and control group (Table 7) (Fig 7b).

The mean value of mean corpuscular haemoglobin concentration (MCHC) of TB seropositive group was found to be 27.79 ± 0.18 g/dl. Out of 32 seropositive, eight (25 per cent) seropositive elephants had MCHC below 27 g/dl. Ten (31.25 per cent) seropositive elephants had MCHC ranging 27 g/dl to 28 g/dl and 14 (43.75

per cent) seropositive elephants had MCHC higher than 28 g/dl. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean MCHC of TB seropositive and control group (Table 7) (Fig 7b).

The mean value of platelet count (PLT) of TB seropositive group was found to be 1388.25 ± 139.47 $\times 10^3$µl. Out of 32 seropositive, eight (25 per cent) seropositive elephants had PLT below than 900 $\times 10^3$µl. 11 (34.37 per cent) seropositive elephants had PLT ranging 900 × 10^3µl to 2000 $\times 10^3$µl and six (18.75 per cent) seropositive elephants had PLT higher than 2000 $\times 10^3$µl. Statistical analysis using independent 't' test revealed that there were no significant difference between mean PLT of TB seropositive and control group (Table 7) (Fig 7b).

The mean value of erythrocyte sedimentation rate (ESR) of TB seropositive group was found to be 4.85 ± 0.15 mm/hr. Out of 32 seropositive, four (12.5 per cent) seropositive elephants had ESR below 4 mm/hr. 12 (37.5 per cent) seropositive elephants had ESR ranging 4 mm/hr to 5 mm/hr and 16 (50 per cent) seropositive elephants had ESR higher than 5 mm/hr. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean ESR of TB seropositive and control group (Table 7)(Fig 7b)

4.2.2 Serum biochemical evaluation

The mean value of blood urea nitrogen (BUN) of TB seropositive group was found to be 11.87 ± 0.1 mg/dl. Out of 32 seropositive, 19 (59.37 per cent) seropositive elephants had BUN ranging between 11 to 12 mg/dl and 13 (40.62 per cent) seropositive elephants had BUN higher than 12mg/dl. Statistical analysis using independent 't' test revealed that there was no significant difference between the mean BUN of TB seropositive and control group (Table 8)(Fig 8a).

The mean value of creatinine of TB seropositive group was found to be 2.08 ± 0.08 mg/dl. Out of 32 seropositive, 18 (56.25 per cent) seropositive elephants had creatinine ranging 1 mg/dl to 2 mg/dl and 14 (43.75 per cent) seropositive elephants had creatinine higher than 2 mg/dl. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean creatinine level of TB seropositive and control group (Table 8) (Fig 8a).

The mean value of total bilirubin of TB seropositive group was found to be 0.91 ± 0.08 mg/dl. Out of 32 seropositive, 18 (56.25 per cent) seropositive elephants had total bilirubin ranging from 0.1 mg/dl to 1 mg/dl and 14 (43.75 per cent) seropositive elephants had total bilirubin higher than 1 mg/dl. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean total bilirubin of TB seropositive and control group (Table 8) (Fig 8a).

The mean value of direct bilirubin concentration of TB seropositive group

was found to be 0.3 ± 0.03 mg/dl. Out of 32 seropositive, three (9.37 per cent) seropositive elephants had direct bilirubin below 0.1 mg/dl. 13 (40.62 per cent) seropositive elephants had direct bilirubin ranging from 0.1 mg/dl to 0.3 mg/dl and 16 (50 per cent) seropositive elephants had direct bilirubin higher than 0.3 mg/dl. Statistical analysis using independent‘t’ test, revealed that there were no significant difference between the mean direct bilirubin of TB seropositive and control group (Table 8) (Fig 8a).

The mean value of indirect bilirubin of TB seropositive group was found to be 0.61 ± 0.07 mg/dl. Out of 32 seropositive, 15 (46.87 per cent) seropositive elephants had indirect bilirubin below 0.5 mg/dl. 11 (34.37 per cent) seropositive elephants had indirect bilirubin ranging from 0.5 mg/dl to 1.00 mg/dl and six (18.75 per cent) seropositive elephants had indirect bilirubin higher than 1.00 mg/dl. Statistical analysis using independent ‘t’ test revealed that there were no significant difference between the mean indirect bilirubin of TB seropositive and control group (Table 8) (Fig 8a).

The mean value of glucose of TB seropositive group was found to be 157.35 ± 14.96 mg/dl. Out of 32 seropositive, eight (25 per cent) infected elephants had glucose below 100 mg/dl. 14 (43.75 per cent) seropositive elephants had glucose ranging 100 mg/dl to 200 mg/dl and ten (31.25 per cent) seropositive elephants had glucose higher than 200 mg/dl. The mean glucose level of TB seropositive group was found to be higher than control group. Statistical analysis using independent ‘t’ test revealed that there were significant difference ($p < 0.05$) in mean glucose level between TB seropositive and control group (Table 8) (Fig 8a).

The mean value of total protein concentration of TB seropositive group was found to be 9.1 ± 0.28 g/dl. Out of 32 seropositive, 19 (59.3 per cent) infected elephants had glucose below 10 g/dl. 12 (37.5 per cent) seropositive elephants had total protein ranging from 10 g/dl to 11 g/dl and one (3.12 per cent) seropositive elephants had total protein higher than 11 g/dl. The mean value of total protein in TB seropositive group is lower than that of control group. Statistical analysis using independent ‘t’ test revealed that there were significant difference ($p < 0.05$) in mean total protein level between TB seropositive and control group (Table 8) (Fig 8a).

The mean value of albumin of TB seropositive group was found to be 2.43 ± 0.12 g/dl. Out of 32 seropositive, seven (21.8 per cent) seropositive elephants had albumin below 2 g/dl. 20 (62.5 per cent) infected elephants had albumin ranging from 2 g/dl to 3 g/dl and five (15.6 per cent) seropositive elephants had albumin higher than 3 g/dl. The mean value of albumin in TB seropositive group is lower than that of control group. Statistical analysis using independent ‘t’ test revealed

that there were significant difference ($p < 0.05$) in mean albumin level between TB seropositive and control group (Table 8) (Fig 8a).

The mean value of globulin of TB seropositive group was found to be 6.67 ± 0.18 g/dl. Out of 32 seropositive, eight (25 per cent) seropositive elephants had globulin below 6 g/dl. 23 (71.87 per cent) infected elephants had globulin ranging from 6 g/dl to 8 g/dl and one (3.12 per cent) seropositive elephants had globulin higher than 8 g/dl. On statistical analysis using independent 't' test, revealed that there were no significant difference between the mean globulin of TB seropositive and control group (Table 8) (Fig 8a).

The mean value of albumin globulin ratio of TB seropositive group was found to be 0.36 ± 0.02. Out of 32 seropositive, seven (21.9 per cent) seropositive elephants had albumin globulin ratio below 0.3, 3 (40.6 per cent) seropositive elephants had globulin ranging from 0.3 to 0.4 and 12 (37.5 per cent) seropositive elephants had globulin higher than 0.4. The mean value of albumin globulin ratio in TB seropositive group is lower than that of control group. Statistical analysis using independent 't' test, revealed that there were significant difference ($p < 0.05$) in mean albumin globulin ratio level between TB seropositive and control group (Table 8) (Fig 8b).

The mean value of cholesterol of TB seropositive was found to be 33.64 ± 1.07 mg/dl. Out of 32 seropositive, nine (28.1 per cent) seropositive elephants had cholesterol below 20 mg/dl. 14 (43.75 per cent) seropositive elephants had cholesterol ranging from 30 mg/dl to 40 mg/dl and five (15.62 per cent) seropositive elephants had cholesterol higher than 40 mg/dl. Statistical analysis using independent 't' test revealed that there were no significant difference between the mean cholesterol of TB seropositive and control group (Table 8) (Fig 8b).

The mean value of aspartate aminotransferase (AST) of TB seropositive was found to be 23.4 ± 1.32 U/l. Out of 32 seropositive, 16 (50 per cent) seropositive elephants had AST below 21 U/l. Eight (25 per cent) seropositive elephants had AST ranging from 21 U/l to 30 U/l and eight (25 per cent) seropositive elephants had AST higher than 31 U/l. The mean glucose level of TB seropositive group was found to be higher than control group. Statistical analysis using independent't' test revealed that there were significant difference ($p < 0.05$) in mean albumin globulin ratio level between TB seropositive and control group (Table 8) (Fig 8b).

The mean value of alanine aminotransferase (ALT) of TB seropositive was found to be 17.4 ± 1.09 U/l. Out of 32 seropositive, four (12.5 per cent) seropositive elephants had ALT below 10 U/l. 15 (46.8 per cent) seropositive elephants had ALT ranging between 11 U/l to 20 U/l and 11 (34.37 per cent) infected elephants had ALT higher than 20 U/l. Statistical analysis using independent't' test revealed that

there were no significant difference between the mean ALT of TB seropositive and control group (Table 8) (Fig 8b).

The mean value of alkaline phosphatase (ALP) of TB seropositive group was found to be 262.23 ± 14.47 U/l. Out of 32 seropositive, three (9.37 per cent) elephants had ALP below 150 U/l, 24 (75 per cent) seropositive elephants had ALP ranging from 150 U/l to 350 U/l and five (15.62 per cent) seropositive elephants had ALP higher than 350 U/l. Statistical analysis revealed no significant difference between the mean ALP of TB seropositive and control group (Table 8) (Fig 8b).

4.2.3 Urine evaluation

4.2.3.1 Colour of urine

Out of 32 TB seropositive elephants, 12 (37.5 per cent) elephants had straw coloured urine, four (12.5 per cent) had amber coloured urine and 16 (50 per cent) had dark yellow colour. Statistical analysis revealed no significant difference in colour of the urine between TB seropositive and control group (Table 9) (Fig 11).

4.2.3.2 Appearance of urine

In TB seropositive group of 32 elephants, 29 (90.6 per cent) elephants had clear urine and three (9.37 per cent) had turbid urine. Statistical analysis revealed that there were significant difference ($p < 0.05$) in the appearance of the urine between TB seropositive and control group (Table 9) (Fig 12).

4.2.3.3 Detection of urine protein

In the urine of TB seropositive and healthy group all samples showed negative results for sulfosalicylic acid test. (Table 9).

4.2.3.4 Evaluation of urine using Dipstick

4.2.3.4.1 Leukocytes

In the urine of TB seropositive and control group trace amount of leukocytes were found in all 86 (100 per cent) elephants (Table 9) (Fig 9).

4.2.3.4.2 Nitrite

In the urine of TB seropositive group, out of 32 elephants nitrite was absent in nine (28.12 per cent) seropositive elephants and was present in 23 (71.8 per cent) seropositive elephants. Statistical analysis using chi square test revealed that there were significant difference ($p < 0.05$) in nitrite (presence or absence) between TB seropositive and control group (Table 9) (Fig 9).

4.2.3.4.3 Urobilinogen

Urobilinogen was absent in all 32 (100 per cent) elephants belonging to the TB seropositive and control group (Table 9).

4.2.3.4.4 Protein

There were trace amount of protein in 4 (12.5 per cent) elephants, moderate amount of protein was noticed in 4 (12.5 per cent) elephants belonging to TB seropositive group and 24 (75 per cent) elephants had no protein in urine (Table 9) (Fig 9).

4.2.3.4.5 pH of the urine

The mean of urine pH value of TB seropositive group was found to be 7.92 ± 0.21. Out of 32 seropositive, nine (28.2 per cent) seropositive elephants had pH below 7. Nine (28.2 per cent) seropositive elephants had pH ranging 7 to 8 and 14 (43.75 per cent) seropositive elephants had urine pH higher than 8. Statistical analysis using independent‘t’ test revealed that there were no significant difference in mean urine pH of TB seropositive and the control group (Table 10) (Fig 10).

4.2.3.4.6 Blood

Blood was absent in all 32 (100 per cent) elephants belonging to the TB seropositive and control group (Table 9).

4.2.3.4.7 Specific gravity

The mean of specific gravity of TB seropositive group was found to be 1.012 ± 0.009. Out of 32 seropositive, 24 (75 per cent) seropositive elephants had specific gravity between 1.005 to 1.02 and Eight (25 per cent) infected elephants had specific gravity 1.03. On statistical analysis using independent‘t’ test, revealed that there were no significant difference in mean specific gravity of TB seropositive and the control group (Table 10) (Fig 10).

4.2.3.4.8 Ketone bodies

Ketones were absent in all 32 (100 per cent) elephants belonging to the TB seropositive and control group (Table 9).

4.2.3.4.9 Bilirubin

In the urine of TB seropositive group, out of 32 elephants bilirubin was absent in 30 (93.75 per cent) seropositive elephants and was present in two (6.25 per cent) seropositive elephants (Table 9) (Fig 9).

4.2.3.4.10 Glucose

Glucose was absent in all 32 (100 per cent) elephants belonging to the TB

seropositive and control group (Table 9).

4.3 MOLECULAR DIAGNOSIS BY POLYMERASE CHAIN REACTION

Trunk wash samples from 32 seropositive elephants were subjected to PCR targeting gene IS*6110*, at 245bp size amplicon to confirm the presence of *Mycobacterium tuberculosis* (Plate 5). Out of 32 seropositive elephants, 25 (78.2 per cent) elephants were confirmed positive for *Mycobacterium tuberculosis* and 7 (21.8 per cent) were negative for *Mycobacterium tuberculosis* (Table 11) (Fig 13).

Table 11. Detection of *Mycobacterium tuberculosis* from trunk wash sample using PCR

4.3.1 Sequencing of PCR product and phylogenetic analysis

The trimmed FAST-ALL (FASTA) sequence was obtained from sequencing one representative sample from 25 PCR positives samples (TB positive samples) (Table 12).

On blasting the acquired trimmed sequence (E2) in National centre for biotechnology information (NCBI) showed that the attained sequence shows 98 per cent query cover and 100 per cent identity for E2 with other published *Mycobacterium tuberculosis* sequences in the database (Plate 6).

After custom sequencing and phylogenetic analysis revealed that the isolate obtained were *Mycobacterium tuberculosis*. The sequence showed homology with *Mycobacterium tuberculosis* strain 1-0013P6C4 chromosome (Plate 7).

4.4 PROTEOMIC PROFILING OF URINE FOR IDENTIFICATION OF BIOMARKERS

The urine of six TB confirmed elephants (positive for DPP VetTB assay, LF-LAM, AFB and PCR) and six healthy elephants (negative for DPP VetTB assay, LF-LAM, AFB and PCR) were selected for proteomic analysis. Six TB confirmed elephants were grouped under TB infected group and six healthy elephants were grouped under control group.

4.4.1 Quantification of urine protein

Protein concentration of TB infected elephants and healthy elephants were calculated by Bradford assay. The concentration of the urine protein was found to be in the range 3.49- 6.36 µg/µl (Table 13) (Fig 14).

4.4.2 Quality analysis of urine protein by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The precipitated urine protein of TB infected and control elephants was subjected to SDS- PAGE. TB infected elephants demonstrated major band

formation between 75- 100kDa, minor bands between 25-37kDa, 150kDa and 250kDa. On the contrary, there were no clear bands visible for healthy elephants (Plate 8).

4.4.3 High Resolution Liquid Chromatography Mass Spectrometry (HR-LCMS)

The protein precipitated from urine samples of TB infected and control elephants were subjected to high resolution liquid chromatography mass spectrometry analysis. Proteins identified in TB infected and control group were listed in the (Table 13) (Table 14) (Table 15) (Table 16).

Input proteins were submitted for accession number. For analysis the acquired proteins were uploaded in Gene Ontology (GO) consortium which is the database for the function of genes. Using (Protein Analysis through Evolutionary Relationship) PANTHER classification system identified proteins were classified based on subfamilies, molecular function, biological process, cellular component and pathways (Table 17 to 21) (Fig 19 and 20). Further after GO enrichment analysis the relationship of the proteins of interest with that of existing proteins in the database were determined in the phylogenetic tree. A hierarchical clustering tree summarizing the correlation among significant pathways listed in the enrichment tab. Pathways with many shared genes are clustered together (Fig 9 to 14). Bigger dots in the phylogenetic tree indicate the significant p - values.

Protein – protein interaction was determined by STRING consortium version 11. The physical and functional associations between proteins were obtained by comparing with the aggregated existing protein database in the consortium. Both the figures (Plate 15, 16) showed interactions with the confidence score ≥ 0.9.

Plate 11. DPP VetTB assay - Negative reaction

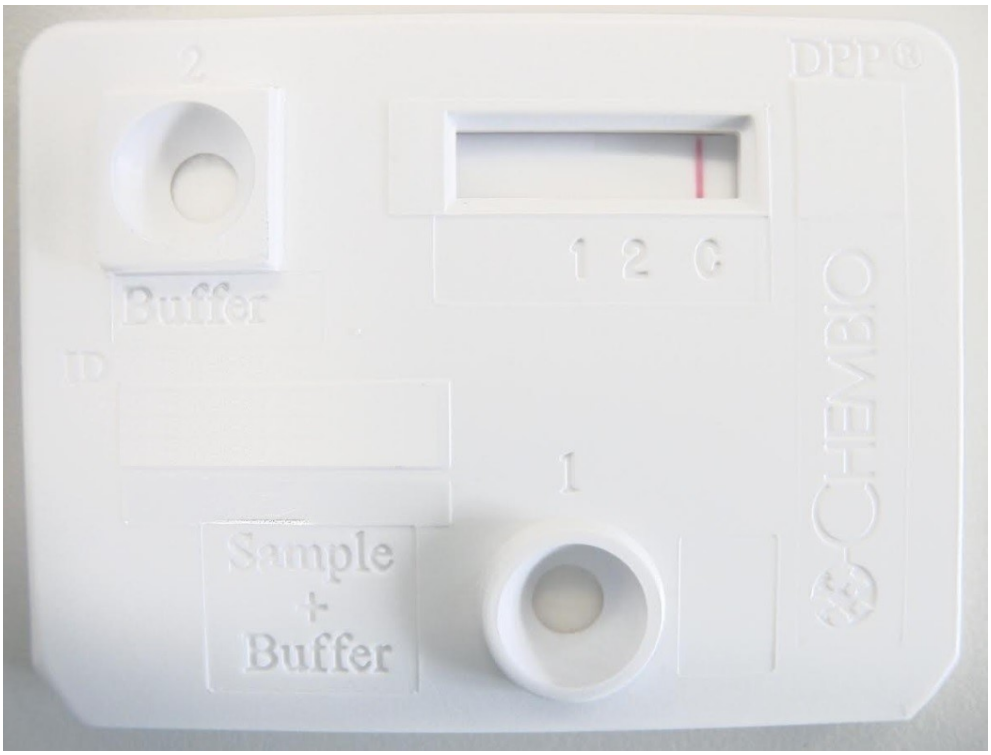


Plate 12. DPP VetTB assay - Positive reaction for TB

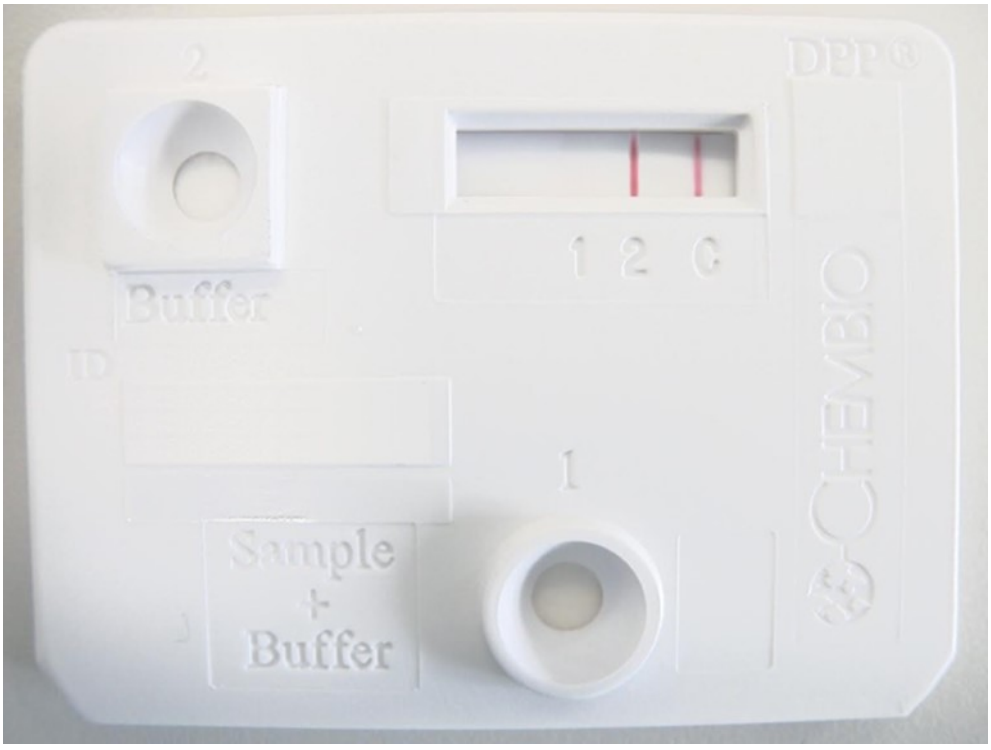


Table 1. Seroprevalence of tuberculosis of captive Asian elephants of Kerala

Sl. No	Seroprevalence	No. of elephants tested	Percentage
1	Positive	32	37.2
2	Negative	54	62.8
	Total	86	100

