UNIVERSITI PUTRA MALAYSIA

CELL MEDIATED AND HUMORAL RESPONSE DETECTION AGAINST *Mycobacterium* spp IN CAPTIVE GIBBONS IN MALAYSIA

NG POOI GIN

lp
FPV 2023 64

CELL MEDIATED AND HUMORAL RESPONSE DETECTION AGAINST *Mycobacterium* spp IN CAPTIVE GIBBONS IN MALAYSIA

NG POOI GIN

A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfillment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

Serdang, Selangor Darul Ehsan.

December 2023

CERTIFICATION

It is hereby certified that we have read this project paper entitled "Cell Mediated and Humoral Response Detection Against *Mycobacterium* spp in Captive Gibbons in Malaysia", by Ng Pooi Gin and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 - Project.

DR. AZLAN CHE'AMAT

DVM, MVSc (UPM), PhD (UCLM, Spain)

Senior Lecturer,

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Main Supervisor)

DR. SITI KHAIRANI BEJO

DVM, MSc, PhD (UPM)

Associate Professor,

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Co-Supervisor)

DR. AZALEA HANI OTHMAN

DVM (UPM), MPhil (Queensland), PhD (UPM)

Senior Lecturer,

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Co-Supervisor)

ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge and sincerely thank my final year project main supervisor, Dr. Azlan Che' Amat for his ongoing mentorship throughout the project. His dynamism and humble approach to research is very inspiring. It is an honour privilege to work under his invaluable guidance. Not forget to mention my co-supervisors, Prof Dr Siti Khairani Bejo and Dr Azalea Hani Othman for their constructive feedback and valuable insights which significantly benefit my final year project. Their encouragement plays a crucial part along the journey.

I would also like to express my deepest gratitude towards Dr. Ridhwan, Dr. Zahidah, Dr. Pei Ting, Ms Mariani and Ms Asyikin who gave me opportunities to conduct my project on their respective facilities. Without their help and support, the completion of the project would not have been possible.

I am also thankful to my colleagues and friends, Dr. Atiqah, Dr. Sajidah, Sathishwaran and Zul Ammar who offered unwavering supports to me during the project sampling. Their company along the sampling process had been my anchor in this challenging times. I enjoyed working with them.

A special thanks goes to my family for their understandings and unfailing mental support throughout the project. Their belief in me has kept me motivated throughout the project.

Lastly, I'd like to extend my gratitude to Faculty of Veterinary Medicine, Universiti Putra Malaysia for providing the research grant to conduct my project. The collective contributions of these individuals and institutions have been indispensable to the success of the project. Thank you for being an integral part of this academic

endeavour. I am blessed to have crossed paths with such remarkable individuals in my life.

Sincerely,

_____

Ng Pooi Gin

CONTENTS

LIST OF TABLES **PAGE**

LIST OF FIGURES **PAGE**

ABSTRAK

Abstrak ini adalah kertas kerja yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Tahun Akhir

PENGESANAN TINDAK BALAS SEL MEDIATED DAN HUMORAL TERHADAP *Mycobacterium* spp DALAM KALANGAN UNGKA KURUNGAN DI MALAYSIA

By

NG POOI GIN

2023

Penyelia: Dr Azlan Che' Amat

Penyelia bersama: Assoc. Prof. Dr Siti Khairani Bejo, Dr Azalea Hani Othman

Kejadian tuberkulosis (TB) pada primat bukan manusia (NHPs) yang dalam kurungan adalah signifikan di seluruh dunia, menekankan kepentingan penyaringan penyakit ini. Walau bagaimanapun, amalan ini jarang dilakukan di Malaysia. Oleh itu, objektif utama kajian ini adalah untuk menyaring spesies NHPs tempatan yang terancam, khususnya "lar gibbon" (*Hylobates lar*) untuk penyakit TB. Kajian ini dilakukan dengan menggunakan kaedah persampelan secara kebetulan. Ungka yang disampel