Fig 1. Seroprevalence of tuberculosis in captive Asian elephants in Kerala

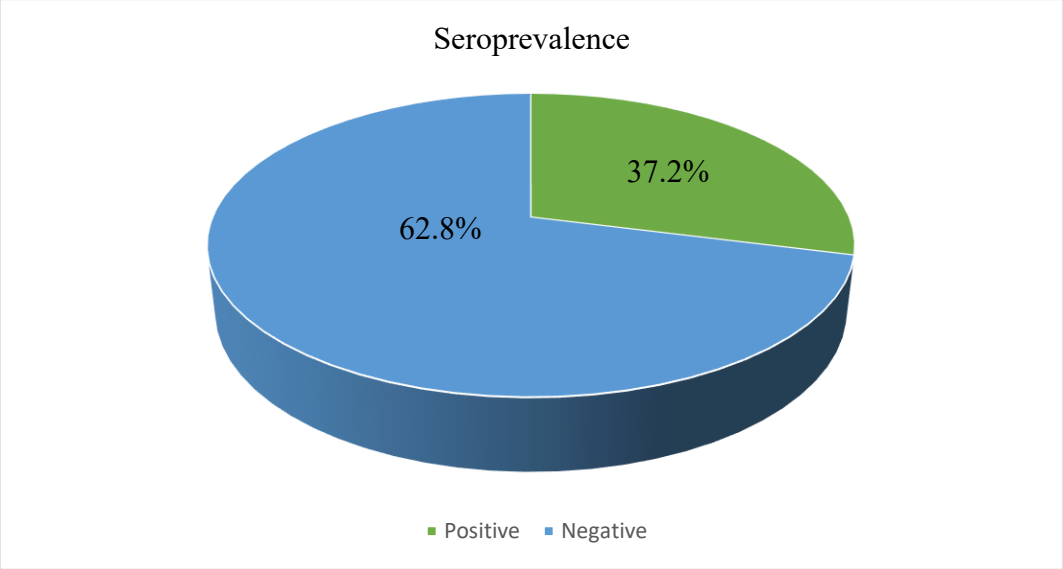


Plate 13. LF-LAM – Negative reaction

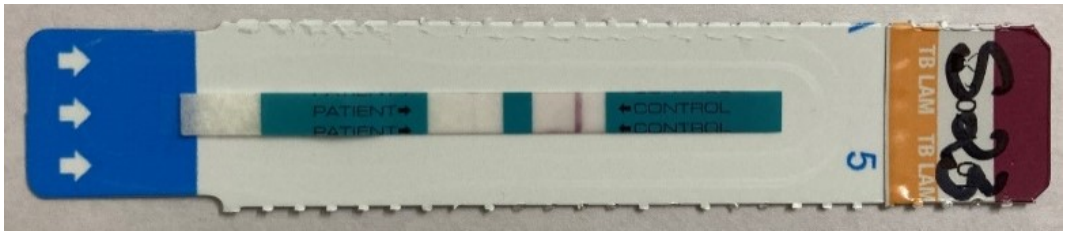


Plate 14. LF-LAM – Positive reaction

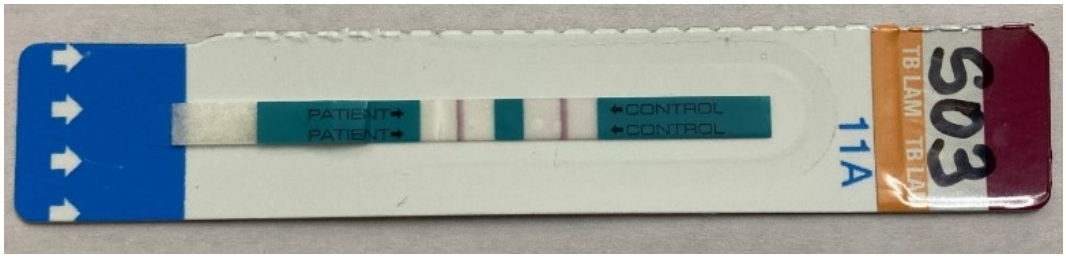


Table 2. LAM Antigen detection of mycobacterium in captive Asian elephants in Kerala

Sl .No	LF- LAM assay	No. of elephants tested	Percentage
1	Positive	29	33.7
2	Negative	57	66.3
	Total	86	100

Fig 2. LAM Antigen detection of mycobacterium in captive Asian elephants

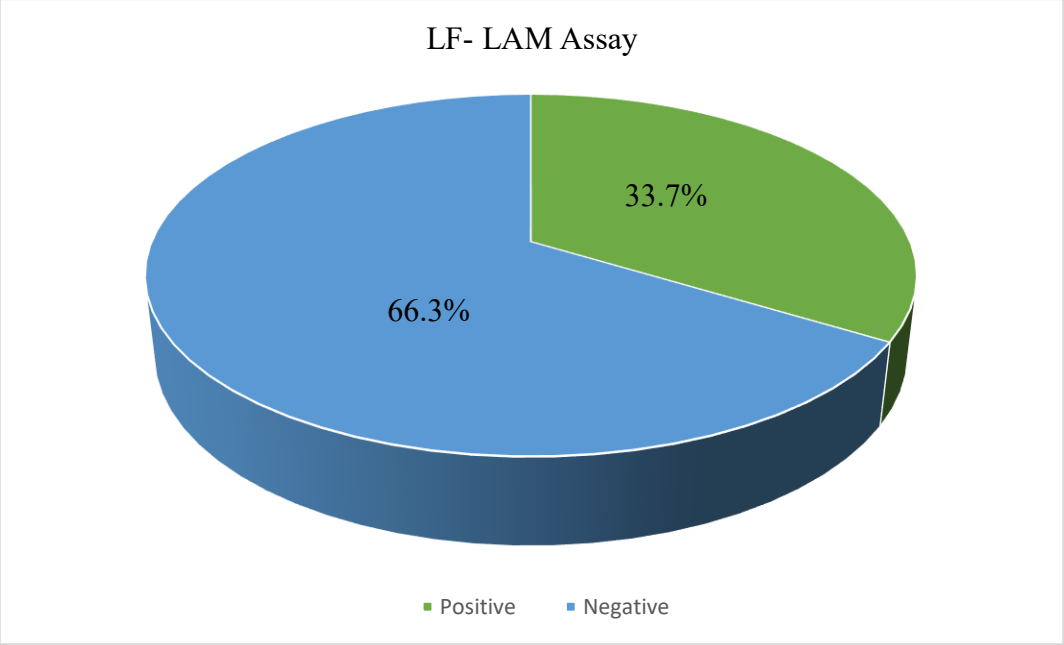


Table 3. Comparison of LF-LAM assay results with DPP VetTB Assay

LF-LAM result	**DPP VetTB Assay**					
	Negative		**Positive**		**Total**	
	Frequency	**Per cent**	**Frequency**	**Per cent**	**Frequency**	**Per cent**
Negative	54	100	3	9.4	57	66.3
Positive	0	0	29	90.6	29	33.7
Total	54	100	32	100	86	100

Fig 3. Comparison of LF-LAM assay with DPP VetTB assay

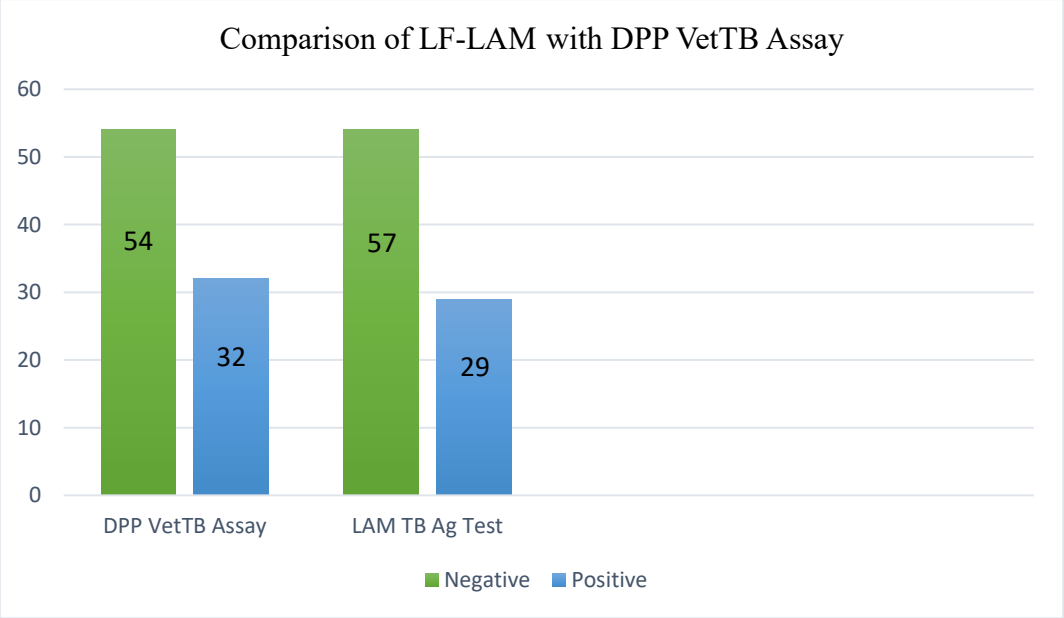


Table 4. Comparison of results of LF- LAM with AFB staining method

LF-LAM Assay	AFB staining result					
	Negative		Positive		Total	
	Frequency	Per cent	Frequency	Per cent	Frequency	Per cent
Negative	57	74.0	0	0	57	66.3
Positive	20	26.0	9	100	29	33.7
Total	57	100	29	100	86	100

Fig 4. Comparison of LF-LAM assay with AFB staining

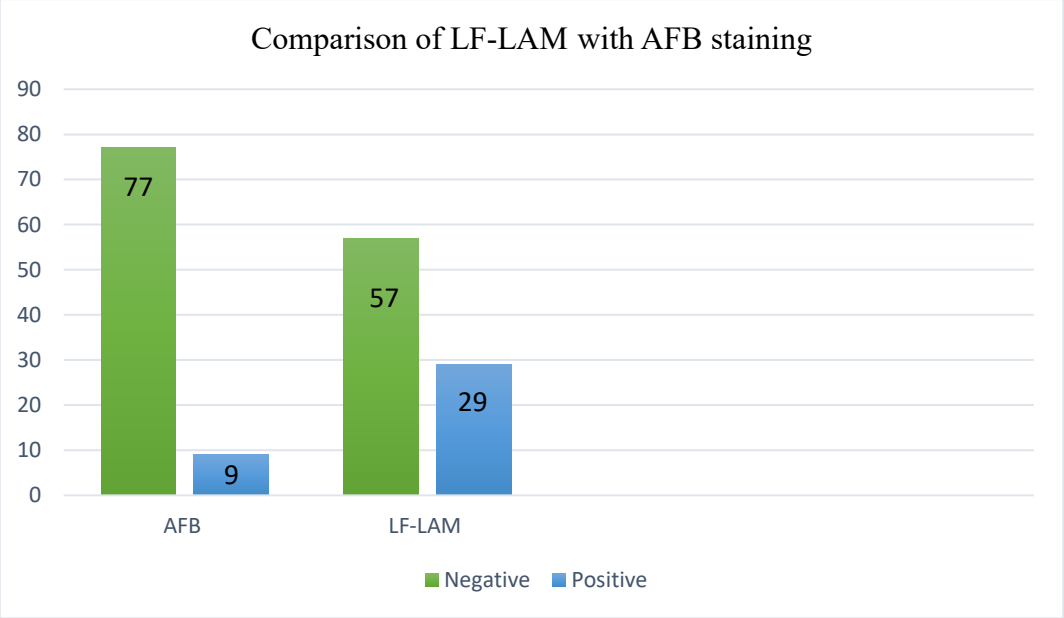


Table 5. Detection of acid-fast bacilli in captive Asian elephants

Sl. No	AFB Result	No. of elephants tested	Percentage
1	Positive	9	10.46
2	Negative	77	89.54
	Total	86	100

Fig 5. Detection of acid-fast bacilli in captive Asian elephants

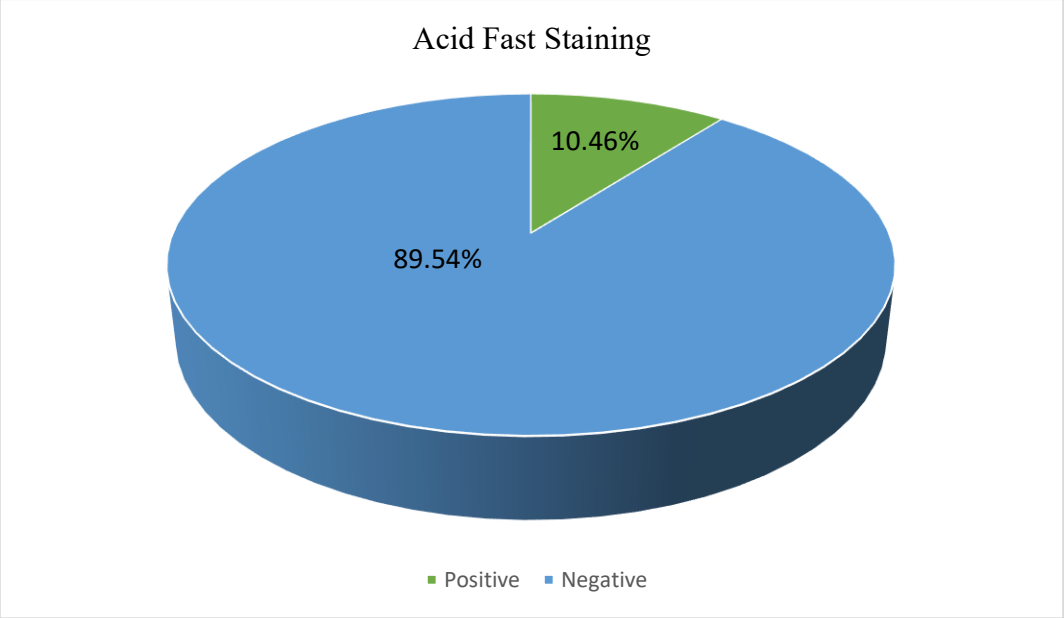


Table 6. Comparison of AFB staining results with DPP VetTB assay

AFB result	DPP VetTB Assay					
	Negative		Positive		Total	
	Frequency	Per cent	Frequency	Per cent	Frequency	Per cent
Negative	54	100	23	71.9	77	89.5
Positive	0	0	9	28.1	9	10.5
Total	54	100	32	100	86	100

Fig 6. Comparison of acid-fast staining with DPP VetTB assay

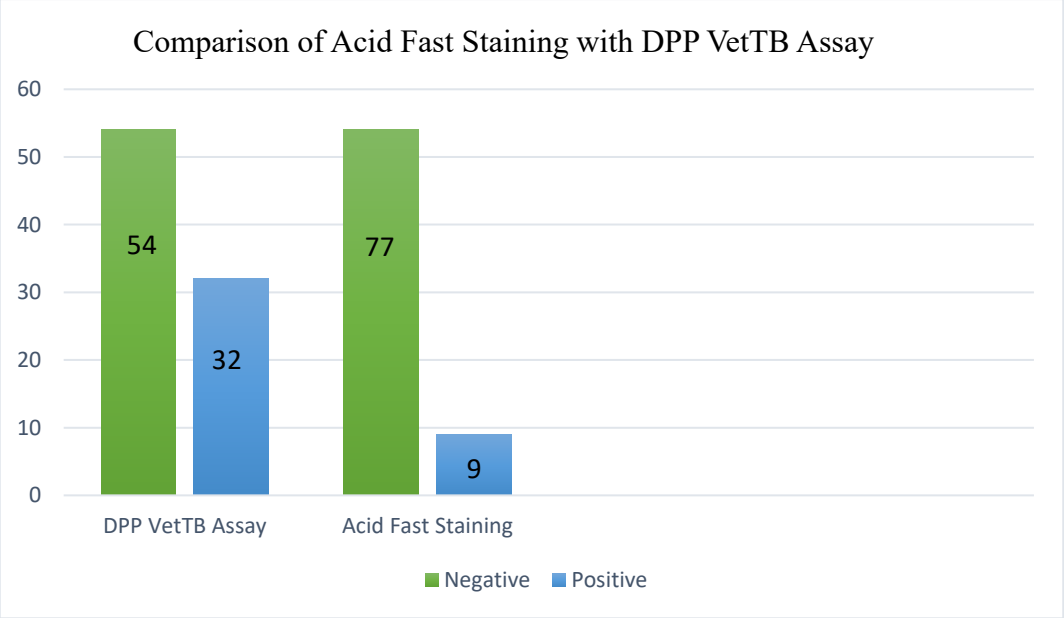


Table: 7 Comparison of haematological parameters between TB seropositive and control group

Sl. No.	Parameter	Unit	Observation		t value	p value
			TB Seropositive n = 32	Control group n = 32		
1	Total Leukocyte Count	$10^3/\mu l$	25.15 ± 0.5	24.14 ± 0.5	1.387^{ns}	0.170
2	Lymphocytes	$10^3/\mu l$	14.43 ± 0.5	14.15 ± 0.64	0.339^{ns}	0.736
3	Monocytes	$10^3/\mu l$	3.08 ± 0.15	3.39 ± 0.13	1.625^{ns}	0.109
4	Granulocytes	$10^3/\mu l$	5.64 ± 0.33	6.61 ± 0.37	1.963^{ns}	0.054
5	Lymphocytes	%	62.15 ± 1.5	58.18 ± 1.76	1.713^{ns}	0.092
6	Monocytes	%	13.67 ± 0.87	14.36 ± 0.69	0.625^{ns}	0.534
7	Granulocytes	%	24.18 ± 1.06	27.46 ± 1.43	1.837^{ns}	0.071
8	Total Erythrocyte Count	$10^6/\mu l$	3.34 ± 0.13	3.45 ± 0.11	0.683^{ns}	0.497
9	Haemoglobin	g/dl	11.73 ± 0.59	12.02 ± 0.44	0.398^{ns}	0.692
10	Haematocrit	%	41.97 ± 2.01	43.54 ± 1.52	0.625^{ns}	0.535
11	Mean Corpuscular Volume	fL	124.25 ± 1.6	125.56 ± 1.14	0.665^{ns}	0.509
12	Mean Corpuscular Haemoglobin	Pg	34.59 ± 0.6	34.6 ± 0.45	0.013^{ns}	0.99
13	Mean Corpuscular Haemoglobin Concentration	g/dl	27.79 ± 0.18	27.52 ± 0.16	1.114^{ns}	0.269
14	Platelet Count	$10^3/\mu l$	1388.25 ± 139.47	1067.22 ± 93.08	1.915^{ns}	0.06
15	Erythrocyte Sedimentation Rate	mm/hr	4.85 ± 0.15	4.65 ± 0.1	1.119^{ns}	0.267

ns non-Significant (P>0.05)

Fig 7a. Comparison of haematological Parameters between TB seropositive and control group

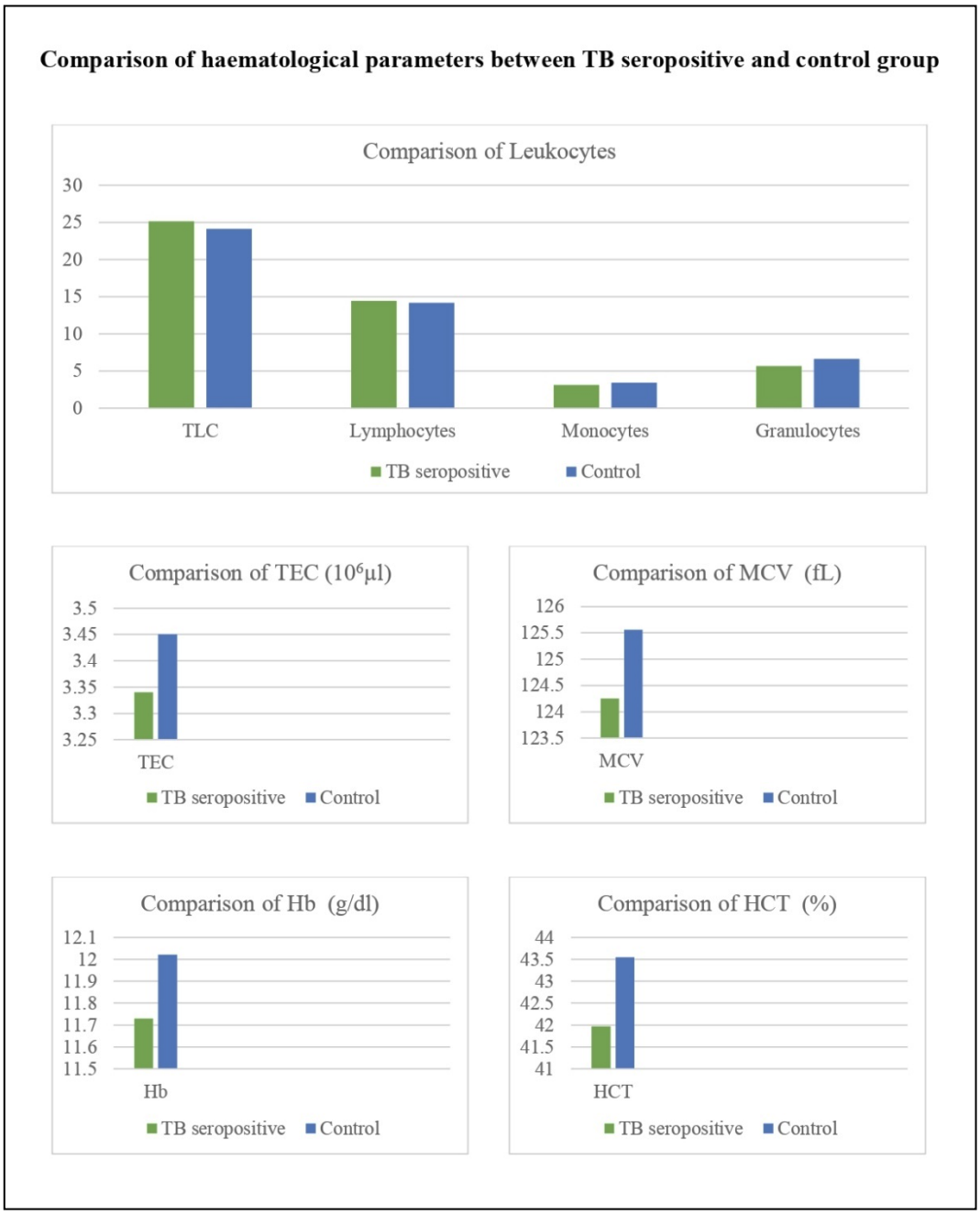


Fig 7b. Comparison of haematological Parameters between TB seropositive and control group

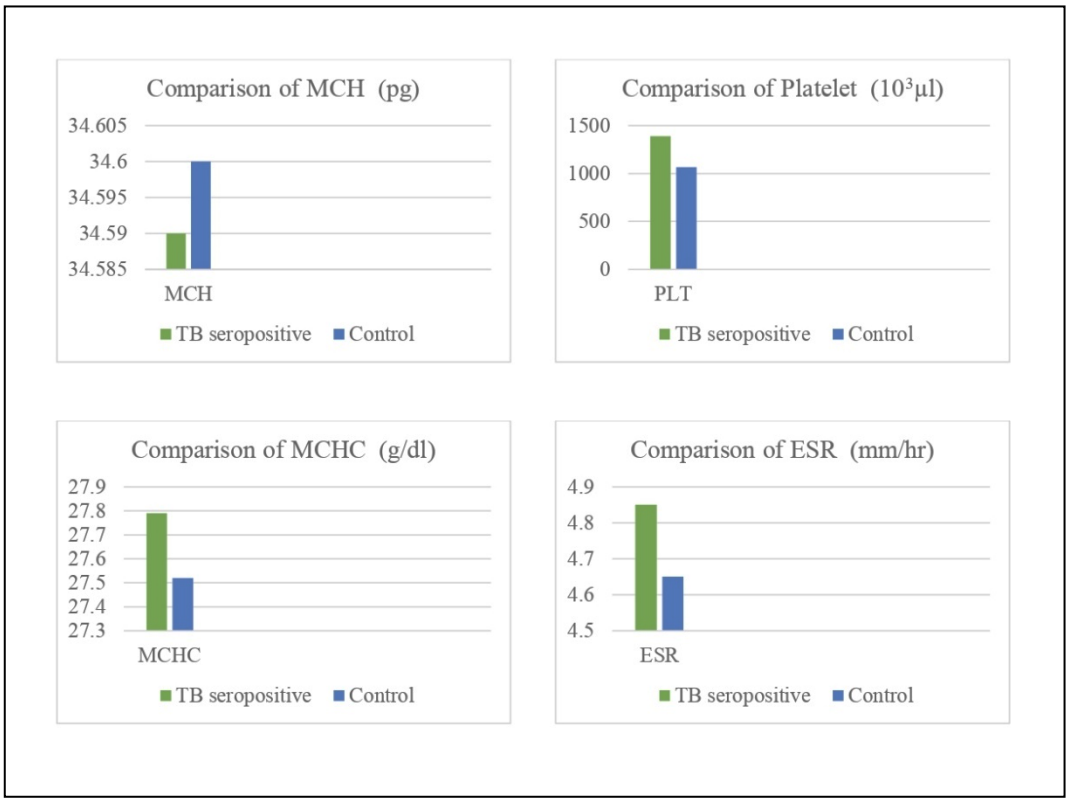


Table 8. Comparison of Serum Biochemical Parameters between TB seropositive and control group

Sl. No.	Parameters	Unit	Observation			
			TB seropositive group n = 32	Control group n = 32	t-value	p-value
1	Blood Urea Nitrogen	mg/dl	11.87 ± 0.1	11.3 ± 0.29	1.876^{ns}	0.068
2	Creatinine	mg/dl	2.08 ± 0.08	1.88 ± 0.06	1.973^{ns}	0.053
3	Total Bilirubin	mg/dl	0.91 ± 0.08	0.81 ± 0.07	0.998^{ns}	0.322
4	Direct Bilirubin	mg/dl	0.3 ± 0.03	0.25 ± 0.02	1.539^{ns}	0.129
5	Indirect Bilirubin	mg/dl	0.61 ± 0.07	0.54 ± 0.06	0.781^{ns}	0.438
6	Glucose	mg/dl	157.35 ± 14.96	124.62 ± 5.53	2.053*	0.047
7	Total Protein	g/dl	9.1 ± 0.28	9.83 ± 0.15	2.275*	0.027
8	Albumin	g/dl	2.43 ± 0.12	2.88 ± 0.07	3.131**	0.003
9	Globulin	g/dl	6.67 ± 0.18	6.96 ± 0.15	1.219^{ns}	0.227
10	A/G Ratio	-	0.36 ± 0.02	0.42 ± 0.02	2.746**	0.008
11	Cholesterol	mg/dl	33.64 ± 1.07	30.5 ± 1.26	1.899^{ns}	0.062
12	Aspartate Aminotransferase	U/l	23.4 ± 1.32	20.17 ± 0.89	2.036*	0.046
13	Alanine Aminotransferase	U/l	17.4 ± 1.09	15.91 ± 1.13	0.949^{ns}	0.346
14	Alkaline Phosphatase	U/l	262.23 ± 14.47	254.85 ± 20.44	0.294^{ns}	0.769

*** Significant at 0.01 level; * Significant at 0.05 level; ns non-Significant (P>0.05)*

Fig 8a. Comparison of Serum Biochemical Parameters between TB seropositive and control group

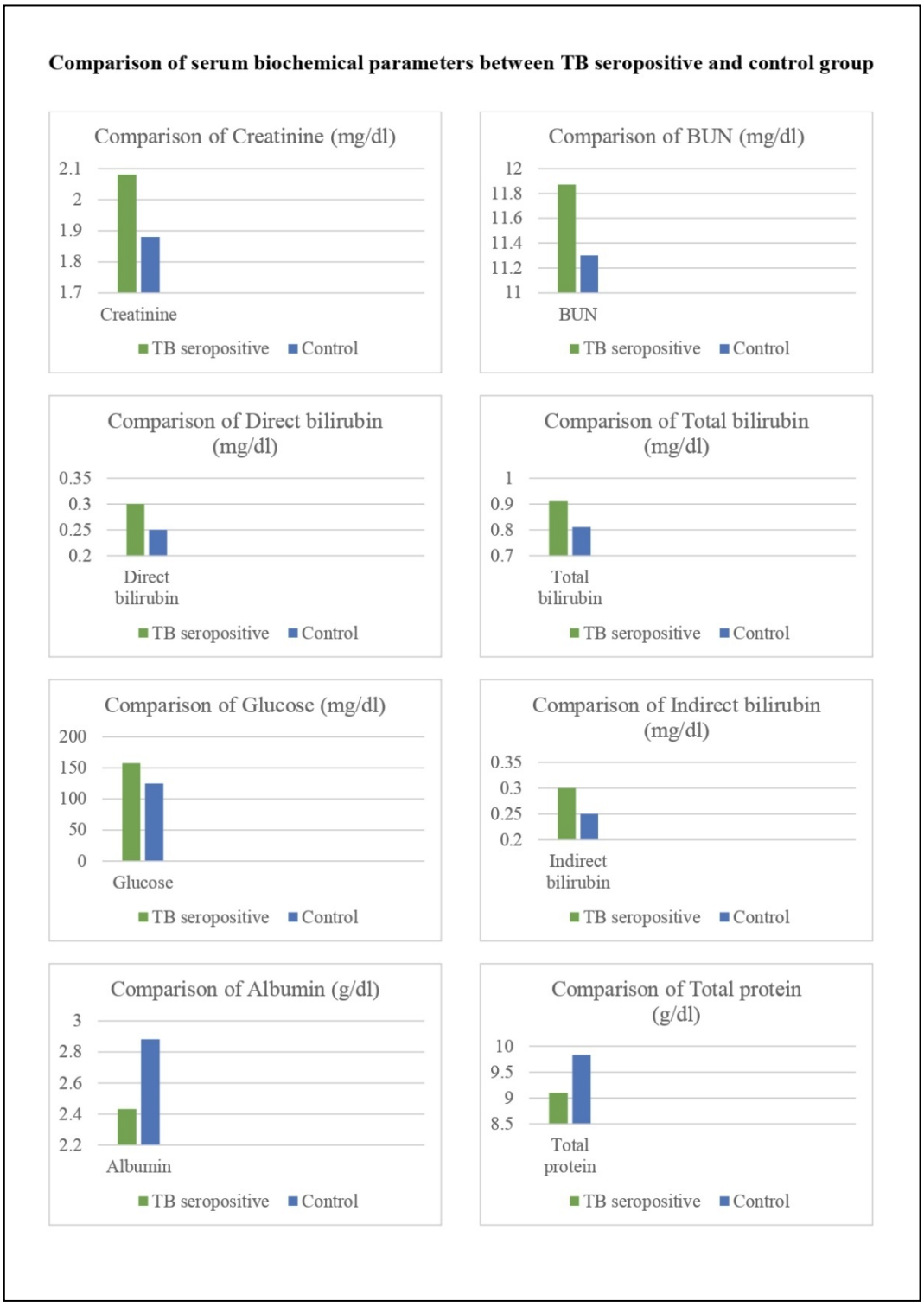


Fig 8b. Comparison of Serum Biochemical Parameters between TB seropositive and control group

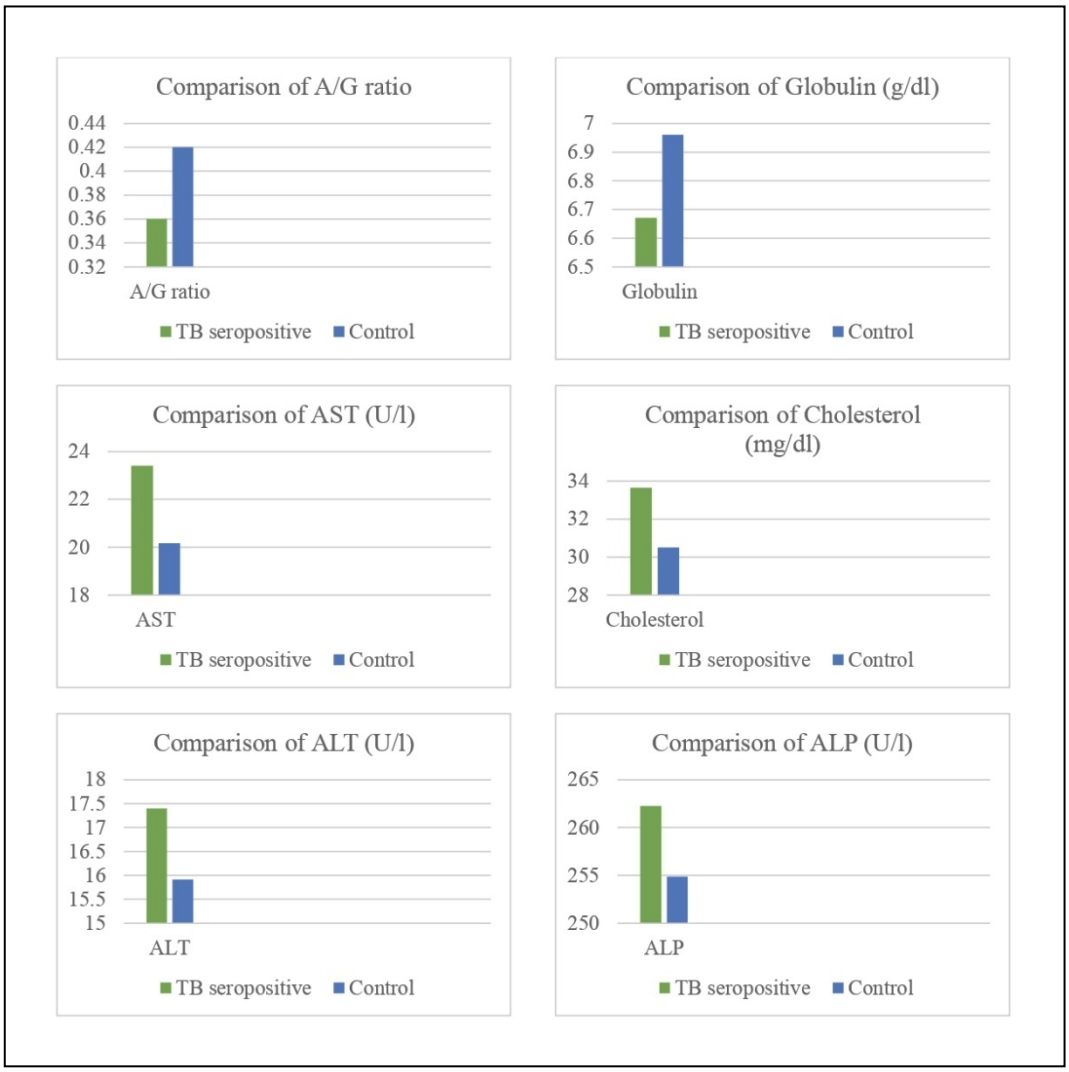


Table 9. Comparison of Urine test parameters between TB infected and control group

Variable	**Category**	**TB seropositive group n = 32**		**Control group n = 32**		**χ^2 Value (p-value)**
Leukocytes	15+	32	100	32	100	-
Nitrite	Negative	9	28.1	19	59.4	6.349* (0.012)
	Positive	23	71.9	13	40.6	
Urobilinogen	Negative	32	100	32	100	-
Protein	0.3+	4	12.5	1	3.1	-
	0.15+	4	12.5	0	0	
	Negative	24	75	31	96.9	
Blood	Negative	32	100	32	100	-
Ketones	Negative	32	100	32	100	-
Bilirubin	17+	2	6.3	0	0	-
	Negative	30	93.8	32	100	
SSA- Test	Negative	32	100	32	100	-
	Positive	0	0	0	0	
Glucose	Negative	32	100	32	100	-
Colour	Straw	4	12.5	4	12.5	1.788^{ns} (0.409)
	Amber	16	50	11	34.4	
	Dark yellow	12	37.5	17	53.1	
Appearance	Clear	29	90.6	21	65.6	4.480* (0.034)
	Turbid	3	9.4	11	34.4	

** Significant at 0.05 level; ns non-significant*

Fig 9. Comparison of Urine dipstick test parameters between TB seropositive and control group

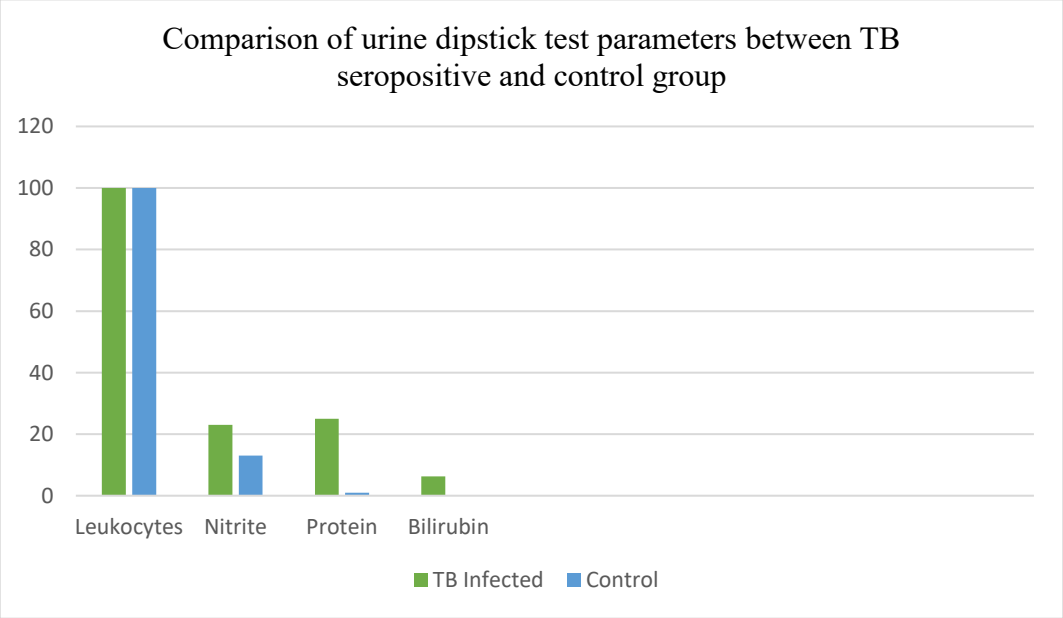


Fig 10. Comparison of pH and specific gravity in TB seropositive and control group

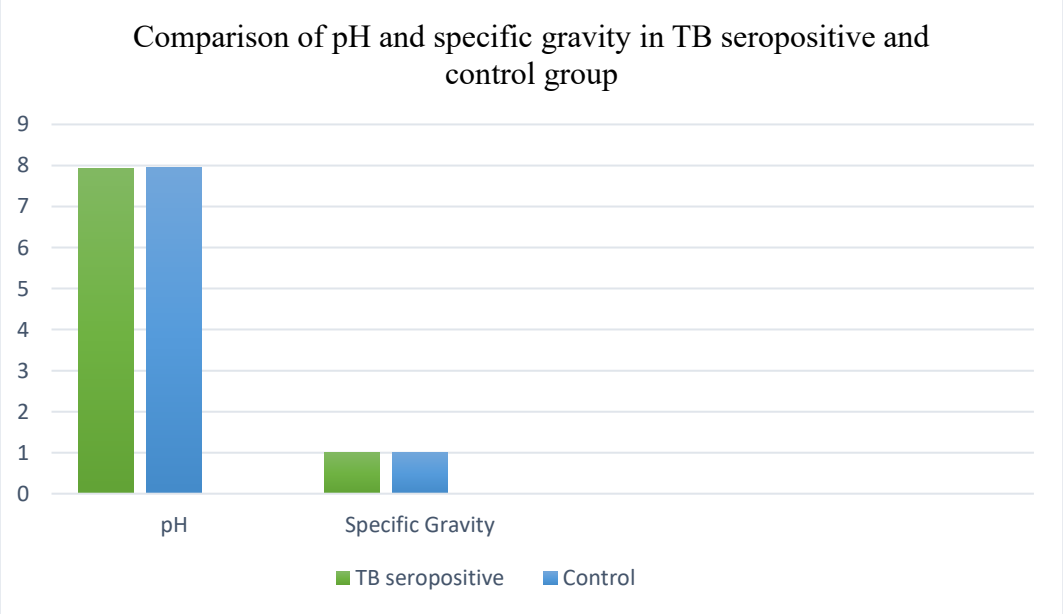


Fig 11. Comparison of colour of urine in TB seropositive and control group

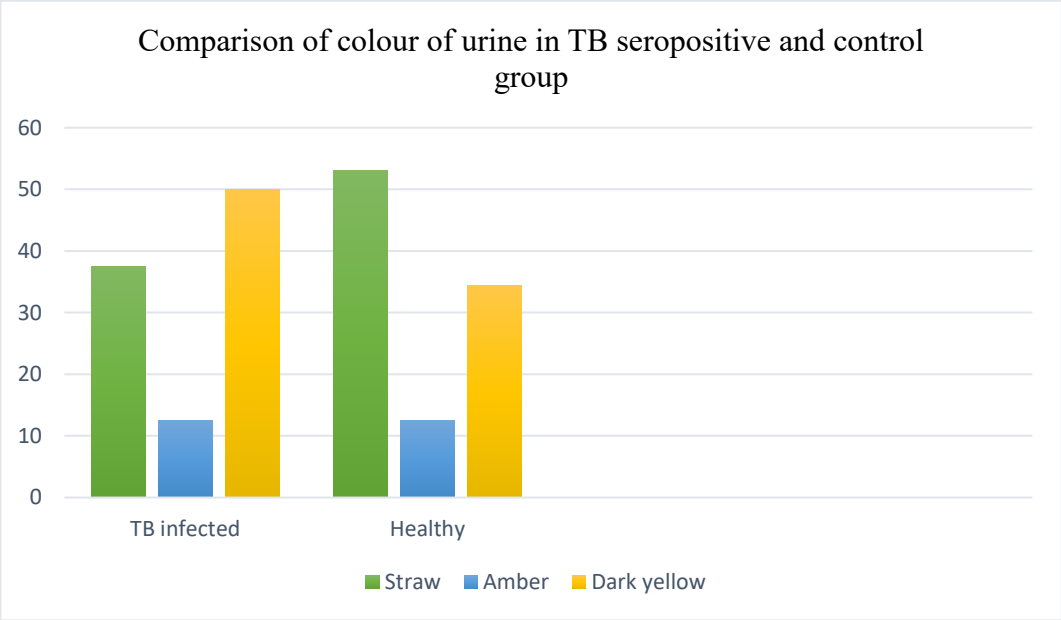


Fig 12. Comparison of appearance of urine in TB seropositive and control group

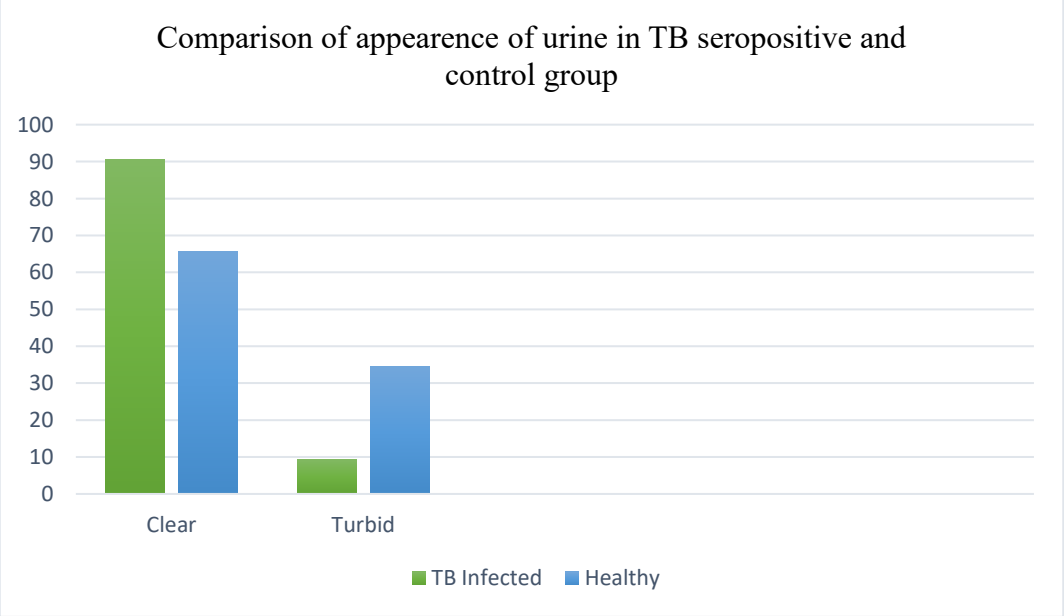


Table 10. Comparison of pH and specific gravity between TB infected and control group

Variable	TB seropositive	Control	t-value	p-value
pH	7.92 ± 0.21	7.94 ± 0.15	0.060 ^{ns}	0.953
Specific gravity	1.012 ± 0.009	1.015 ± 0.007	1.681 ^{ns}	0.098

ns non-significant

Table 11. Detection of *Mycobacterium tuberculosis* from trunk wash sample using PCR

Sl. No	Test Result	No. of elephants	Percentage
1	Positive	25	78.2
2	Negative	7	21.8
	Total	32	100

Fig 13. Detection of *Mycobacterium tuberculosis* from trunk wash sample using PCR

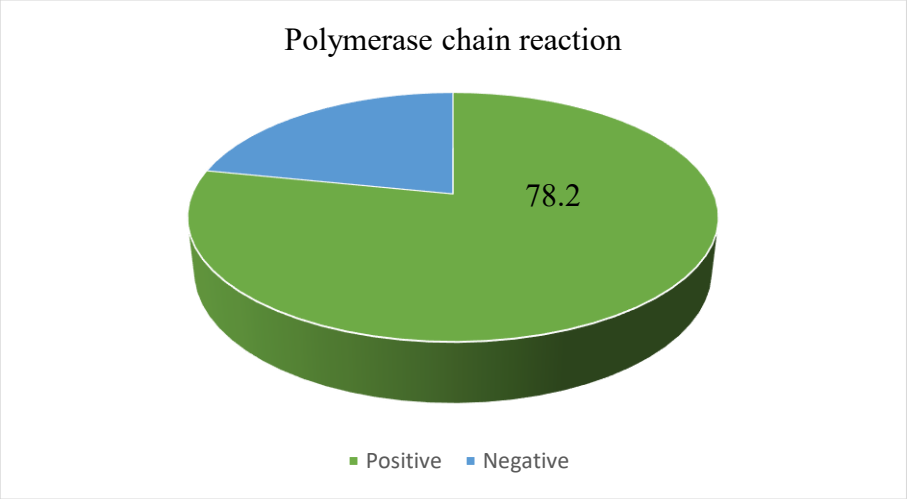
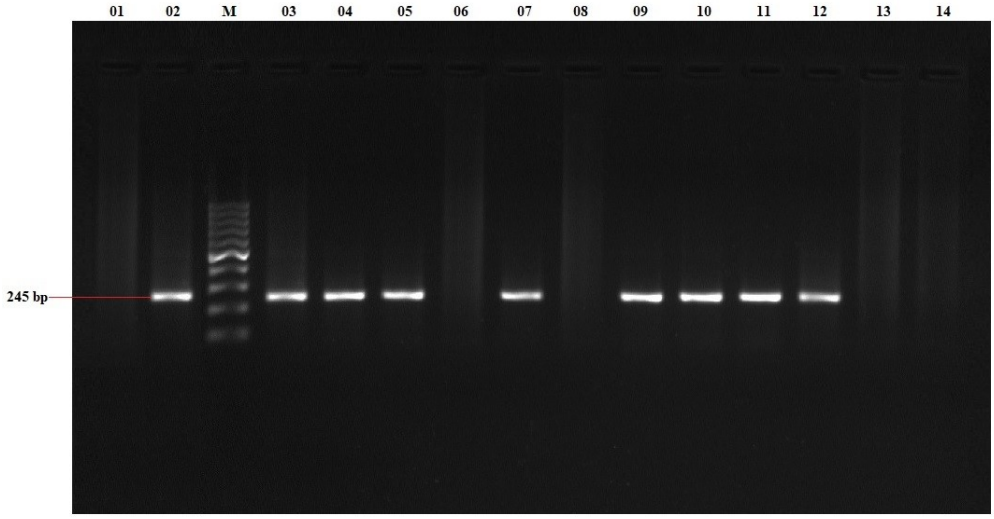


Plate 16. Agarose gel electrophoresis of IS*6110* specific PCR of *Mycobacterium tuberculosis,* expressed at 245bp.