dalam kajian ini adalah dari Malaya Gibbon Rehabilitation Project (GReP) (n=11), Zoo Melaka (n=3), dan Zoo Taiping (n=2). Untuk menilai TB, ujian tuberkulin (TST) dijalankan dengan menyuntikkan "mammalian old tuberculin" (MOT) dan "bovine tuberculin purified protein derivatives" (bPPD) secara "intradermal" ke dalam kelopak mata kanan dan kiri masing-masing. Ujian Chembio Dual-Path Platform (DPP®) VetTB digunakan untuk mengesan antibodi TB dalam darah. Ujian ini menggunakan antigen MPB83 dan CFP10/ESAT-6 daripada *Mycobacterium bovis* dan *Mycobacterium tuberculosis* masing-masing. Pemerhatian dan penilaian tindak balas kelopak mata dilakukan pada 24, 48, dan 72 jam selepas pelaksanan TST dengan menggunakan sistem skoring TB . Secara keseluruhan, semua ungka tidak menunjukkan sebarang tindak balas dalam TST (skor 0). Walau bagaimanapun, dua ungka (12.5%) menunjukkan tindak balas skor 2 pada kelopak mata kiri dengan tanda kemerahan kelopak mata dan pembengkakan yang bertahap ringan. Dalam pengesanan antibodi TB, 18.75% (3/16) ungka diuji positif. Khususnya, seekor ungka menunjukkan tindak balas positif terhadap MPB83, seekor lagi menunjukkan tindak balas positif terhadap CFP10/ESAT-6, dan seekor lagi menunjukkan tindak balas positif terhadap kedua-dua MPB83 dan CFP10/ESAT-6. Ketiga-tiga ungka yang diuji positif mempunyai sejarah kontak rapat dengan manusia, menunjukkan kemungkinan pendedahan kepada *Mycobacterium* spp daripada manusia. Walaupun tiada tanda klinikal diperhatikan pada mana-mana ungka, terdapat kemungkinan bahawa mereka boleh menjadi pembawa penyakit asymptomatik. Keputusan TST perlu diinterpretasikan dengan berhati-hati kerana hanya sekali TST telah dijalankan. Keputusan ujian itu patut dianggap sebagai jankitan TB laten atau tindak balas silang dengan *Mycobacterium* spp lain. Oleh itu, ujian saringan TB perlu dijalankan dengan

lebih lanjut. Kajian ini menggambarkan kepentingan untuk menjalankan amalan penyaringan TB dalam NHPs dan penjaganya. Langkah-langkah keselamatan biologi mesti diambil ketika menangani kes TB yang disyaki dalam kalangan NHP kurungan. Kajian kohort prospektif terhadap ungka-ungka yang diuji positif dalam ujian antibodi sangat disyorkan untuk memantau ungka-ungka tersebut atas perkembangan tanda klinikal TB.

Kata kunci: Tuberkulosis, Primat bukan manusia, Ujian tuberkulin, Antibodi, *Mycobacterium* spp.

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Final Year Project

CELL MEDIATED AND HUMORAL RESPONSE DETECTION AGAINST *Mycobacterium* spp IN CAPTIVE GIBBONS IN MALAYSIA

By

NG POOI GIN

2023

Supervisor: Dr Azlan Che' Amat

Co-supervisor: Assoc. Prof. Dr Siti Khairani Bejo, Dr Azalea Hani Othman

The occurrence of tuberculosis (TB) in captive non-human primates (NHPs) has been significant worldwide, emphasizing the importance of screening. However, this practice rarely conducted in Malaysia, thus the primary objective of this study was to screen endangered native NHP species, specifically lar gibbons (*Hylobates lar*) for TB. This was accomplished by employing convenient sampling method, which included gibbons from the Malaya Gibbon Rehabilitation Project (GReP) (n=11), Malacca Zoo (n=3) and Taiping Zoo (n=2). To assess for TB, a tuberculin skin test

(TST) was conducted by injecting mammalian old tuberculin (MOT) and bovine tuberculin purified protein derivatives (bPPD) intradermally into the right and left palpebral respectively. The Chembio Dual-Path Platform (DPP®) VetTB assay was used to detect TB-specific antibodies in the blood. This assay utilizes MPB83 and CFP10/ESAT-6 antigens from both *Mycobacterium bovis* and *Mycobacterium tuberculosis* respectively. Observation and grading of eyelid reactions were conducted at 24, 48 and 72 hours post-intradermal TST using a standardized scoring method. Overall, all the lar gibbons did not show any reaction to the intradermal TST (score 0). However, two lar gibbons (12.5%) exhibiting score 2 reactions on left palpebral with various degrees of erythema and minimal swelling. For the TB-specific antibody detection, 18.75% (3/16) of the lar gibbons tested seropositive. Specifically, one exhibited a reaction to MPB83, another to CFP10/ESAT-6, and one reacted positively to both MPB83 and CFP10/ESAT-6. Gibbons that tested seropositive had a documented history of prolonged close human contact, indicating a potential exposure to mycobacterium. Although no clinical signs were observed in any of the gibbons, there is a possibility that they could become asymptomatic carriers. The intradermal TST should be interpreted with care due to only single screening test was conducted, however results should be considered presumptive of latent carriers or cross reaction with other mycobacteria and screening test should be further conducted. The overall findings illustrate the need to enhance compliance with TB screening practices in both captive NHPs and care handlers. Biosafety precautions must be taken when handling suspected TB cases in NHPs. A prospective cohort studies on seropositive-tested animals is highly recommended to enable close monitoring on the development of clinical signs and TB-like lesions.