Lane 1: Negative control

Lane 2: Positive control

Lane M: DNA ladder (100bp)

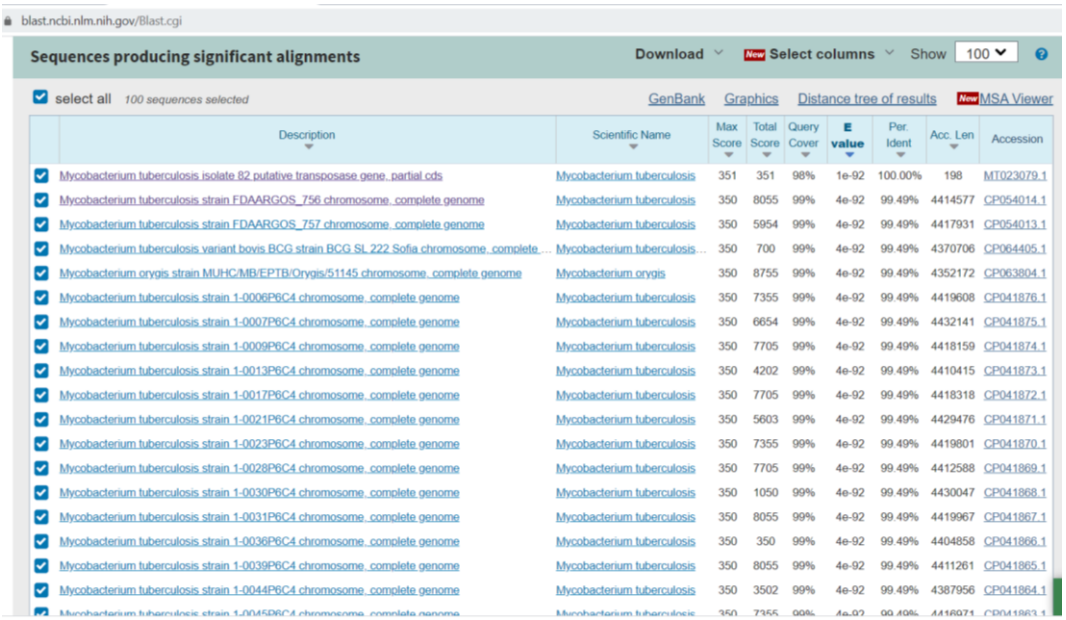
Lane 3-5, 7, 9-12: Positive samples with 245bp product

Lane 6, 8, 13, 14: Negative samples

Table 12. Trimmed FASTA sequence for positive sample

Sample No.	**Trimmed FASTA Sequence**
E2	TCGGCCTGTCCGGGACCACCCGCGGCAAAGCCCGCAGGA CCACGATCGCTGATCCGGCCACAGCCCGTCCCGCCGATCT CGTCCAGCGCCGCTTCGGACCACCAGCACCTAACCGGCTG TGGGTAGCAGACCTCACCTATGTGTCGACCTGGGCAGGGT TCGCCTACGTGGCCTTTGTCACCGACGCCTACGCACGT

Plate 17. BLAST results for representative sequence



blast.ncbi.nlm.nih.gov/Blast.cgi

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Mycobacterium tuberculosis isolate 82 putative transposase gene, partial cds	Mycobacterium tuberculosis	351	351	98%	1e-92	100.00%	198	MT023079.1
Mycobacterium tuberculosis strain FDAARGOS_756 chromosome, complete genome	Mycobacterium tuberculosis	350	8055	99%	4e-92	99.49%	4414577	CP054014.1
Mycobacterium tuberculosis strain FDAARGOS_757 chromosome, complete genome	Mycobacterium tuberculosis	350	5954	99%	4e-92	99.49%	4417931	CP054013.1
Mycobacterium tuberculosis variant bovis BCG strain BCG SL 222 Sofia chromosome, complete...	Mycobacterium tuberculosis...	350	700	99%	4e-92	99.49%	4370706	CP064405.1
Mycobacterium orygis strain MUHC/MB/EPTB/Orygis/51145 chromosome, complete genome	Mycobacterium orygis	350	8755	99%	4e-92	99.49%	4352172	CP063804.1
Mycobacterium tuberculosis strain 1-0006P6C4 chromosome, complete genome	Mycobacterium tuberculosis	350	7355	99%	4e-92	99.49%	4419608	CP041876.1
Mycobacterium tuberculosis strain 1-0007P6C4 chromosome, complete genome	Mycobacterium tuberculosis	350	6654	99%	4e-92	99.49%	4432141	CP041875.1
Mycobacterium tuberculosis strain 1-0009P6C4 chromosome, complete genome	Mycobacterium tuberculosis	350	7705	99%	4e-92	99.49%	4418159	CP041874.1
Mycobacterium tuberculosis strain 1-0013P6C4 chromosome, complete genome	Mycobacterium tuberculosis	350	4202	99%	4e-92	99.49%	4410415	CP041873.1
Mycobacterium tuberculosis strain 1-0017P6C4 chromosome, complete genome	Mycobacterium tuberculosis	350	7705	99%	4e-92	99.49%	4418318	CP041872.1
Mycobacterium tuberculosis strain 1-0021P6C4 chromosome, complete genome	Mycobacterium tuberculosis	350	5603	99%	4e-92	99.49%	4429476	CP041871.1
Mycobacterium tuberculosis strain 1-0023P6C4 chromosome, complete genome	Mycobacterium tuberculosis	350	7355	99%	4e-92	99.49%	4419801	CP041870.1
Mycobacterium tuberculosis strain 1-0028P6C4 chromosome, complete genome	Mycobacterium tuberculosis	350	7705	99%	4e-92	99.49%	4412588	CP041869.1
Mycobacterium tuberculosis strain 1-0030P6C4 chromosome, complete genome	Mycobacterium tuberculosis	350	1050	99%	4e-92	99.49%	4430047	CP041868.1
Mycobacterium tuberculosis strain 1-0031P6C4 chromosome, complete genome	Mycobacterium tuberculosis	350	8055	99%	4e-92	99.49%	4419967	CP041867.1
Mycobacterium tuberculosis strain 1-0036P6C4 chromosome, complete genome	Mycobacterium tuberculosis	350	350	99%	4e-92	99.49%	4404858	CP041866.1
Mycobacterium tuberculosis strain 1-0039P6C4 chromosome, complete genome	Mycobacterium tuberculosis	350	8055	99%	4e-92	99.49%	4411261	CP041865.1
Mycobacterium tuberculosis strain 1-0044P6C4 chromosome, complete genome	Mycobacterium tuberculosis	350	3502	99%	4e-92	99.49%	4387956	CP041864.1

Plate 18. Phylogenetic tree

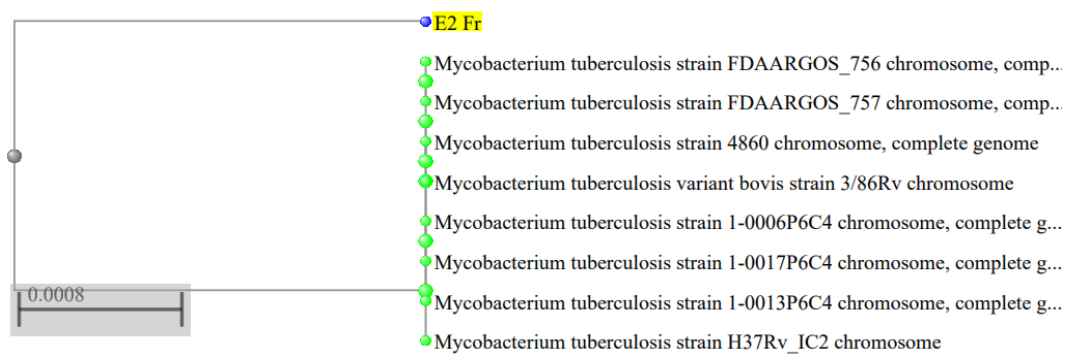


Table 13. Concentration of protein and optical density

	Concentration (µg/µL)	**Absorbance at 595nm**
Standard Protein	0.625	0.805
	1.25	0.956
	2.5	1.131
	5	1.304
	10	1.444
Test Proteins	4.49	0.853
	4.83	0.924
	4.62	0.883
	5.99	1.146
	5.88	1.124
	5.32	1.017
	6.36	1.216
	6.12	1.17
	3.49	0.667
	4.48	0.857
	3.77	0.721
	4.77	0.912

Fig 14. Graph representing concentration and optical density of standard and test proteins

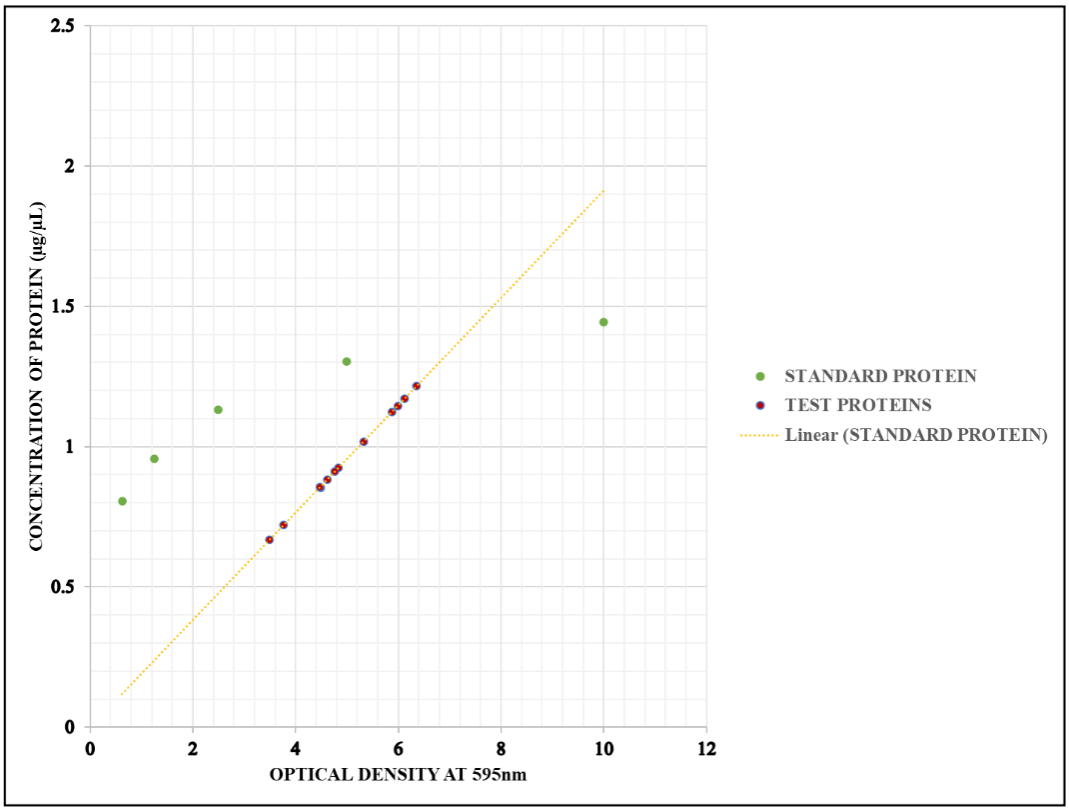
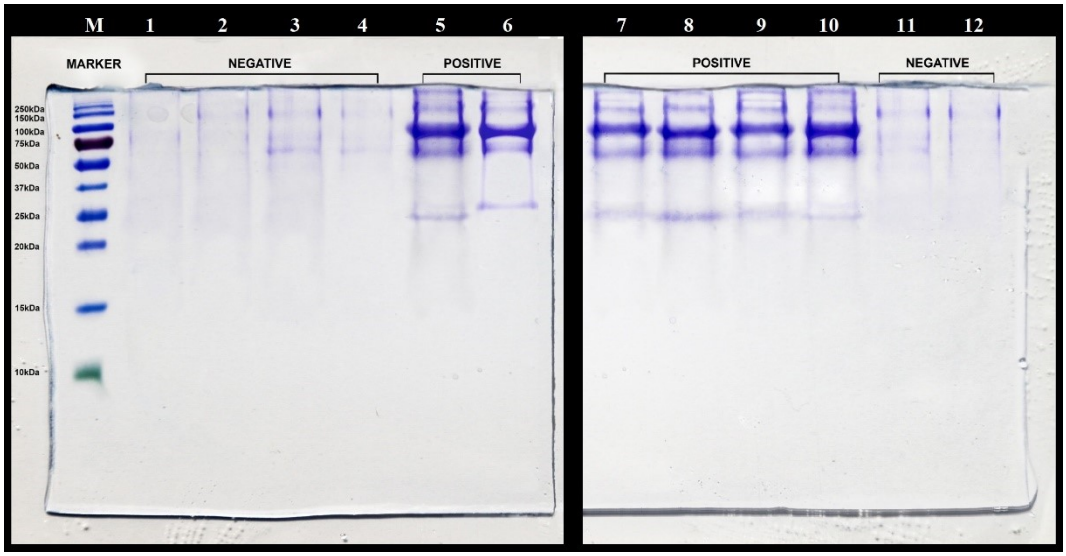


Plate 19. SDS-PAGE of urine proteins in healthy and TB infected elephants



Lane M: Marker (10-250kDa)

Lane 1- 4, 11- 12: Urine proteins of healthy elephants

Lane 5- 10: Urine proteins of TB infected elephants

Table 14. Identified proteins in TB infected group

Sl. No	**List of Proteins Found in TB Infected Group**
1	WAP domain-containing protein OS=Loxodonta africana OX=9785 PE=4 SV=1
2	Histone H2B OS=Loxodonta africana OX=9785 GN=HIST1H2BK PE=3 SV=1
3	Keratin 10 OS=Loxodonta africana OX=9785 GN=KRT10 PE=3 SV=1
4	Enolase 1 OS=Loxodonta africana OX=9785 GN=ENO1 PE=3 SV=1
5	Keratin 5 OS=Loxodonta africana OX=9785 GN=KRT5 PE=3 SV=1
6	Uncharacterised protein OS=Callorhinchus milii OX=7868 PE=4 SV=1

7	Actin beta OS=Loxodonta africana OX=9785 GN=ACTB PE=3 SV=1
8	Hemoglobin subunit alpha OS=Elephas maximus OX=9783 GN=HBA-T2 PE=2 SV=1
9	Resistin OS=Loxodonta africana OX=9785 GN=RETN PE=4 SV=1
10	Alpha-amylase OS=Loxodonta africana OX=9785 GN=LOC100661902 PE=3 SV=1
11	Galactosylgalactosylxylosylprotein3-beta-glucuronosyltransferase OS=Callorhinchus milii OX=7868 PE=3 SV=1
12	Keratin 79 OS=Loxodonta africana OX=9785 GN=KRT79 PE=3 SV=1
13	Alpha-1-B glycoprotein OS=Loxodonta africana OX=9785 GN=A1BG PE=4 SV=1
14	Uromodulin OS=Loxodonta africana OX=9785 GN=UMOD PE=4 SV=1
15	Heat shock protein 90 alpha family class B member 1 OS=Loxodonta africana OX=9785 GN=HSP90AB1 PE=3 SV=1
16	Fibrinogen gamma chain OS=Loxodonta africana OX=9785 GN=FGG PE=4 SV=1
17	Fibronectin 1 OS=Loxodonta africana OX=9785 GN=FN1 PE=4 SV=1
18	Alpha-amylase OS=Loxodonta africana OX=9785 GN=LOC100661620 PE=3 SV=1
19	Peptidase S1 domain-containing protein OS=Loxodonta africana OX=9785 GN=LOC100659862 PE=3 SV=1
20	Yip1 domain family member 3 OS=Loxodonta africana OX=9785 GN=YIPF3 PE=4 SV=1
21	Fibrinogen beta chain OS=Loxodonta africana OX=9785 GN=FGB PE=4 SV=1
22	EGF containing fibulin extracellular matrix protein 1 OS=Loxodonta africana OX=9785 GN=EFEMP1 PE=4 SV=1
23	Granulin precursor OS=Loxodonta africana OX=9785 GN=GRN PE=4 SV=1

24	Albumin OS=Loxodonta africana OX=9785 GN=ALB PE=4 SV=1
25	Keratin 77 OS=Loxodonta africana OX=9785 GN=KRT77 PE=3 SV=1
26	Transket_pyr domain-containing protein OS=Callorhinchus milii OX=7868 PE=4 SV=1
27	Interleukin 1 receptor accessory protein (Fragment) OS=Callorhinchus milii OX=7868 PE=2 SV=1
28	Prothrombin OS=Loxodonta africana OX=9785 GN=F2 PE=3 SV=1
29	Sulfhydryl oxidase OS=Loxodonta africana OX=9785 GN=QSOX1 PE=4 SV=1
30	Inter-alpha-trypsin inhibitor heavy chain 4 OS=Loxodonta africana OX=9785 GN=ITIH4 PE=4 SV=1
31	Latent transforming growth factor beta binding protein 4 OS=Loxodonta africana OX=9785 GN=LTBP4 PE=4 SV=1
32	Alpha-2-macroglobulin OS=Loxodonta africana OX=9785 GN=A2M PE=4 SV=1
33	Fibrillin 1 OS=Loxodonta africana OX=9785 GN=FBN1 PE=4 SV=1
34	Complement C3 OS=Loxodonta africana OX=9785 GN=C3 PE=4 SV=1

Table 15. Identified proteins in control group

Sl. No	**List of Proteins Found in control Group**
1	Beta-globin (Fragment) OS=Mirounga leonina OX=9715 PE=4 SV=1
2	WAP domain-containing protein OS=Loxodonta africana OX=9785 PE=4 SV=1
3	Histone H4 OS=Loxodonta africana OX=9785 PE=3 SV=1
4	Histone H2B OS=Loxodonta africana OX=9785 GN=HIST1H2BK PE=3 SV=1

5	Keratin 10 OS=Loxodonta africana OX=9785 GN=KRT10 PE=3 SV=1
6	Enolase 1 OS=Loxodonta africana OX=9785 GN=ENO1 PE=3 SV=1
7	Hemoglobin subunit beta OS=Trichechus inunguis OX=9777 GN=HBB PE=1 SV=1
8	Keratin 5 OS=Loxodonta africana OX=9785 GN=KRT5 PE=3 SV=1
9	Uncharacterised protein OS=Loxodonta africana OX=9785 PE=3 SV=1
10	Cofilin 2 OS=Loxodonta africana OX=9785 GN=CFL2 PE=3 SV=1
11	Uncharacterised protein OS=Callorhinchus milii OX=7868 PE=4 SV=1
12	Cystatin B OS=Loxodonta africana OX=9785 GN=CSTB PE=3 SV=1
13	Hemoglobin subunit alpha OS=Trichechus inunguis OX=9777 GN=HBA PE=1 SV=1
14	Actin beta OS=Loxodonta africana OX=9785 GN=ACTB PE=3 SV=1
15	Pyruvate kinase OS=Loxodonta africana OX=9785 GN=PKM PE=3 SV=1
16	Hemoglobin subunit alpha OS=Elephas maximus OX=9783 GN=HBA-T2 PE=2 SV=1
17	Heat shock protein family D (Hsp60) member 1 OS=Loxodonta africana OX=9785 GN=HSPD1 PE=3 SV=1
18	Resistin OS=Loxodonta africana OX=9785 GN=RETN PE=4 SV=1
19	Stathmin OS=Loxodonta africana OX=9785 GN=STMN1 PE=3 SV=1
20	Glutathione S-transferase pi 1 OS=Loxodonta africana OX=9785 GN=GSTP1 PE=3 SV=1
21	Tubulin alpha chain OS=Callorhinchus milii OX=7868 GN=LOC103172121 PE=3 SV=1
22	Alpha-amylase OS=Loxodonta africana OX=9785 GN=LOC100661902 PE=3 SV=1
23	Proteasome subunit alpha type OS=Loxodonta africana OX=9785 GN=PSMA6 PE=3 SV=1
24	Calreticulin OS=Loxodonta africana OX=9785 GN=CALR PE=3 SV=1

25	Heterogeneous nuclear ribonucleoprotein A2/B1 OS=Loxodonta africana OX=9785 GN=HNRNPA2B1 PE=4 SV=1
26	ATP synthase subunit beta OS=Callorhinchus milii OX=7868 GN=atp5f1b PE=3 SV=1
27	Keratin 79 OS=Loxodonta africana OX=9785 GN=KRT79 PE=3 SV=1
28	Alpha-1-B glycoprotein OS=Loxodonta africana OX=9785 GN=A1BG PE=4 SV=1
29	Uromodulin OS=Loxodonta africana OX=9785 GN=UMOD PE=4 SV=1
30	Transmembrane protein 26 OS=Loxodonta africana OX=9785 GN=TMEM26 PE=4 SV=1
31	Heat shock protein 90 alpha family class B member 1 OS=Loxodonta africana OX=9785 GN=HSP90AB1 PE=3 SV=1
32	Tr-type G domain-containing protein OS=Loxodonta africana OX=9785 PE=4 SV=1
33	WD_REPEATS_REGION domain-containing protein OS=Callorhinchus milii OX=7868 PE=4 SV=1
34	Fibrinogen gamma chain OS=Loxodonta africana OX=9785 GN=FGG PE=4 SV=1
35	Fibronectin 1 OS=Loxodonta africana OX=9785 GN=FN1 PE=4 SV=1
36	Glyceraldehyde-3-phosphate dehydrogenase OS=Loxodonta africana OX=9785 PE=3 SV=1
37	Uncharacterised protein OS=Callorhinchus milii OX=7868 GN=cct2 PE=3 SV=1
38	Phosphoglycerate kinase OS=Loxodonta africana OX=9785 GN=PGK2 PE=3 SV=1
39	Alpha-amylase OS=Loxodonta africana OX=9785 GN=LOC100661620 PE=3 SV=1
40	T-complex protein 1 subunit delta OS=Loxodonta africana OX=9785 GN=CCT4 PE=3 SV=1
41	Tubulin beta chain OS=Loxodonta africana OX=9785 GN=TUBB

	PE=3 SV=1
42	Uncharacterised protein OS=Loxodonta africana OX=9785 GN=HNRNPH1 PE=4 SV=1
43	Elongation factor Tu OS=Loxodonta africana OX=9785 GN=TUFM PE=3 SV=1
44	T-complex protein 1 subunit gamma OS=Loxodonta africana OX=9785 GN=CCT3 PE=3 SV=1
45	Peptidase S1 domain-containing protein OS=Loxodonta africana OX=9785 GN=LOC100659862 PE=3 SV=1
46	Yip1 domain family member 3 OS=Loxodonta africana OX=9785 GN=YIPF3 PE=4 SV=1
47	Fibrinogen beta chain OS=Loxodonta africana OX=9785 GN=FGB PE=4 SV=1
48	EGF containing fibulin extracellular matrix protein 1 OS=Loxodonta africana OX=9785 GN=EFEMP1 PE=4 SV=1
49	Granulin precursor OS=Loxodonta africana OX=9785 GN=GRN PE=4 SV=1
50	Albumin OS=Loxodonta africana OX=9785 GN=ALB PE=4 SV=1
51	Keratin 77 OS=Loxodonta africana OX=9785 GN=KRT77 PE=3 SV=1
52	Gelsolin OS=Loxodonta africana OX=9785 GN=GSN PE=4 SV=1
53	Transket_pyr domain-containing protein OS=Callorhinchus milii OX=7868 PE=4 SV=1
54	Uncharacterised protein OS=Callorhinchus milii OX=7868 GN=LOC103180319 PE=3 SV=1
55	Complement C5 OS=Loxodonta africana OX=9785 GN=C5 PE=4 SV=1
56	Interleukin 1 receptor accessory protein (Fragment) OS=Callorhinchus milii OX=7868 PE=2 SV=1
57	Prothrombin OS=Loxodonta africana OX=9785 GN=F2 PE=3 SV=1
58	Polymeric immunoglobulin receptor OS=Loxodonta africana OX=9785

	GN=PIGR PE=4 SV=1
59	Sulfhydryl oxidase OS=Loxodonta africana OX=9785 GN=QSOX1 PE=4 SV=1
60	Inter-alpha-trypsin inhibitor heavy chain 1 OS=Loxodonta africana OX=9785 GN=ITIH1 PE=4 SV=1
61	AP-3 complex subunit beta OS=Loxodonta africana OX=9785 GN=AP3B1 PE=3 SV=1
62	Inter-alpha-trypsin inhibitor heavy chain 4 OS=Loxodonta africana OX=9785 GN=ITIH4 PE=4 SV=1
63	Uncharacterised protein OS=Loxodonta africana OX=9785 PE=4 SV=1
64	Latent transforming growth factor beta binding protein 4 OS=Loxodonta africana OX=9785 GN=LTBP4 PE=4 SV=1
65	Alpha-2-macroglobulin OS=Loxodonta africana OX=9785 GN=A2M PE=4 SV=1
66	Collagen type XIV alpha 1 chain OS=Loxodonta africana OX=9785 GN=COL14A1 PE=4 SV=1
67	Fibrillin 1 OS=Loxodonta africana OX=9785 GN=FBN1 PE=4 SV=1
68	Complement C3 OS=Loxodonta africana OX=9785 GN=C3 PE=4 SV=1

Table 16. List of proteins absent in TB infected group in comparison with control group

Sl. No	**Proteins Not Found in TB Infected Group**
1	Beta-globin (Fragment) OS=Mirounga leonina OX=9715 PE=4 SV=1
2	Histone H4 OS=Loxodonta africana OX=9785 PE=3 SV=1
3	Hemoglobin subunit beta OS=Trichechus inunguis OX=9777 GN=HBB PE=1 SV=1

4	Uncharacterised protein OS=Loxodonta africana OX=9785 PE=3 SV=1
5	Cofilin 2 OS=Loxodonta africana OX=9785 GN=CFL2 PE=3 SV=1
6	Cystatin B OS=Loxodonta africana OX=9785 GN=CSTB PE=3 SV=1
7	Hemoglobin subunit alpha OS=Trichechus inunguis OX=9777 GN=HBA PE=1 SV=1
8	Pyruvate kinase OS=Loxodonta africana OX=9785 GN=PKM PE=3 SV=1
9	Heat shock protein family D (Hsp60) member 1 OS=Loxodonta africana OX=9785 GN=HSPD1 PE=3 SV=1
10	Stathmin OS=Loxodonta africana OX=9785 GN=STMN1 PE=3 SV=1
11	Glutathione S-transferase pi 1 OS=Loxodonta africana OX=9785 GN=GSTP1 PE=3 SV=1
12	Tubulin alpha chain OS=Callorhinchus milii OX=7868 GN=LOC103172121 PE=3 SV=1
13	Proteasome subunit alpha type OS=Loxodonta africana OX=9785 GN=PSMA6 PE=3 SV=1
14	Calreticulin OS=Loxodonta africana OX=9785 GN=CALR PE=3 SV=1
15	Heterogeneous nuclear ribonucleoprotein A2/B1 OS=Loxodonta africana OX=9785 GN=HNRNPA2B1 PE=4 SV=1
16	ATP synthase subunit beta OS=Callorhinchus milii OX=7868 GN=atp5f1b PE=3 SV=1
17	Transmembrane protein 26 OS=Loxodonta africana OX=9785 GN=TMEM26 PE=4 SV=1
18	Tr-type G domain-containing protein OS=Loxodonta africana OX=9785 PE=4 SV=1
19	WD_REPEATS_REGION domain-containing protein OS=Callorhinchus milii OX=7868 PE=4 SV=1
20	Glyceraldehyde-3-phosphate dehydrogenase OS=Loxodonta africana OX=9785 PE=3 SV=1
21	Uncharacterised protein OS=Callorhinchus milii OX=7868 GN=cct2

	PE=3 SV=1
22	Phosphoglycerate kinase OS=Loxodonta africana OX=9785 GN=PGK2 PE=3 SV=1
23	Keratin 3 OS=Loxodonta africana OX=9785 GN=KRT3 PE=3 SV=1
24	T-complex protein 1 subunit delta OS=Loxodonta africana OX=9785 GN=CCT4 PE=3 SV=1
25	Tubulin beta chain OS=Loxodonta africana OX=9785 GN=TUBB PE=3 SV=1
26	Uncharacterised protein OS=Loxodonta africana OX=9785 GN=HNRNPH1 PE=4 SV=1
27	Elongation factor Tu OS=Loxodonta africana OX=9785 GN=TUFM PE=3 SV=1
28	T-complex protein 1 subunit gamma OS=Loxodonta africana OX=9785 GN=CCT3 PE=3 SV=1
29	G3UMD4
30	Uncharacterised protein OS=Callorhinchus milii OX=7868 GN=LOC103180319 PE=3 SV=1
31	Complement C5 OS=Loxodonta africana OX=9785 GN=C5 PE=4 SV=1
32	Polymeric immunoglobulin receptor OS=Loxodonta africana OX=9785 GN=PIGR PE=4 SV=1
33	Inter-alpha-trypsin inhibitor heavy chain 1 OS=Loxodonta africana OX=9785 GN=ITIH1 PE=4 SV=1
34	AP-3 complex subunit beta OS=Loxodonta africana OX=9785 GN=AP3B1 PE=3 SV=1
35	Uncharacterised protein OS=Loxodonta africana OX=9785 PE=4 SV=1
36	Collagen type XIV alpha 1 chain OS=Loxodonta africana OX=9785 GN=COL14A1 PE=4 SV=1

Table 17. List of proteins absent in control group and present in TB infected group

SL. No.	Protein Exclusively Present In TB Infected Group
1	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase OS=Callorhinchus milii OX=7868 PE=3 SV=1

Table 18. Proteins identified in TB infected group based on functionality

Sl. No.	Classification Based on Functionality of Proteins	No. of Proteins Identified in TB Infected Group
1	cellular process (GO:0009987)	57
2	response to stimulus (GO:0050896)	48
3	metabolic process (GO:0008152)	41
4	biological regulation (GO:0065007)	40
5	immune system process (GO:0002376)	31
6	localization (GO:0051179)	16
7	interspecies interaction between organisms (GO:0044419)	15
8	signaling (GO:0023052)	15
9	multicellular organismal process (GO:0032501)	14
10	biological adhesion (GO:0022610)	9
11	developmental process (GO:0032502)	2
12	locomotion (GO:0040011)	1

Table 19. Proteins identified in control group based on functionality

Sl. No.	**Classification Based on Functionality of Proteins**	**No. of Proteins Identified in control Group**
1	cellular process	52
2	response to stimulus	42
3	metabolic process	39
4	biological regulation	35
5	immune system process	25
6	localization (GO:0051179)	14
7	multicellular organismal process	14
8	interspecies interaction between organisms	12
9	signaling	11
10	biological adhesion	7
11	developmental process (GO:0032502)	2
12	locomotion (GO:0040011)	1

Table 20. Comparison of proteins in TB infected and control group based on biological process

Sl. No	Biological Process	No. of Proteins in TB Infected Group	No. of Proteins in control Group
1	Cellular process	57	52
2	Response to stimulus	48	42
3	Metabolic process	41	39
4	Biological regulation	40	35
5	Immune system process	31	25
6	Localization	16	14
7	Multicellular organismal process	14	14
8	Interspecies interaction between organisms	15	12
9	Signaling	15	11
10	Biological adhesion	9	7
11	Developmental process	2	2
12	Locomotion	1	1

Fig 15. Comparison of proteins in TB infected and control group based on biological process

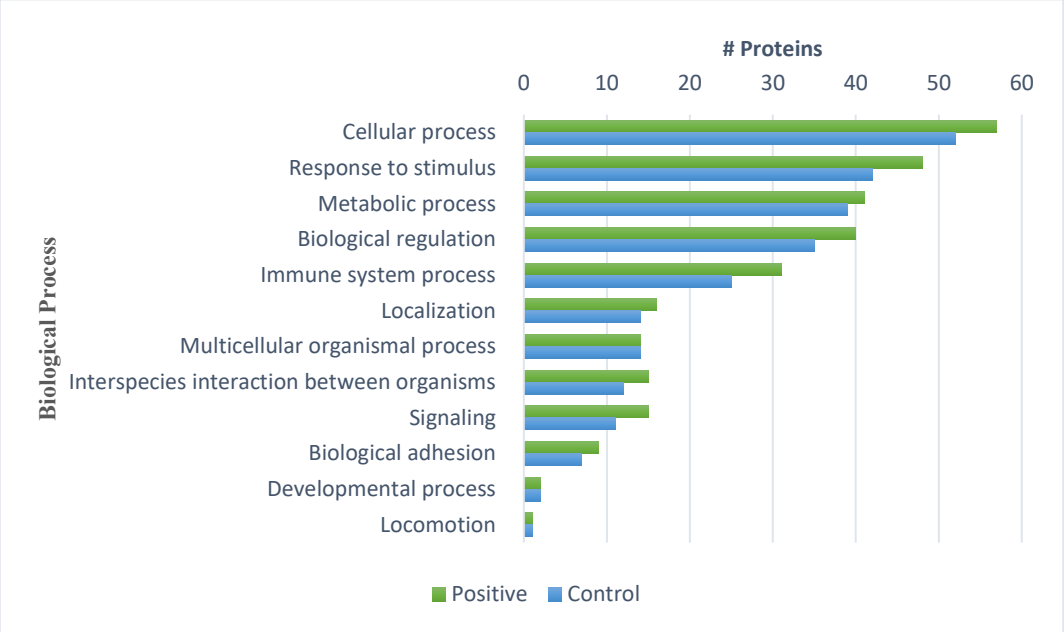


Plate 20. Phylogenetic tree of biological process in control group. Bigger blue dot depicts higher significance in p – value.

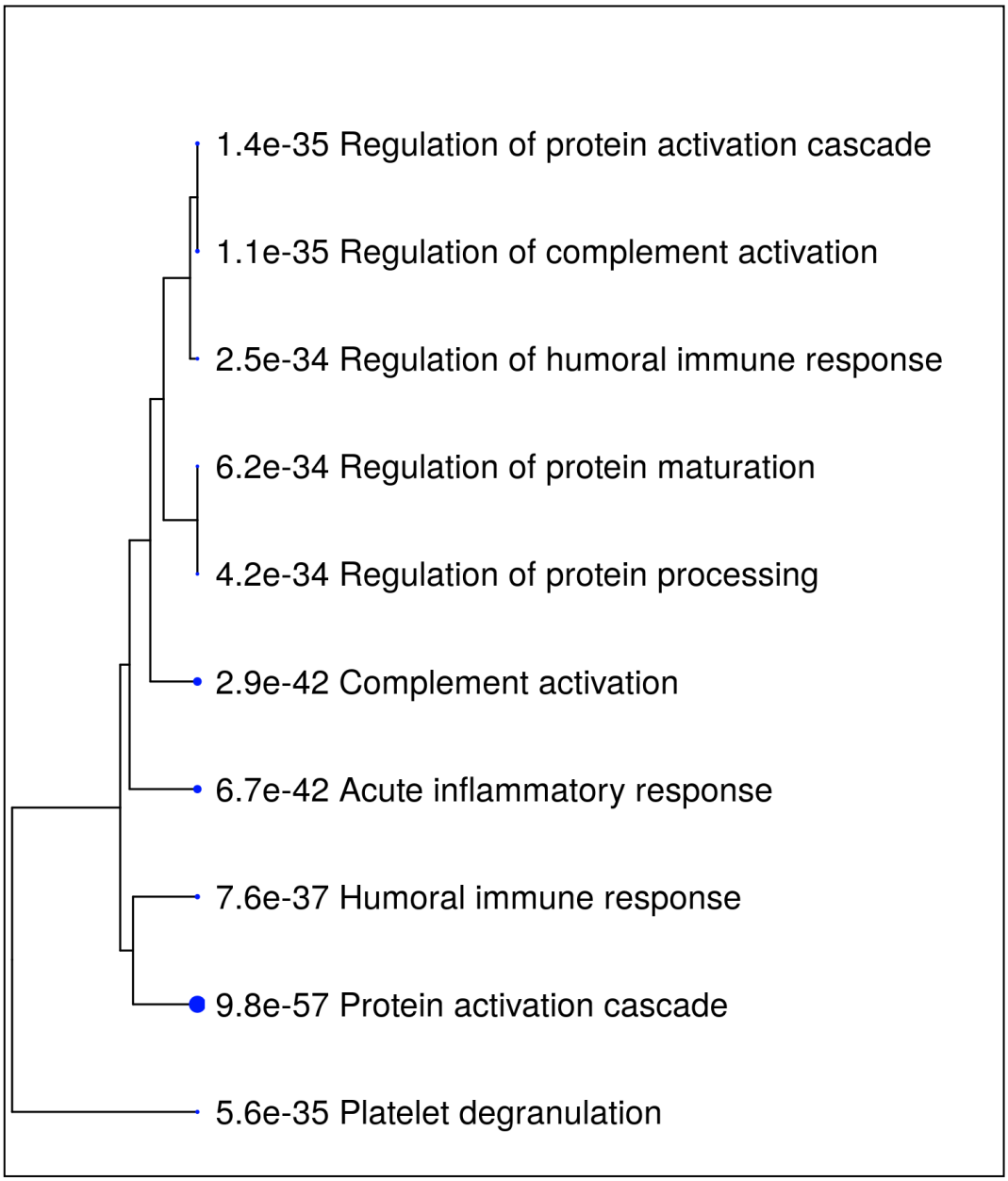


Plate 21. Phylogenetic tree of biological process in TB infected group. Bigger blue dot depicts higher significance in p – value.

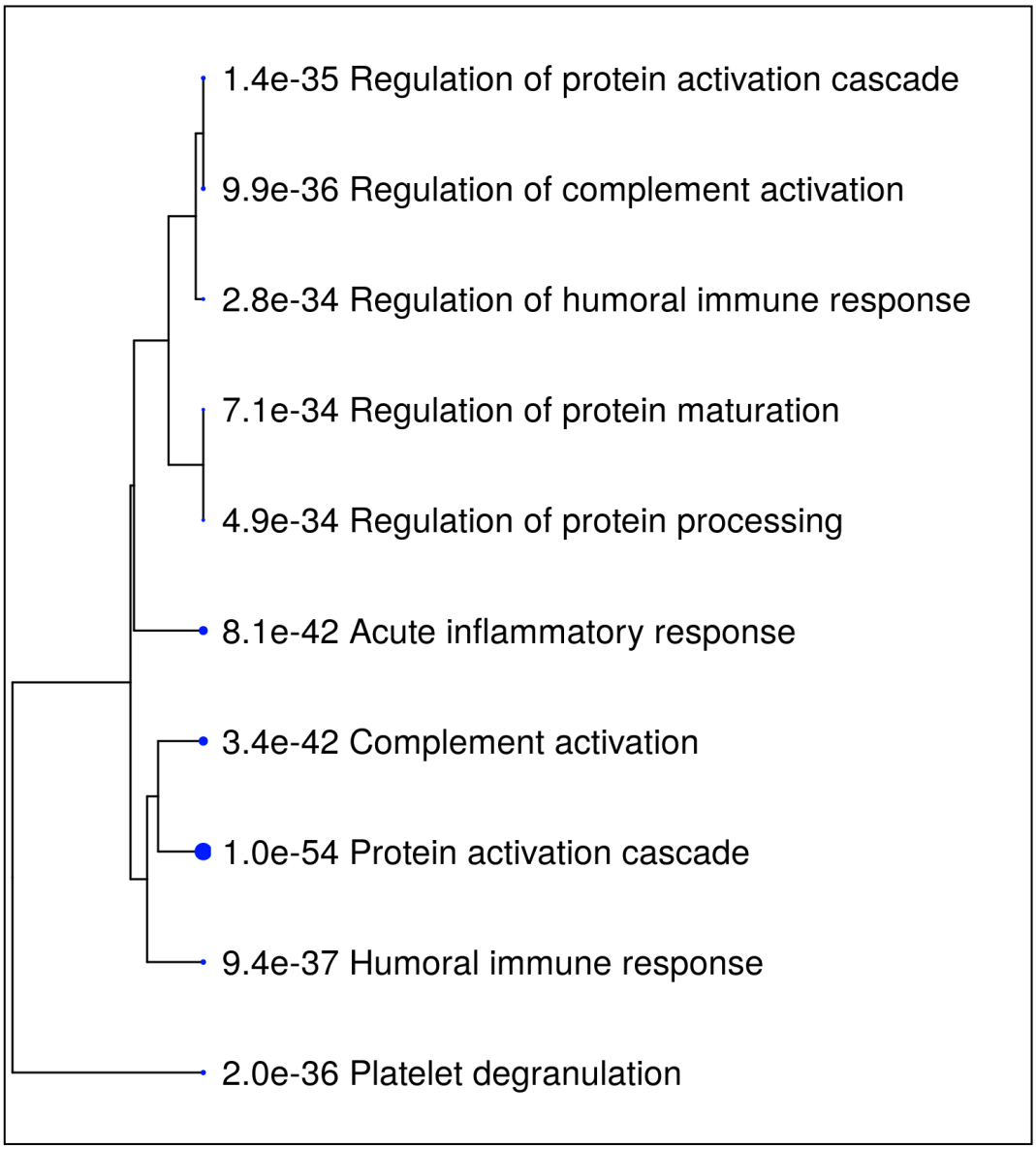


Table 21. Comparison of proteins in TB infected and control group based on molecular function

Sl. No	Molecular Function	No. of Proteins in TB Infected Group	No. of Proteins in control Group
1	Binding	43	40
2	Catalytic activity	34	34
3	Molecular function regulator	11	11
4	Transporter activity	4	4
5	Structural molecule activity	3	3
6	Molecular transducer activity	1	1
7	Molecular adaptor activity	1	1

Fig 16. Comparison of proteins in TB infected and control group based on molecular function

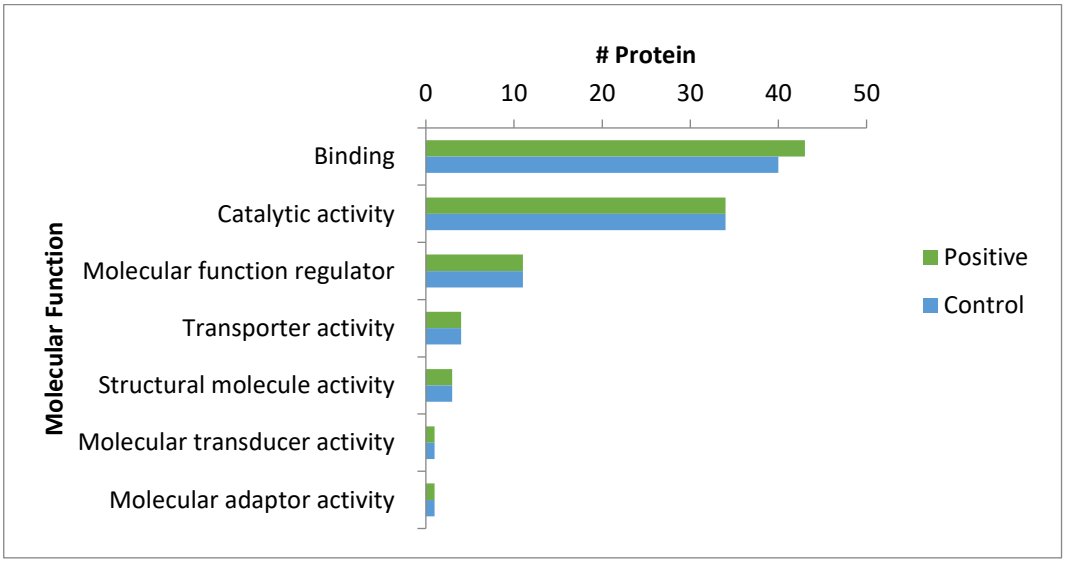


Plate 22. Phylogenetic tree of molecular function in control group. Bigger blue dot depicts higher significance in p – value.