Keywords: Tuberculosis, Non-human primates, Intradermal tuberculin skin test, Antibodies, *Mycobacterium* spp

1.0 INTRODUCTION

Gibbons, also known as lesser apes, have gained significant attention from conservationists over the decades. They are classified as 'endangered' in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Brockelman & Geissmann, 2020). According to Fan (2016), there was no documented observations of the Yunnan subspecies of lar gibbons in China, leading to its formal declaration of extinction. In Malaysia, there has been no previous estimation of population size of lar gibbons. Malaysia's tropical rainforests are home to five species of gibbons, which account quarter of the 20 species found in Southeast Asia. These species include lar gibbon, agile gibbon, siamang, abbot's gibbon, and north Bornean gibbon (Geissmann, 2007). Habitat loss due to deforestation and the illegal wildlife pet trade are the main factors contributing to the decline in gibbon population in Malaysia (Geissmann, 2003). The presence of gibbons in the pet trade not only deprive the gibbons of living in their natural habitats, but also increase the wildlife-human interface, potentially facilitating the transmission of zoonotic diseases. The growing of the challenges faced by gibbons in Malaysia has led to the flourished conservation efforts, such as the establishment of Malaya Gibbon Rehabilitation Project (GReP) and Borneo GReP. These centres serve as temporary shelters for rescued or surrendered gibbons.

Vervenne (2004) reported that tuberculosis (TB) outbreaks most often occurred in zoological facilities, primate rehabilitation and non-human primates (NHPs) research centres. TB has been detected in wild macaques (*Macaca fascicularis*) and capuchin monkeys (*Cebus sapajus*) in Asia but there is a lack of

studies on other NHPs in Malaysia (Wilbur et al., 2012; Rosenbaum et al., 2015). The etiological agent of TB is *Mycobacterium tuberculosis complex* (MTBC) which primarily consists of *M. tuberculosis, M. bovis, M. africanum, M. canetti* and *M. microti* (Biet et al., 2005). Among these five species, NHPs are often affected by *M. tuberculosis*, followed by *M. bovis* (Biet et al., 2005). On the other hand, *M. africanum*, *M. canetti*, and *M. microti* are infrequently encountered (Thorel, 1980). *Mycobacterium* spp. is a gram-positive, rod-shaped, slow-growing acid-fast bacillus (AFB) with a unique fatty acid termed mycolic acid in the impermeable cell wall which aids in invading macrophages and hiding from the host immune system (Zhao et al., 2015). On the other hand, *M. avium-intracellulare complex* (MAC) is an atypical, non-pathogenic mycobacterium that can produce TB-like mycobacteriosis and often sensitizes animals in the tuberculin skin test, giving false-positive results (Vervenne, 2004). The MAC is an environmental non-tuberculous mycobacterium that can cause Johne's disease in cattle and opportunistic infections in immunodeficient NHPs, especially those infected with simian immunodeficiency virus (SIV) (Mansfield and Lackner, 1997; Biet et al., 2005).

Tuberculosis can be active or latent infection. When immunocompetent hosts are exposed to MTBC, they recruit T cells and macrophages to contain the pathogen and induce a dormant state, resulting latent infection without any apparent clinical signs (Parrish et al., 1998). Animals with latent TB are not contagious and may remain dormant for their lifetime or may be reactivated after several years or when the host is immunocompromised. Animals infected with active TB usually show non-specific clinical signs, such as coughing, chronic weight loss, dull hair coat, paralysis, or

general depression (Mätz-Rensing et al., 2015). Pulmonary TB is the predominant form of the disease characterized by intermittent coughing and chronic weight loss (Garcia et al., 2004), while clinical signs of extrapulmonary TB vary depending on the specific organs affected. Cutaneous TB presents as non-healing wounds, draining ulcers or fistulous tracts presented together with lymphadenopathy (Mätz-Rensing et al., 2015). Cerebral TB may present as epileptic seizures (Machotka et al., 1975), while vertebral TB causes paraplegia or kyphosis (Fox et al., 1974). Severe diarrhoea can be the consequence of intestinal TB (Simmons and Gibson, 2012).