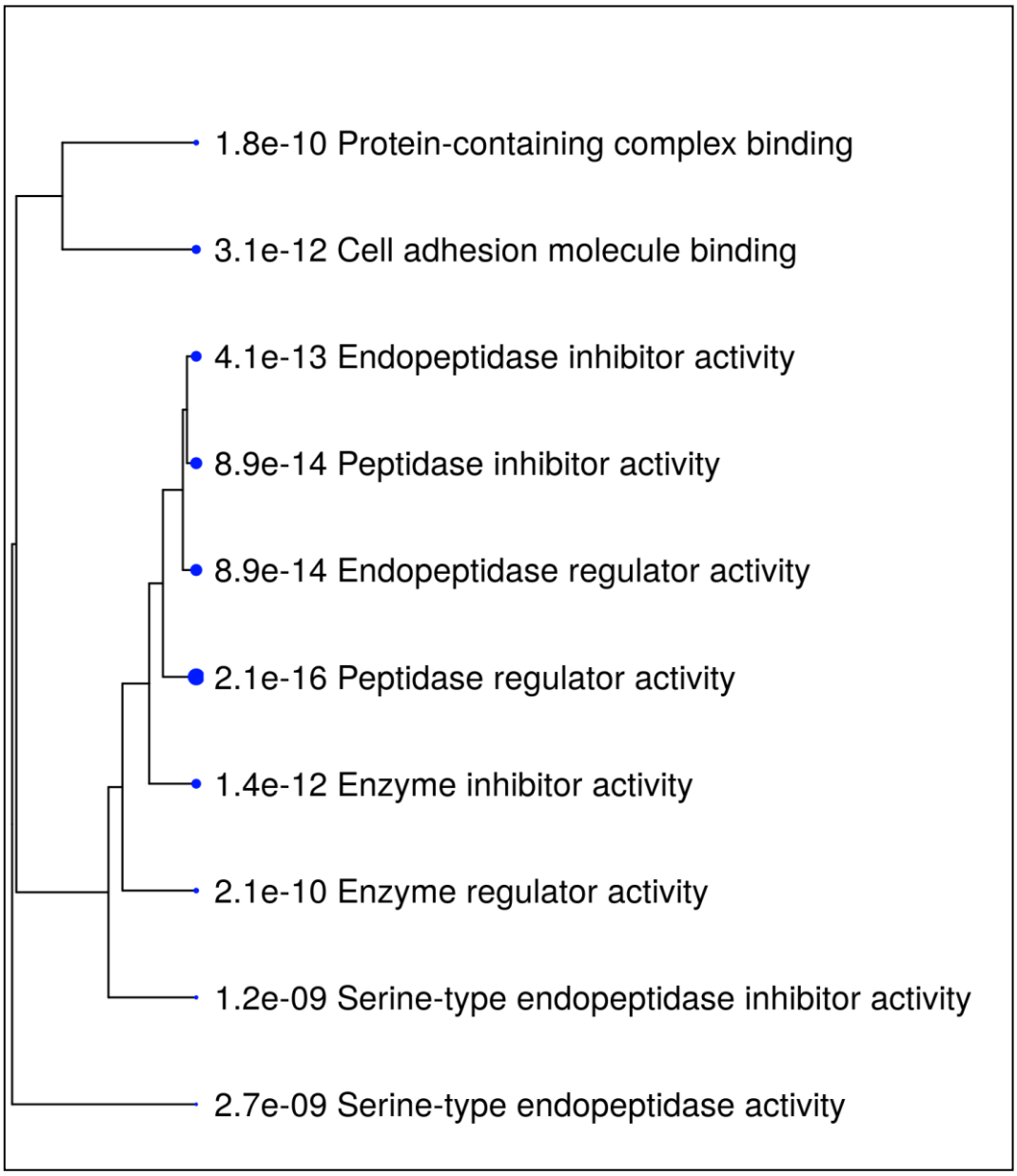


Plate 23. Phylogenetic tree of molecular function in control group. Bigger blue dot depicts higher significance in p – value.

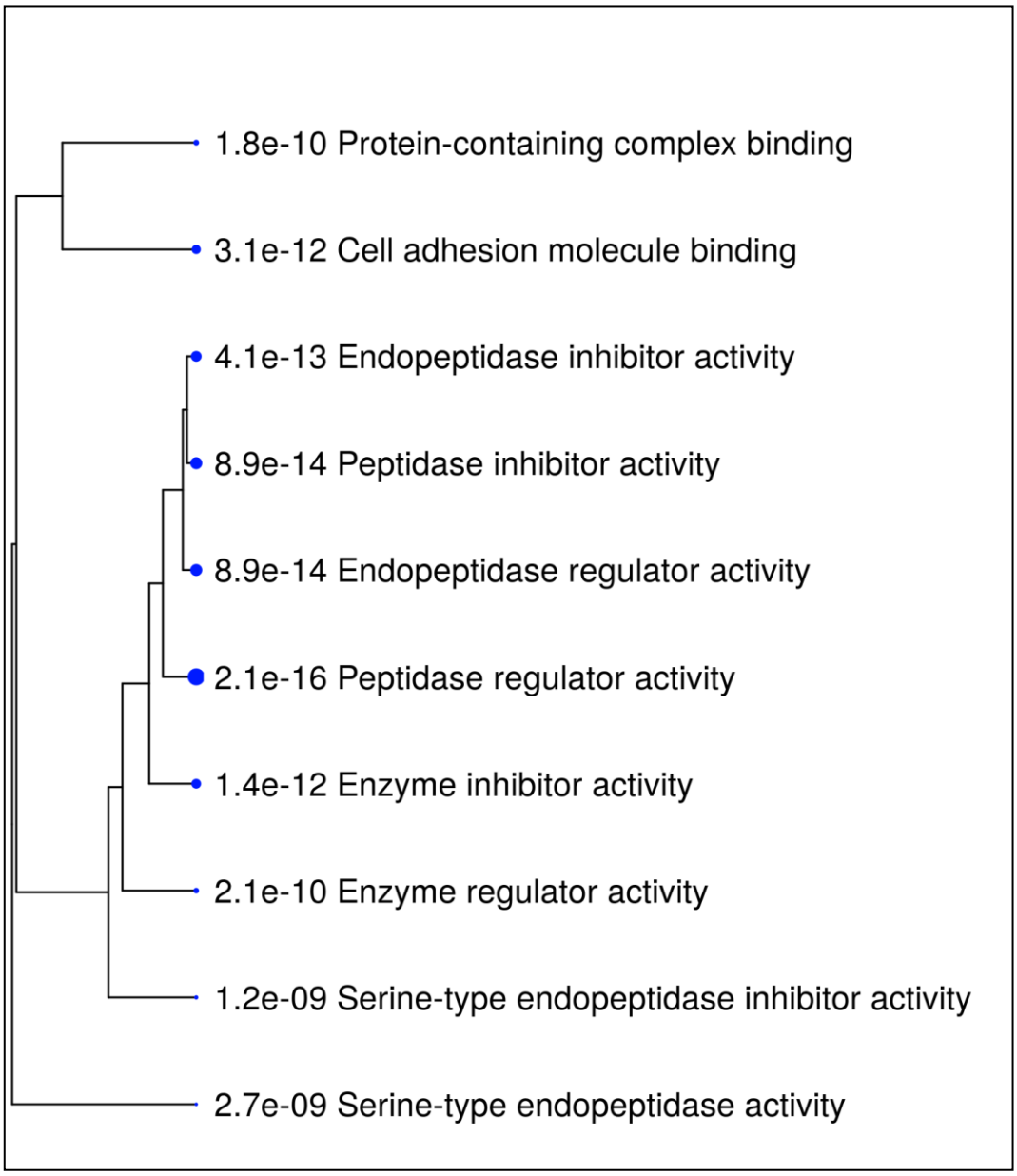


Plate 24. Phylogenetic tree of molecular function in TB infected group. Bigger blue dot depicts higher significance in p – value.

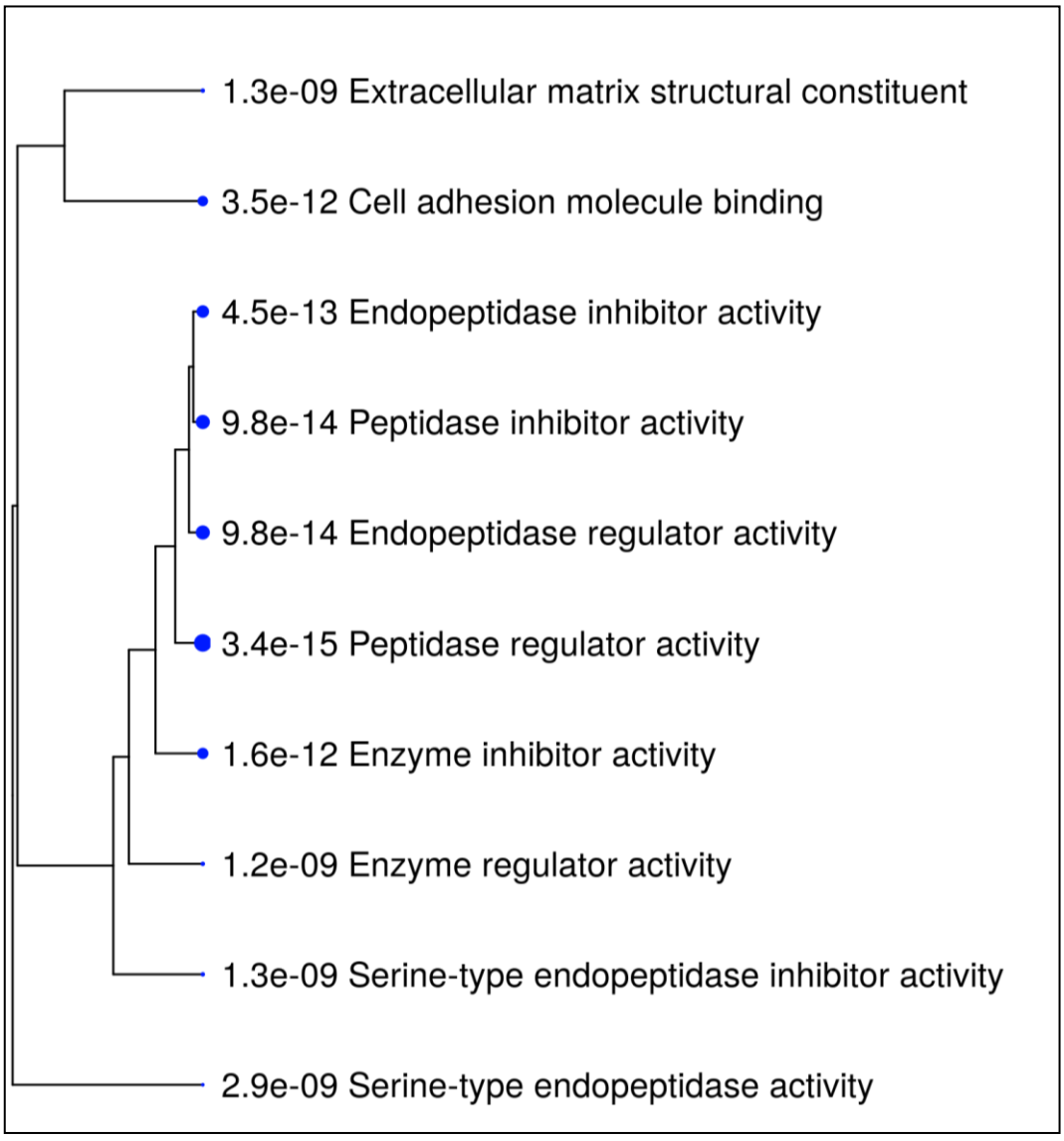


Table 22. Comparison of proteins in TB infected and control group based on cellular component

Sl. No	Cellular Component	No. of Proteins In TB Infected Group	No. of Proteins in control Group
1	Cellular anatomical entity	98	89
2	Protein-containing complex	17	18
3	Intracellular	19	16

Fig 17. Comparison of proteins in TB infected and control group based on cellular component

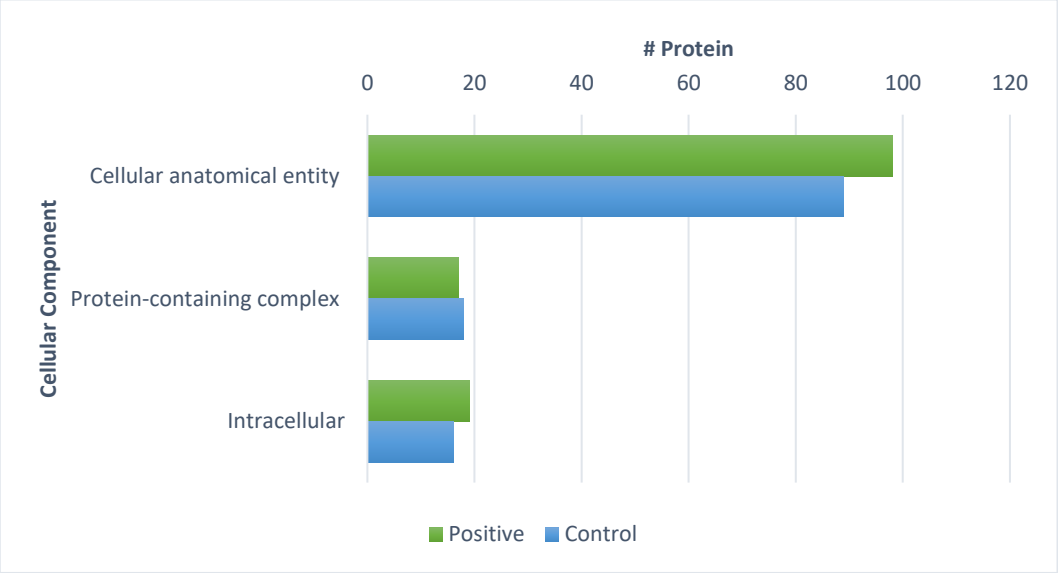


Plate 25. Phylogenetic tree of cellular component in control group. Bigger blue dot depicts higher significance in p – value.

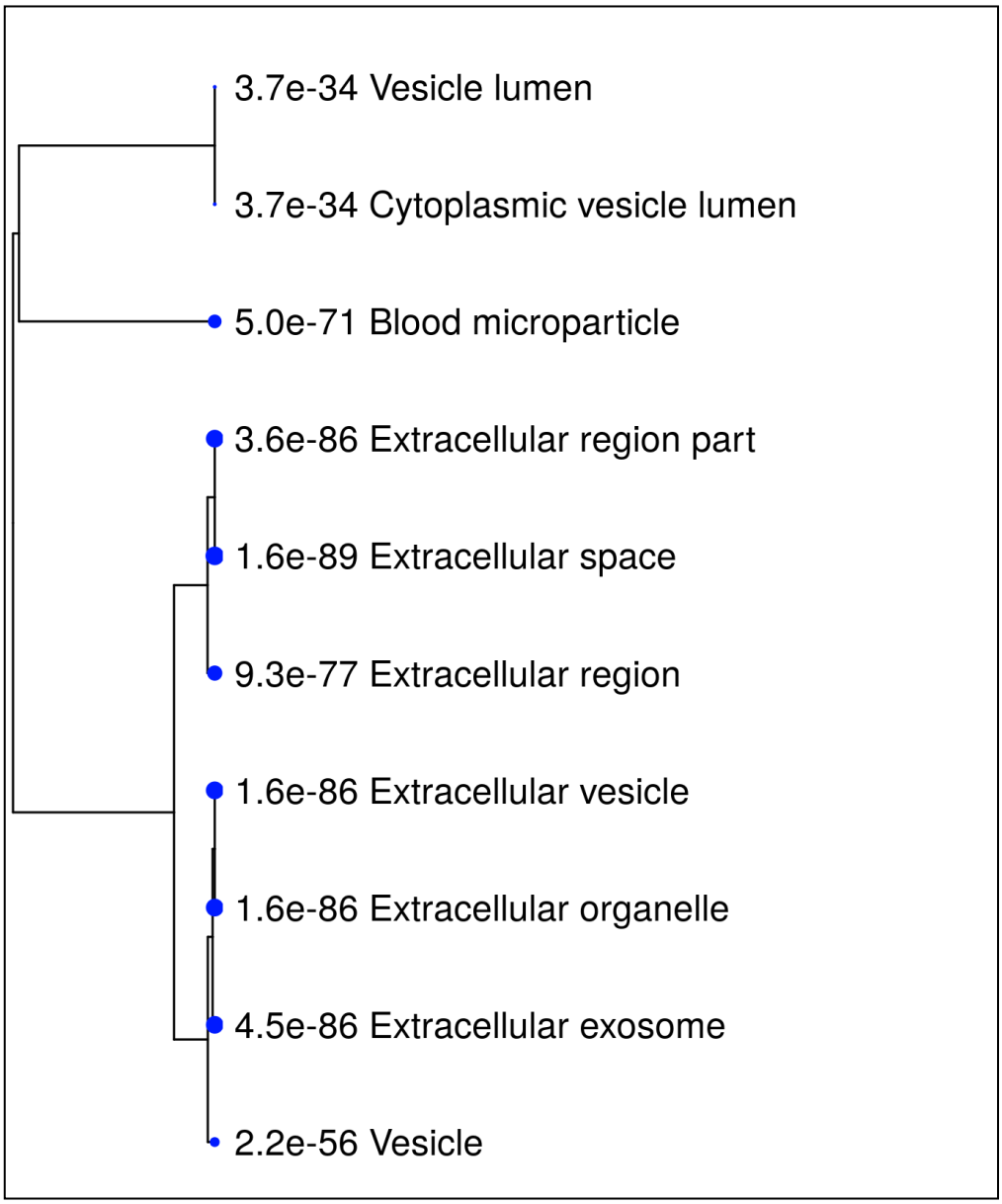


Plate 26. Phylogenetic tree of cellular component in TB infected group. Bigger blue dot depicts higher significance in p – value.

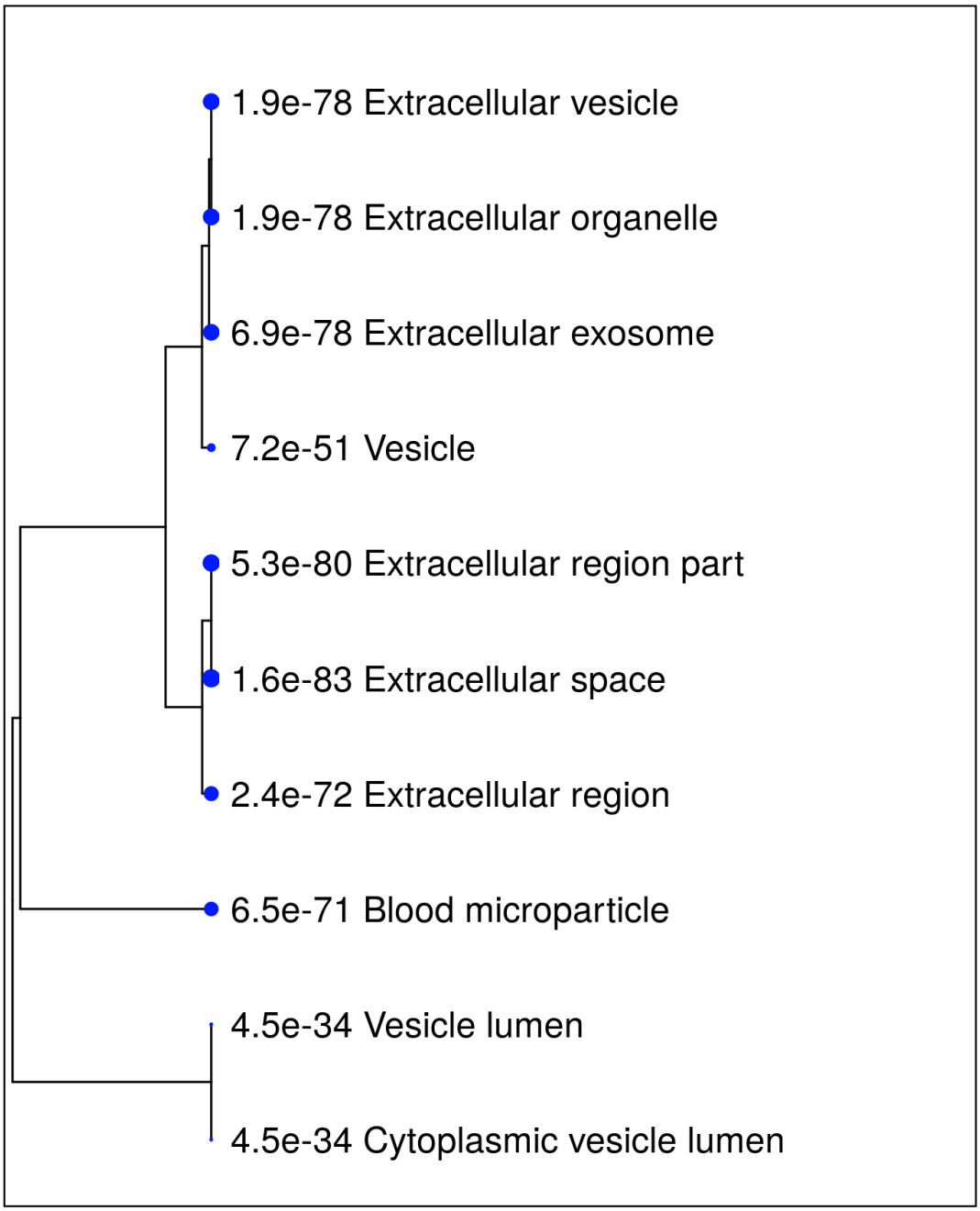


Plate 27. Protein – protein interaction in control group

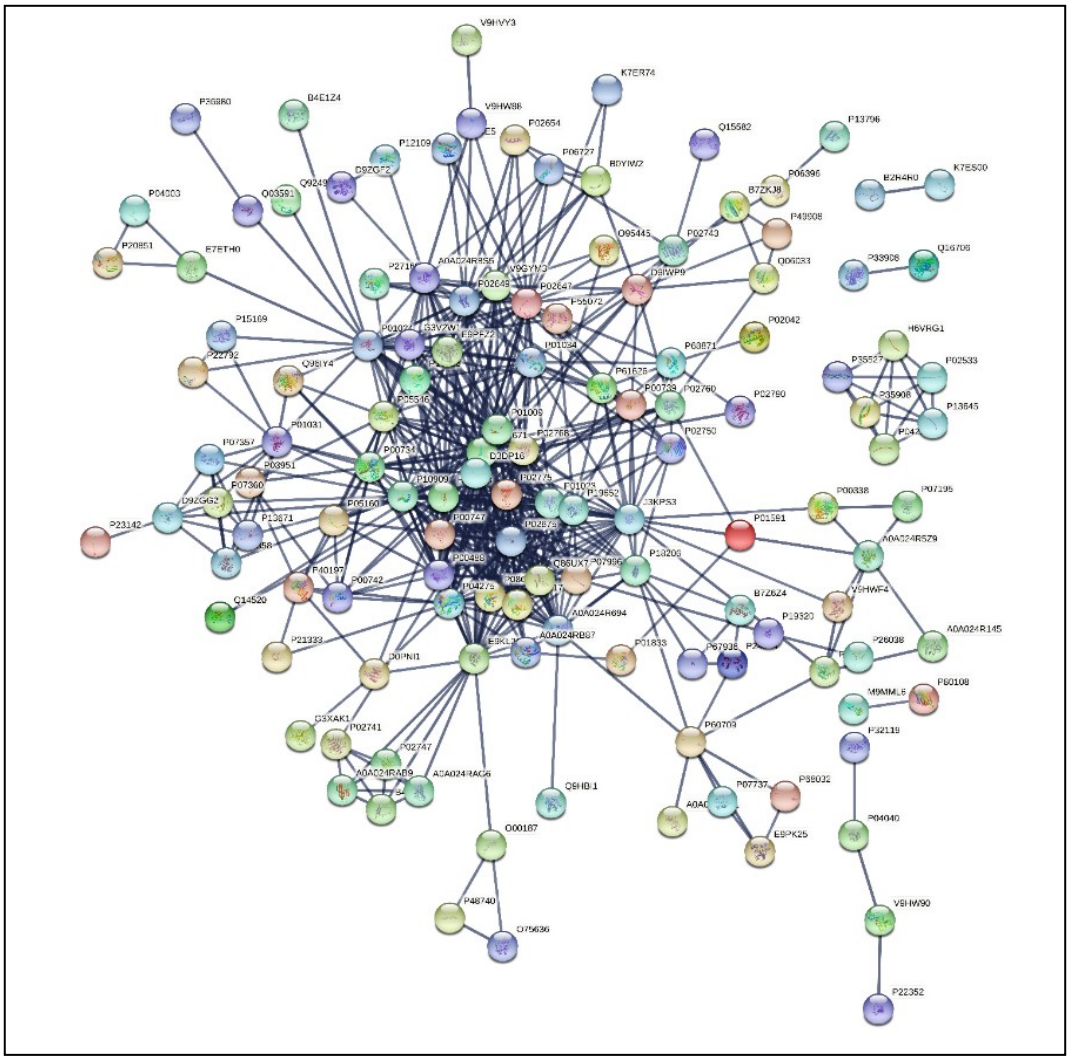
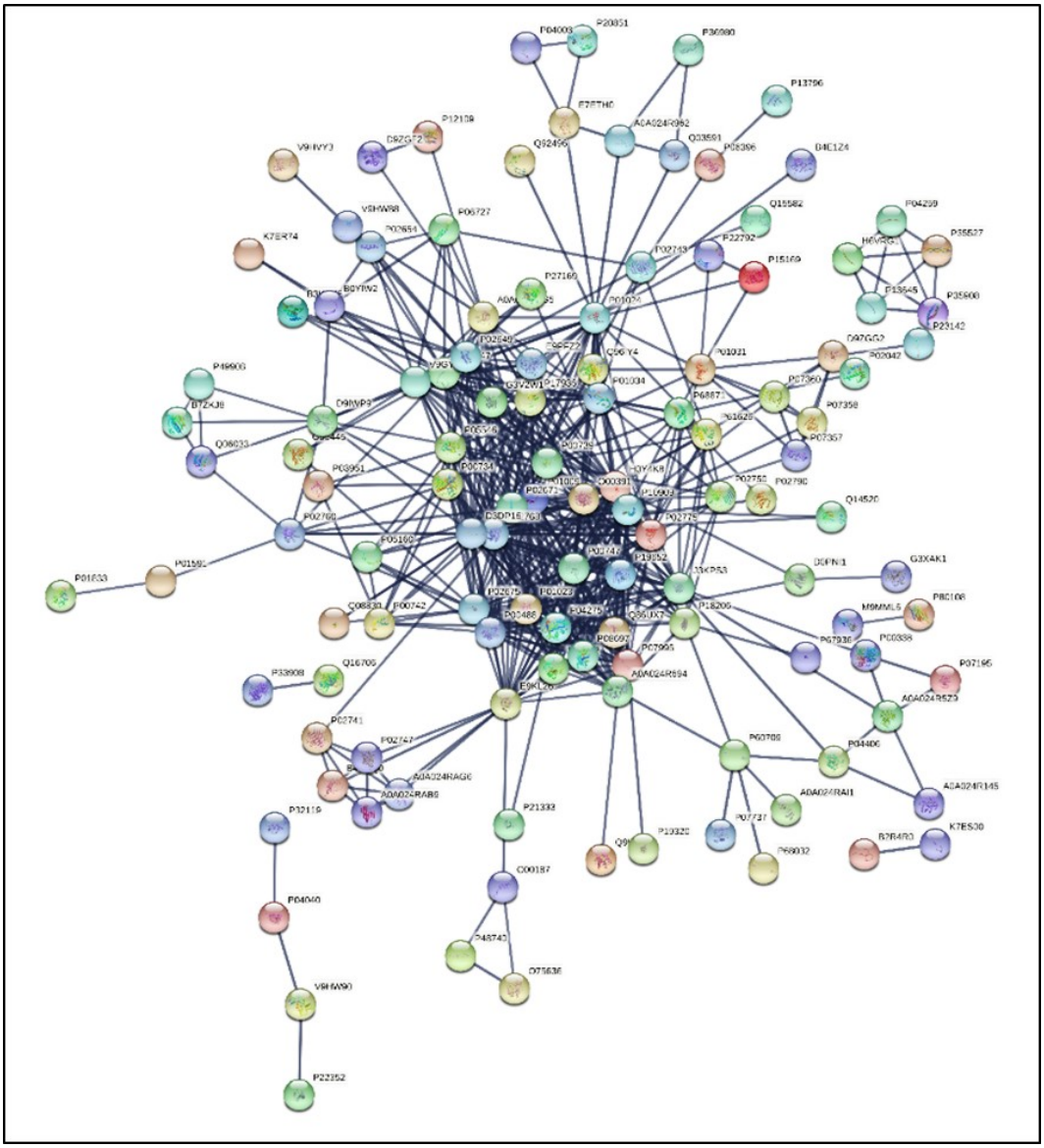


Plate 28. Protein – protein interaction in TB infected group



Discussion

5. DISCUSSION

The present study was conducted in the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Pookode, Wayanad during the period of September 2019 to December 2020. A total of 86 captive Asian elephants from Kerala formed the subject of the study.

5.1 SCREENING USING LATERAL FLOW ASSAYS

5.1.1 Screening using DPP® (Dual Path Platform) VetTB - Elephant assay kits by Chembio Diagnostic Systems, INC.

Sera from 86 elephants were screened for tuberculosis using DPP VetTB assay. Sera of 32 out of 86 elephants were seropositive for tuberculosis. Seroprevalence of 37.2 per cent was obtained in the study, this was in agreement with the study conducted by Abraham *et al.* (2008), Greenwald *et al.* (2009), Mikota and Maslow (2011), Rosen *et al.* (2018) and Kerr *et al.* (2019) where all the authors obtained a seroprevalence greater than 15 per cent. In comparison with the previously published reports, present study has relatively higher seroprevalence and this may be attributable to the lack of regular TB screening in elephant population, lack of awareness among elephant owners about the subclinical TB infection in their animals, which could result in higher transmission rate among elephants.

In the present study, DPP VetTB assay was used for screening of elephants for tuberculosis. It was a novel USDA approved lateral flow kit currently used for the screening of tuberculosis in elephants (Greenwald *et al.,* 2009). Dual path platform Vet TB assay detects antibodies for CFP10/ESAT-6 antigen in whole blood or serum. Greenwald *et al.* (2009) in their study, found that all the elephants included in the TB infected group had antibodies for CFP10/ESAT-6 antigen and there was no seroreactivity in non TB group which indicated that there was 100 per cent specificity and sensitivity for DPP VetTB assay. Lyashchenko *et al.* (2012) in his study proved that DPP VetTB assay has the potential to detect tuberculosis infection months before the actual excretion of the organism. Specificity and sensitivity of the test was found to be 100 and 90-100 per cent respectively. Angkawanish *et al.* (2010) also indicated the high test accuracy for DPP VetTB assay.

5.1.2 Screening using Alere Determine^{TM} TB LAM Ag assay

Urine samples from 86 elephants were screened for mycobacterium using LAM TB Ag test (LF-LAM). Urine samples of 29/86 elephants (33.7 per cent) were positive and 57/86 elephants (66.3 per cent) were negative for Lipoarabinomannan (LAM) antigen of mycobacterium organisms. In comparison with DPP VetTB

assay, LF- LAM assay demonstrated sensitivity of 90.63 per cent and specificity of 100 per cent. The positive predictive and negative predictive values were 100 per cent and 94.7 per cent respectively. Further, the test had high kappa statistical and accuracy values which were 0.924 (p - value <0.001) and 95.51 per cent respectively. These values indicated that LF-LAM assay could be as efficient as DPP VetTB assay and could be used as an alternative for DPP VetTB assay.

In comparison with AFB smear microscopy, LF-LAM assay had sensitivity of 100 per cent, specificity 74.03 per cent, Positive Predictive value of 31.0, Negative Predictive value of 100.0, accuracy of 76.74 and Kappa Statistics value of 0.374; p-value <0.001. These values indicate that LAM assay has higher sensitivity that traditional smear microscopy. These findings were in accordance with previously published report by Elsawy and Redwan (2012). The LAM detection assay by Alere Determine LF-LAM assay was found to have increased sensitivity compared to other LAM detection assay by Chemogen, Although both assays use the same polyclonal antibodies, the differences in the manufacturing process *viz* matrix composition, pH and viscosity had considerable impact on the performance of the test (Minion *et al.,* 2011). The positivity in LAM assay is directly proportional to the bacillary burden in the body (Minion *et al.,* 2011; Agha *et al.,* 2013).

However in a study conducted by Minion *et al.* (2011) in their systemic review and meta-analysis of LAM in humans found that the sensitivity of the LAM assays in microbiologically confirmed cases were variable ranging 13 – 93 per cent and specificity 87 – 99 per cent. Nakiyingi *et al.* (2014) found a sensitivity of 38 per cent and specificity of 100 per cent. The variations in the sensitivity in present study and previously published studies could be attributed to the variable LAM detection methods, disease severity, patient population and specimen handling.

5.1.3 Screening using acid fast staining technique

Trunk wash smears of all 86 elephants were screened for acid fast bacilli. Smear of 9/86 (10.46 per cent) elephants demonstrated acid fast organisms. On comparison with DPP VetTB assay, acid fast staining shows sensitivity of 28.13 per cent, specificity 100 per cent, positive predictive value 100 per cent, negative predictive value 70.1 per cent, accuracy 73.26 per cent and kappa statistics value of 0.329 (p-value <0.001). The sensitivity of smear microscopy obtained in the study was comparatively less when compared to the previously published studies by Qureshi *et al.* (2019) and Schluger, (2018) in human sputum samples. The sensitivities obtained in those studies were 47.6 per cent and 38.8 per cent respectively. Mathew *et al.* (2002) reported that on comparison of AFB of sputum

smear and culture, smear held a sensitivity of 67.5 per cent (95 per cent Cl, 60.6 to 73.9) and specificity of 97.5 per cent (95% CI, 97.0 to 97.9). The specificity of smear microscopy in the present study was similar to previously published report by Mathew *et al.* (2002). The low sensitivity of smear microscopy might be due to the difference in the severity of infection among elephants or might be due to the intermittent shedding of the organisms. In order to demonstrate a positive result in smear microscopy approximately 5,000 to 10,000 bacilli would be required (Qureshi *et al.,* 2019). Reduced number of bacilli in the samples could be a factor for the low sensitivity.

5.2 CLINICAL PATHOLOGY

5.2.1 Haematological evaluation

Blood samples were collected for haematological evaluation on the day of presentation. The values obtained were statistically analysed. The mean and their standard error for various parameters were tabulated. In the present study the mean values of Total erythrocyte count (TEC), Haemoglobulin (Hb), Haematocrit (HCT) and Mean corpuscular volume (MCV) in TB seropositive group were comparatively low compared to control group. This might be attributable to the production of cytokines, Interleukin-1 (IL-1), IL-2 and IL-6, where these factors causes diversion of iron into their iron store in the reticuloendothelial system. Hence the availability of iron in plasma would be reduced which could result in low Hb, TEC and HCT (Abdelkareem *et al.,* 2015). However, in haematological parameters studied, there was no statistical significant difference between the TB seropositive and control group. This observation is in accordance with the study conducted by Ranjini *et al.* (2021) in captive Asian elephants. This was not in agreement with the previously publishes studies (Abdelkareem *et al.* 2015, Rohini *et al.,* 2016, Abay *et al.,* 2018) in TB infected humans. They found that in TB confirmed patients, Hb, TEC and HCT were significantly low.

The individual mean values of Total leukocyte count (TLC), lymphocytes and platelets (PLT) were higher in TB infected group when compared to control group. This might be due to the body immune defence mechanism to infection. There can be increase in polymorphonuclear cells and macrophages which can contribute to the leucocytosis, lymphotocytosis, thrombocytosis and neutrophila. However, there was no statistical significant difference in TLC, lymphocytes and platelets between TB seropositive and control group. This finding was in contrast with Yaranal *et al.*, 2013; Abdelkareem *et al.*, 2015; Rohini *et al.*, 2016 and Rathod *et al.,* 2017; Ranjini *et al.,* 2021 where tuberculosis confirmed patients showed significant increase in TLC, lymphocyte count and platelet count.

In a study conducted by Banerjee *et al.* (2015) reported that ESR could be

considered as a sensitive parameter for any inflammatory response. In infections like tuberculosis the Erythrocyte sedimentation rate (ESR) value could be higher due to the pro-inflammatory reaction, increase in fibrinogen and immunoglobulins. The present study also exhibited an elevation in the mean value of ESR in TB seropositive group in comparison with control group. However there was no statistical difference between these groups. This was in agreement with the study conducted by Ranjini *et al.* (2021) in TB infected elephants. These findings were in contrast with the previously published reports (Oliva *et al.*, 2008; Rohini *et al.,* 2016 and Kahase *et al.,* 2020) where authors obtained marked statistical difference in ESR value in TB infected patients.

In the present study overall statistically significant difference does not exist in haematological parameters in TB seropositive and control group could be due to the influence of the sample size.

5.2.2 Serum biochemical evaluation

The obtained serum biochemistry values were statistically analysed. The mean and standard error of the various serum biochemical parameters were tabulated (Table 7). The mean values of total protein, albumin and globulin in TB seropositive group were lower than the control group, which in turn resulted in low albumin globulin ratio. There were significant statistical differences in total protein, albumin and albumin globulin ratio which was in agreement with the previously published by reports (Wong and Saha*,* 1989; Okolie, 2016; Ranjini *et al*., 2021). The authors reported that in TB infected patients there were decline in total protein, albumin and albumin globulin ratio and was statistically relevant. The rise in serum protein level could be due to altered immunity, edema, inflammatory response, decreased antioxidant activity in the body (Okolie, 2016).

There was no statistical significant difference in Blood urea nitrogen (BUN), serum cholesterol, total bilirubin, indirect and direct bilirubin, ALT, ALP values between TB seropositive and control group. This result was in agreement with the study conducted by Giri *et al*. (2007) and Ranjini *et al*. (2021). It was found that BUN, serum cholesterol, total bilirubin, indirect and indirect bilirubin, ALT, ALP values had no correlation with the TB in Asian elephants (Giri *et al*., 2007; Ranjini *et al*., 2021). However the mean cholesterol level in TB seropositive group was found to be lower than the control group, this was in accordance with the study by Miner *et al*. (2009). In their study on role of cholesterol in TB infection reported that cholesterol was crucial for the persistence of mycobacterium. There was a marked elevation in the mean glucose value in TB seropositive group and statistically significant difference were noticed between the TB seropositive and control groups. This was in accordance with the study by Jawad *et al.* (1995) who

reported glucose intolerance in TB patients. The study showed glucose rise in 49 per cent of patients with active tuberculosis. Statistically significant difference was noticed in AST values in TB seropositive and control groups, this could be due to some underlying hepatic dysfunction or it could be an isolated finding (Kerr, 2002).

5.2.3 Urine evaluation

A detailed urine evaluation was carried out in 32 TB seropositive elephants and 32 healthy elephants. Analysis of various urine test parameters pertaining to the disease TB is a least explored area until now in humans and animals. This could be the first report on estimation of urine parameters with respect TB in elephants. Urine samples were collected on the day of presentation and evaluated. The values acquired where statistically analysed separately for TB seropositive and healthy elephants. The mean and chi-square test significance for the various urine parameters were tabulated.

5.2.3.1 Colour of urine

Out of 32 seropositive elephants, 12 (37.5 per cent) elephants had straw coloured urine, four (12.5 per cent) had amber coloured urine and 16 (50 per cent) had dark yellow coloured urine. Statistical analysis revealed no significant difference in colour of the urine between TB seropositive and control group. Normal healthy elephants could possess variation in colour of the urine ranging from straw to dark yellow (Benedict, 1936; Simon, 1958; Ranjini *et al.,* 2021). Therefore it can be concluded that variation in colour may not have any association with the TB condition.

5.2.3.2 Appearance of urine

In TB seropositive group of 32 elephants, 29 (90.6 per cent) elephants had clear urine and three (9.37 per cent) had turbid urine. Statistical analysis revealed that there were significant difference ($p < 0.05$) in the appearance of the urine between TB seropositive and control group. Depending upon the presence or absence of crystals or mucus cells in urine the appearance of it may vary in healthy elephants (Benedict, 1936; Simon, 1958; Ranjini *et al.,* 2021). Therefore the association between clear urine and the disease TB may not have a diagnostic importance.

5.2.3.3 Detection of urine protein

In the urine of TB seropositive and control group, all samples showed negative results for SSA test. The SSA test implicate the presence of protein in the urine. In the previously published reports Wiedner *et al.* (2009) and Ranjini *et al.* (2021) there were complete absence of turbidity indicating absence of protein in all

the samples.

5.2.3.4 Evaluation using Dipstick

All elephants in both TB seropositive and control group were subjected to urine dipstick analysis. Elephants belonging to both seropositive and control group had trace amount of leukocytes, absence of urobilinogen, ketones, blood and glucose. This was in agreement with Wiedner *et al.* (2009) and Ranjini *et al.* (2021). On the contrary in a study conducted in human TB patients it was found that among 90 newly diagnosed TB patients 65 per cent of patients were confirmed with glycosuria using dipstick method (Restrepo and Schleringer, 2013). The absence of glucose in seropositive elephants indicates that unlike in human, in elephants TB does not cause glycosuria. However further studies with more samples are required to confirm this result.

There were presence of bilirubin in two elephants belonging to TB seropositive group and bilirubin was absent in 93.75 per cent of elephants in TB seropositive group. This was in accordance with the study by Osborne *et al.* (1972) where trace amount of bilirubin was considered as normal in healthy elephants. It indicates that there might not be any correlation between the presence of bilirubin and TB infection.

The specific gravity of urine in TB seropositive and healthy elephants were 1.012±0.009 and 1.015±0.007 respectively. The range of specific gravity noticed in previously published reports (Benedict *et al.,* 1936; Wiedner *et al.*, 2009 and Ranjini *et al.* 2021) were between 1.019 – 1.025 which was similar to the values obtained in present study. It indicates that there might not be any correlation between the specific gravity and TB infection.

Apparently healthy elephants has slightly alkaline urine. The present study obtained mean pH of 7.92 ± 0.21 in TB seropositive group and 7.94 ± 0.15 in control group. The above obtained values of pH was in accordance with the pH values acquired by Wiedner *et al.* (2009) and Ranjini *et al.* (2021) in normal healthy elephants. It indicates that there might not be any correlation between the pH and TB infection.

In the urine of TB seropositive group, 23/32 (71.8 per cent) showed presence of nitrite. Statistical analysis using chi square test revealed that there was a significant difference ($p < 0.05$) in the presence or absence of nitrite between TB seropositive and control group. This was in contrast with the study by Behandus *et al.* (2016) where all TB infected human patients were negative for nitrite in dipstick test. In the presence of bacteria, nitrates in the urine are converted to nitrites therefore nitrites indicate presence of bacteria in the urine.

There were trace amount of protein in 4 (12.5 per cent) elephants, moderate amount of protein was noticed in 4 (12.5 per cent) elephants belonging to TB seropositive group and 24 (75 per cent) elephants had no protein in urine. Presence of mild to moderate amount of protein could be attributable to the diet, physiological condition of the animal, inflammatory response and could also indicate faulty glomerular filtration.

5.3 MOLECULAR DIAGNOSIS USING POLYMERASE CHAIN REACTION

Trunk wash samples from 32 seropositive elephants were subjected to PCR targeting gene IS*6110*, at 245bp size amplicon to confirm the presence of *Mycobacterium tuberculosis.* Out of 32 seropositive elephants, 25 (78.2 per cent) elephants were confirmed positive for *Mycobacterium tuberculosis* and 7 (21.8 per cent) were negative for *Mycobacterium tuberculosis*. This finding supports the evidence of active and latent TB in elephants, which was in agreement with the study conducted by Ong *et al.* (2013). In their study they found that three seropositive samples were positive for trunk wash PCR, seven seropositive samples were negative in trunk wash PCR and concluded that there could be active and latent TB in elephants.

In the present study sensitivity obtained was 78.2 per cent, this was in agreement with Cheng *et al.* (2004) who found that the sensitivity of TB PCR in culture confirmed cases was 75.9 per cent. Considering the culture as gold standard test the sensitivity was found to be 78.3 per cent, for pulmonary cases it was 82.3 per cent and for extra pulmonary cases it was 72 per cent.

5.3.1 Sequencing of PCR product and phylogenetic analysis

On blasting the acquired trimmed sequence in National centre for biotechnology information (NCBI) showed that the attained sequence shows 98 per cent query cover and 100 per cent identity with other published *Mycobacterium tuberculosis* sequences in the database. Phylogenetic analysis revealed that the isolates obtained were *Mycobacterium tuberculosis*. The sequence showed homology with *Mycobacterium tuberculosis* strain 1-0013P6C4 chromosome.

5.4 PROTEOMIC PROFILING OF URINE FOR IDENTIFICATION OF BIOMARKERS

Identification of urine protein biomarkers formed an integral part of the present study. Urine as a biological sample has various advantages, needs no trained personnel or equipment, can be obtained in surplus, least invasive and contain plethora of molecules that could be considered as a biomarker. This was a preliminary and first ever study focusing on the elephant urine proteome in healthy as well as tuberculosis infected elephants.

Quality of the urine proteins in tuberculosis infected and control group was evaluated by SDS-PAGE. SDS-PAGE revealed that there were differentiating bands in tuberculosis infected group from that of control group. The difference in band patterns indicate the possibility of using 1D- electrophoresis to assess the quality of precipitated proteins obtained and to identify the differentiating band patterns. The differential band pattern observed in the present study showed similarity with the band pattern observed in rat urine infected with tuberculosis (Mukherjee *et al.,* 2004). In their study on *Mycobacterium tuberculosis* proteins in rats infected with TB, found that unique bands were obtained at 75kDa, 25kDa and 16.5kDa whereas in the present study major bands were formed between 75-100kDa, minor bands between 25-37kDa, 150kDa and 250kDa in TB positive group. Similar differential expression of urinary protein patterns was observed in humans through 2D gel electrophoresis were between 10- 75kDa (Wang *et al.,* 2018). Therefore SDS-PAGE could assist in urine based proteomic diagnosis of tuberculosis once it is standardised based on different stages of tuberculosis and physiological status of healthy animals.

Mass spectrometry based proteomics analysis was a major emerging tool in determining the physiological and pathological role of the body systems in both healthy and diseased condition (Liotta *et al.,* 2003). Furthermore the agile platform had contributed immensely to the field of disease diagnosis and contributed to the discovery of specific biomarkers to confirm the disease condition (Comes *et al.,* 2018). Introduction of MS analysis to the study of diseases helps to have an elaborate outlook on various biomarkers which were critical for the detection and monitoring and treatment specific disease entities (Lim *et al.,* 2004).

Among the biological samples selected for proteomics, urine being the abundant biological fluid and least invasive sample that can be collected makes urine an ideal candidate for biomarker discovery (Brondani *et al.,* 2020; Siebert *et al.,* 2017). Bathala *et al.* (2015) describes urine as an ideal sample for the identification of biomarkers as it is a dynamic biological fluid, the composition changes according to the metabolism of the animal at that point of time. This aids in acquiring a true reflection of the metabolic process in animals.

In the present study, precipitated urine protein of both TB infected and control group were subjected to proteomics analysis. The urine proteins demonstrated differential expression in both conditions. The differential expression of proteins had led to the alteration of pathways. There were higher number of proteins related to immune system process, biological regulation, protein related to response to stimulus and cellular process in TB positive cases indicating more immune related biological activity in elephants having tuberculosis. Acute inflammatory response and complement activation protein were more in positive

case further confirming immune related activity. This was confirmed by higher number of protein activation cascade related proteins in positive cases. Furthermore, for marker discovery, we found 23 protein were exclusively present in health animals and only one protein Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase was exclusively obtained in TB infected group. This was in agreement with Young *et al.* (2014) where they reported the presence of similar biomarker in humans. They reported that urine biomarkers had true potential to be developed as a new point of care test for the diagnosis of TB. They could identify 10 mycobacterial proteins exclusively in the urine of TB confirmed patients, six proteins exclusively in latent TB patients. There were 20 human proteins that could significantly discriminate between TB infected and non TB group. Ovalle *et al.* (2006) reported that Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase was the protein responsible for proteoglycan synthesis, and play a role in glycosaminoglycan biosynthesis. This protein have been reported to be identified as an early TB diagnosis marker in humans (Garra, 2013). The present study was the first report of the presence of Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase protein in urine of elephant with tuberculosis. Since the presence of this enzymatic protein is required for the viability and persistence of the organism further validation of this protein in a diagnostic level could be a milestone in TB diagnosis in elephants.

Summary

SUMMARY

According to the mythological and cultural beliefs, elephants are considered as a sacred symbol of power, spirituality and strength. This belief continues to be a part of Indian culture even today. These majestic pachyderms still form a vital part of the temple and religious festivities. However, there is a decline in the number of Asian elephants in India. Asian elephants are listed as endangered species as per International Union for Conservation of Nature (IUCN). The major causes of decline is due to habitat loss, diseases and poaching. Tuberculosis (TB) had been reported worldwide in captive elephants. The prognosis of the disease in elephants are grave if not diagnosed earlier. In Asian countries, the occurrence of TB is high among captive wild elephants due to the co-existence and close association with humans and other domestic livestock. Infected animals tend to shed the organism in the preclinical period which risks the transmission of the infection to elephants, humans, and other mammals. Definitive antemortem diagnosis of TB in elephants can be challenging and has serious limitations. For the past two decades, the diagnosis of TB predominantly relied on trunk wash culture and acid-fast staining techniques. Trunk wash culture is considered as one of the gold standard test for active TB infection in elephants however, the test has numerous limitations.

Present study highlights on exploring new methodologies for the early diagnosis of TB and assessing the reliability of considering urine as a biological sample for the diagnosis of TB in elephants. Urine was selected as a primary sample because it is least invasive, requires less labour, and easily available from all the elephants. The primary focus of the study was to identify biomarkers from urine.

The study design utilized serological, molecular, and proteomic diagnostic assays for early TB diagnosis in elephants. Seroprevalence of tuberculosis in elephants were evaluated in 86 elephants from Kerala using Chembio DPP VetTB, which revealed 37.2 per cent seroprevalence. All 86 elephants were screened for acid fast bacilli and LAM antigen using trunk wash smear and urine samples respectively. Nine (10.46 per cent) out of 86 elephants were positive for AFB and 29 (33.7 per cent) out of 86 elephants were positive for LAM antigen. On comparison of efficiency of LF-LAM assay with that of DPP VetTB assay, LF-LAM assay had a sensitivity of 90.63 per cent, specificity 100 per cent , positive predictive value 100 per cent, negative predictive value 94.7 per cent, accuracy 95.51 per cent and kappa statistic value 0.924 (p - value <0.001). On comparison of LF-LAM with traditional acid fast staining method, LF- LAM assay had the sensitivity of 100 per cent, specificity 74.03 per cent , positive predictive value 31 per cent, negative predictive value 100 per cent, accuracy 76.74 per cent

and kappa statistic value 0.374 (p - value <0.001).

The 32 Elephants with positive seroreactivity for tuberculosis in DPP VetTB assay were considered as TB seropositive group and 32 elephants which were negative in DPP VetTB assay, LF-LAM, AFB staining were considered as control group. To monitor the haemato-biochemical changes associated with tuberculosis in TB seropositive and control group, blood, serum and urine were subjected to haematological, serum biochemical and urine evaluation. The mean value of glucose in TB seropositive group (157.35 ± 14.96) were higher than that of control group (124.62 ± 5.53). The mean value of total protein (9.1 ± 0.28), albumin (2.43 ± 0.12), albumin globulin ratio (0.36 ± 0.02) and aspartate aminotransferase (23.4 ± 1.32) was lower than that of control group. Hence the parameters like serum glucose, total protein, albumin, albumin globulin ratio and AST showed statistical significant difference between TB seropositive and control group. There were no statistical significant difference in other serum biochemical and haematological parameters. On urine evaluation, there were statistical significant difference in appearance and nitrite component (71.9 per cent) of the urine between the two groups.

Trunk wash sample of 32 seropositive elephants were evaluated using PCR targeting gene IS*6110*, at 245bp size amplicon to confirm the presence of *Mycobacterium tuberculosis.* Out of 32 seropositive elephants, 25 (78.2 per cent) elephants were confirmed positive for *Mycobacterium tuberculosis.* After custom sequencing and phylogenetic analysis revealed that the isolate obtained were *Mycobacterium tuberculosis*.

The urine of six TB confirmed elephants (positive for DPP VetTB assay, LF-LAM, AFB and PCR) and six healthy elephants (negative for DPP VetTB assay, LF-LAM, AFB and PCR) were selected for proteomic analysis. The protein in urine was precipitated, quantified, subjected to SDS-PAGE, enzymatic digestion and HRLCMS analysis. Mass spectrometry data analysis revealed that there were 49 proteins identified in TB infected group, 68 proteins identified in control group and Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase was the protein exclusively present in TB infected group. Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase was identified as a potent biomarker for tuberculosis, responsible for proteoglycan synthesis and it plays a major role in glycosaminoglycan biosynthesis. The present study is the first report of the presence of Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase protein in urine of elephant.

Conclusions derived from the study:

1. In Kerala the seroprevalence of tuberculosis in elephants was 37.2 per cent.

2. Urine could be used a potential biological sample for TB diagnosis in elephants.
3. Potential biomarkers identified from urine were Lipoarabinomannan (LAM) and Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase.
4. Alere Determine TB LAM Ag (LF-LAM) found to be a reliable diagnostic assay with sensitivity of 90.63 per cent, specificity 100 per cent , positive predictive value 100 per cent, negative predictive value 94.7 per cent, accuracy 95.51 per cent and kappa statistic value 0.924 (p - value <0.001) in comparison with that of standard DPP VetTB assay.
5. TB LAM Ag (LF-LAM) is a lateral flow assay that has been used in human field till date, present study was the first study to demonstrate its adoptability to veterinary field.
6. Mass spectrometry data analysis revealed that there were 49 proteins identified in TB infected group, 68 proteins identified in control group.
7. Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase was the protein exclusively present in TB infected group. Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase was identified as a potent biomarker for tuberculosis on proteomics analysis.
8. There were difference in number and abundance of the protein in TB infected and control group which could indicate difference in expression of protein in diseased conditions in comparison with that healthy condition.
9. These identified biomarkers could be further validated and extrapolated to design an animal side diagnostic lateral flow assay.
10. This is the first study of urine protein biomarkers associated with TB in elephants.

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IDENTIFICATION OF URINE BIOMARKERS FOR THE DIAGNOSIS OF TUBERCULOSIS IN ELEPHANTS

RANJINI MANUEL

(18–MVP–19)

ABSTRACT

Abstract of thesis submitted in partial fulfilment of the requirement for the degree of

MASTER OF VETERINARY SCIENCE

(Veterinary Epidemiology and Preventive Medicine)

2021

Faculty of Veterinary and Animal Sciences

Kerala Veterinary and Animal Sciences University

DEPARTMENT OF VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

POOKODE, WAYANAD- 673 576

KERALA, INDIA

ABSTRACT

The present study envisages on utilization of serological, molecular and proteomic diagnostic assays for tuberculosis diagnosis. The usage of urine as a biological sample for the identification of biomarkers for tuberculosis forms the prime focus of the study. The objectives were screening of elephants for tuberculosis using Chembio DPP VetTB assay, proteomic study and identification of biomarkers from urine of healthy and tuberculosis infected elephants.

Seroprevalence of tuberculosis in elephants were evaluated in 86 elephants from Kerala using Chembio DPP VetTB, which revealed 37.2 per cent seroprevalence. All 86 elephants were screened for acid fast bacilli and LAM antigen using trunk wash smear and urine samples respectively. Nine (10.46 per cent) out of 86 elephants were positive for AFB and 29 (33.7 per cent) out of 86 elephants were positive for LAM antigen. On comparison of efficiency of LF-LAM assay with that of DPP VetTB assay, LF-LAM assay had a sensitivity of 90.63 per cent, specificity 100 per cent , positive predictive value 100 per cent, negative predictive value 94.7 per cent, accuracy 95.51 per cent and kappa statistic value 0.924 (p - value <0.001). On comparison of LF-LAM with traditional acid fast staining method, LF- LAM assay had the sensitivity of 100 per cent, specificity 74.03 per cent , positive predictive value 31 per cent, negative predictive value 100 per cent, accuracy 76.74 per cent and kappa statistic value 0.374 (p - value <0.001).

The 32 Elephants with positive seroreactivity for tuberculosis in DPP VetTB assay were considered as TB seropositive group and 32 elephants which were negative in DPP VetTB assay, LF-LAM, AFB staining were considered as control group. To monitor the changes associated with tuberculosis in TB seropositive and control group, blood, serum and urine were subjected to haematological, serum biochemical and urine evaluation. The mean value of glucose in TB seropositive group (157.35 ± 14.96) were higher than that of control group (124.62 ± 5.53). The mean value of total protein (9.1 ± 0.28), albumin (2.43 ± 0.12), albumin globulin ratio (0.36 ± 0.02) and aspartate aminotransferase (23.4 ± 1.32) was lower than that of control group. Hence the parameters like serum glucose, total protein, albumin, albumin globulin ratio and AST showed statistical significant difference between TB seropositive and control group. There were no statistical significant difference in other serum biochemical and haematological parameters. On urine evaluation, there were statistical significant difference in appearance and nitrite component (71.9 per cent) of the urine between the two groups.

Trunk wash sample of 32 seropositive elephants were evaluated using PCR

targeting gene IS*6110*, at 245bp size amplicon to confirm the presence of *Mycobacterium tuberculosis.* Out of 32 seropositive elephants, 25 (78.2 per cent) elephants were confirmed positive for *Mycobacterium tuberculosis.* After custom sequencing and phylogenetic analysis revealed that the isolate obtained were *Mycobacterium tuberculosis*.

The urine of six TB confirmed elephants (positive for DPP VetTB assay, LF-LAM, AFB and PCR) and six healthy elephants (negative for DPP VetTB assay, LF-LAM, AFB and PCR) were selected for proteomic analysis. The protein in urine was precipitated, quantified, subjected to SDS-PAGE, enzymatic digestion and HRLCMS analysis. Mass spectrometry data analysis revealed that there were 49 proteins identified in TB infected group, 68 proteins identified in control group and Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase was the protein exclusively present in TB infected group. Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase was identified as a potent biomarker for tuberculosis, responsible for proteoglycan synthesis and it plays a major role in glycosaminoglycan biosynthesis. The present study is the first report of the presence of Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase protein in urine of elephant.

Acad. Form No. 19

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY

Faculty of Veterinary and Animal Science

PROGRAMME OF RESEARCH WORK FOR THESIS FOR MASTERS DEGREE

1. Title of thesis:

Identification of urine biomarkers for the diagnosis of tuberculosis in elephants

2. (a) Title of the departmental/ KVASU research project of which this forms a part:

Nil

(b) Code No. if any, and order by which departmental /KVASU research project is approved:

Not applicable

3. a) Name of student:

Ranjini Manuel

b) Admission Number:

18-MVP-19

c) Name of the Discipline:

Veterinary Epidemiology and Preventive Medicine

4. a) Name of Major Advisor (Guide):

Dr. Deepa P. M.

b) Designation:

Assistant Professor and Head (i/c) Department of Veterinary Epidemiology and Preventive Medicine,

College of Veterinary and Animal Sciences, Pookode, Wayanad, Kerala 673576

5. Objectives of the study:

1. Screening of elephants for tuberculosis (TB) using Chembio DPP VetTB assay
2. Proteomics study of urine of healthy and diseased elephants
3. Identification of biomarkers for diagnosis of TB in elephants

6. Practical/Scientific utility:

Tuberculosis (TB) is an ancient malady that continues to be a threat to the elephants world-wide. It is a re-emerging zoonotic disease that can be spread among elephants and humans. Compared to wild elephants, captive elephants are more susceptible to TB as they are in constant contact with the human population. Diagnosis of TB

generally rely on trunk wash culture; however due to low sensitivity, slow turnaround time, variable sample quality and significant biosafety facilities, the test encounters serious limitations. Lipoarabinomannan (LAM) is a heat stable glycolipid found in the cell wall of mycobacterium. It serves as an immunogenic virulence factor that is released from metabolically active or degrading bacterial cells during infection. It is a potential biomarker for TB diagnosis which could be confirmed by analysis of urine. The proteomics of the urine in TB affected elephants may reveal more potential biomarkers for the development of novel diagnostic kits for TB. There is a dire need for the development of highly sensitive and specific diagnostic tests for TB in elephants for the early diagnosis of the disease thereby reducing the mortality caused by the disease.

In humans, sputum microscopy and microbiological culture and specific staining are the golden standard methods. Recent researches have looked at urine as clinically relevant biomarkers for TB. Since collection of urine sample is quite easy and non- invasive, the present study envisages on the detection of LAM and identification of the other relevant protein biomarkers using mass spectrometry from the urine of TB affected elephants.

7. Important publications on which the study is based:

Gopinath and Singh (2008) observed that both PCR using mycobacterium genus specific primers targeting *hsp65* gene and culture by BACTEC MGIT960, Lowenstein-Jensen methods from urine samples showed high sensitivity and 100 per cent specificity.

Lipoarabinomannan (LAM) assay in urine sample by ELISA was found to be superior to sputum microscopy among the patients with CD4 cell count less than 100 cells/µL (Lawn *et al*., 2009).

Greenwald *et al*. (2009) described three novel serologic techniques, the ElephantTB Stat-Pak kit, multiantigen print immunoassay and dual-path platform VetTB test for rapid antibody detection in elephants. The serological assays demonstrated 100 per cent sensitivity and 95 to 100 per cent specificity.

Elsawy and Redwan (2012) compared smear microscopy, sputum mycobacterial culture and urine LAM testing by ELISA for diagnosis of TB in human patients and found that sensitivity of LAM test (94.3 per cent) more than sputum smear microscopy (80.8 per cent).

Verma-Kumar *et al.* (2012) used a latent class model to estimate the diagnostic characteristics of an existing rapid serum test and new four in-house ELISA and found that diagnostic sensitivities of four ELISAs were 91.3 to 97.6 per cent and specificity were 89.6 to 98.5 per

cent and concluded high prevalence of asymptomatic Mycobacterium tuberculosis (*M. tuberculosis*) infection in captive Asian elephants of South India.

Agha *et al.* (2013) estimated LAM level in the urine by ELISA in different TB patients and found that LAM level in urine was higher in patients with disseminated disease than pulmonary or extra pulmonary disease. They concluded that LAM levels in urine was positively correlated with the degree of bacillary burden.

Heydari *et al*. (2014) found 48 per cent f patients with positive sputum culture exhibited positivity for TB by urine PCR and 100 percent specificity and observed that urine PCR could be used as diagnostic aid in TB cases.

Young *et al*. (2014) had conducted proteomic study of human urine sample and identified 10 mycobacterial proteins exclusively in urine of definite TB patients, while six mycobacterial proteins in the urine of presumed latent tuberculosis infection (LTBI) patients. Furthermore, seven human proteins were differentially over or under expressed in TB versus non TB group.

Zachariah *et al*. (2017) reported that 3.4 per cent of Asian elephants in Southern part of India undergone post-mortem examination were confirmed to be infected with *M. tuberculosis* by PCR amplification of the targeted bacterial genome and sequencing.

Urine proteomic profiles of pulmonary TB patients identified nineteen differentially expressed proteins in affected patients. Western blotting and quantitative reverse transcription (qRT- PCR) showed that two proteins, mannose binding lectin 2 and a 35 KDa fragment of inter- a- trypsin inhibitor H4, exhibited highest differential expression. On construction of protein- microRNA interaction network, showed significant differential expression of only miR-625-3p and observed a novel urine biomarker combination based on miR-625-3p, mannose binding lectin 2 and inter- a- trypsin inhibitor H4 significantly improve TB diagnosis (Wang *et al*.,2018).

8. Outline of technical programme:

A minimum of 30 captive elephants will be screened for TB by serological assay using Chembio DPP VetTB assay (Lyashchenko *et al*., 2006). Confirmation of the TB will be done using PCR targeting IS*6110* insertion sequence of the *M. Tuberculosis* complex (Kay *et al*., 2011) from trunk wash. From the acquired samples identification of LAM in urine sample will be done by lateral flow assay kit (Determine ^{TM} TB LAM Ag test kit by Abbott Lab, USA) (Thit *et al*., 2017)

Urine samples will be collected from

a minimum of six tuberculosis seropositive animals and six seronegative healthy controls. The protein that is precipitated from the urine samples will be subjected to Liquid chromatography -mass spectrometry (LC-MS) analysis (Young *et al*., 2014) thereby the MS data generated will be studied for identifying the candidate protein exclusively present in tuberculosis patient and absent in healthy patient. Such protein will be a potential biomarker for TB testing. Haematological and serum biochemical parameters of TB affected and healthy control will be analysed. Results obtained will be statistically analysed using SPSS version 24.0.

9. Main items of observation to be made:

1. Seroprevalence of TB
2. Amplicons specific for TB
3. Protein profile
4. Haematological findings:
   a) Total Red Blood Cell Count ($x10^6/\mu L$)

   b) Total Leucocyte Count ($x10^3/\mu L$)

   c) Haemoglobin (g/dL)

   d) Granulocytes (%)

   e) Lymphocytes (%)

   f) Monocytes (%)

   g) Platelet Count ($10^3/\mu L$)

   h) Packed Cell volume (%)

   i) Mean Corpuscular Volume (fL)

   j) Mean Corpuscular Haemoglobin (pg)

   k) Mean Corpuscular Heamoglobin Concentration (g/dL)

5. Serum Biochemical findings:

   a) Alanine amino transferase (U/L)

   b) Aspartate amino transferase (U/L)

   c) Total protein (g/dL)

   d) Albumin (g/dL)

   e) Globulin (g/dL)

   f) A:G ratio

   g) Serum cholesterol (mg/dL)

6. The results obtained will be statistically analysed using SPSS version 24.0

10. Facilities:

(a) Existing:

Facilities for gene expression study and other biochemical study are available at Department of Veterinary Epidemiology and Preventive Medicine and other departments of College of Veterinary and Animal Sciences, Pookode, Wayanad.

(b) Additional facilities required:

Liquid chromatography-mass spectrometry (LC-MS) facility which is not available in the University, will be availed through outsourcing preferably from Rajiv Gandhi Centre for Biotechnology Thriruvananthapuram

11. Duration of study:

Four semesters

12. Financial estimate:

a. Chemicals and biological : Rs.20,000/-

b. Miscellaneous : Rs 5,000/-

Total : Rs 25,000/-

Signature of the Student

Project coordination group to which the proposal will be placed:

Wildlife

Signature of the Major Advisor

Pookode

27.6.2019

Name and Signature of Members of the Advisory Committee

1. Dr. Deepa P.M.
   Assistant Professor and Head (i/c)

2. Prof (Dr.) K. Vijayakumar
   Professor and Head

2. Dr. Janus A.
   Assistant Professor

3. Dr. Rajeshkumar
   Assistant professor

APPENDIX–I

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APPENDIX II

Time frame of work

Semester I

1. Collection of literature
2. Planning of program for research
3. Preparation of synopsis

Semester II

1. Collection of literature
2. Standardisation of collection procedures

Semester III

1. Sample collection
2. Standardisation of PCR
3. Separation of proteins from urine for proteomic analysis.

Semester IV

1. Continuation of clinical sample testing
2. Analysis of data and interpretation of the results
3. Preparation and submission of thesis

CERTIFICATE

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

Place: Pookode

Date: 27.6.2019

Dr. Deepa

P. M.

Assistant
Professor and Head (i/c)

Major Advisor

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