The routine screening test for TB in NHPs is intradermal tuberculin skin test (TST) by injecting mammalian old tuberculin (MOT) or bovine purified protein derivative (bPPD) into eyelids or abdomen (Vervenne, 2004). This test requires three-day observation at 24h, 48h and 72h of swelling at the injection site to detect delayed type hypersensitivity (DTH). However, it often yields false-positive or false negative results. Therefore, it is recommended to do other complementary such as immunochromatographic rapid test, interferon-gamma release assay (IGRA), molecular test, bacterial culture, and chest radiography examination. Due to lack of TB screening practices and no evidence of TB in our captive NHP, this study aims:

i. to report the preliminary occurrence of TB and antibodies against MTBC in captive gibbons in zoological parks and conservation centre in Peninsular Malaysia.

ii. to screen and identify the reaction and score of swelling by using intradermal tuberculin test to complement serological assay.

2.0 LITERATURE REVIEW

2.1 Overview of the epidemiology of animal TB

Tuberculosis (TB) infects domestic animals, wildlife and NHPs. Based on the known epidemiology of MTBC, the two most pathogenic species are *M. tuberculosis* and *M. bovis*. Naturally, humans are the only reservoir hosts for *M. tuberculosis* and also the source of spill over to other animals, and the infected animals retransmit to healthy human populations (Une and Mori, 2007). *M. bovis* is the second major cause of tuberculosis that has broader host range. Cattle, farmed buffalo and goats are the reservoir hosts of *M. bovis* who can spill it over to pigs, dogs, cats, horses and sheep (Biet et al., 2005). It also transmits to a widespread free-ranging and captive wildlife as well as humans. Bovine TB is identified as a notifiable disease in most countries whereby test and slaughter is deployed once it is detected. Other eradication programmes include post mortem meat inspection, pasteurization of milk for consumption, systematic individual cattle testing and movement controls. Open house or extensive livestock management makes the eradication difficult as it may result in potential wildlife-livestock interface, causing development of TB in wildlife reservoir especially the wild boar (*Sus scrofa*) as they can act as a maintenance or spill over host in the ecosystem (Biet et al., 2005). The common route of transmission is via inhalation of the respiratory droplets containing bacilli. Transmission through ingestion of fomites or contaminated milk and direct contact of excretion from infected animals are also possible. Potential reservoir for TB differs from one area to another, hence occurrence status of animal TB is crucial for public health concern on zoonosis and conservation.

Montali et al. (2001) mentioned that naturally-infecting TB in wild elephants and NHPs remote from human settlement is probably non-existent. Captive animals living in proximity of humans are more often affected by TB outbreak and these can be proven by few incidents happened worldwide in spite of reasonable precautions. In 2004, there was a TB outbreak occurred in a facility in Japan which affected four workers and various animals such as Malay gharial, spectacled caiman, chimpanzee, Asian elephant, red ruffed lemur, Abyssinian colobus monkeys and tufted capuchin monkeys (Une and Mori, 2007). There was also a case of captive Malayan tapir (*Tapirus indicus*) confirmed to be infected by *M. tuberculosis* by molecular test in 2010 (Kaewamatawong et al., 2010). German Research Institutes recorded a total of 11 rhesus monkeys (*Macaca mulatta*) tested positive for TB in with suspected indirect contact with a human TB patient in 2015 (Mätz-Rensing et al., 2015).

2.2 Prevalence of wildlife TB in Malaysia

Wildlife disease surveillance in Malaysia used to gain less attention as compared to livestock surveillance due to the lower economic impacts. But recently, studies on TB seroprevalence in captive Asian elephants in National Elephant Conservation Centre (NECC), Pahang was conducted and recorded as 20.4% (10/49) and 23.3% (14/60) respectively in 2013 and 2016. Lekko et al. (2021) further reported that 21 captive elephants from the same groups of elephants that were tested positive in 2013 and 2016 were all seronegative probably due to the improvement in management and treatment given to the elephants. From the same study, 42 wild macaques sampled in Selangor, he reported that all were seronegative, however wild boar had a

seroprevalence status of 16.7% (5/30) which positively correlated with dairy cattle TB cases in the same area. The first surveillance of TB among NHPs in Malaysia's Zoo was done by Lekko et al. (2022) and he stated that two orangutan showed positive reaction in intradermal TST and both were seropositive.

2.3 Performance of different diagnostic tests for TB detection

Every diagnostic test has a number of limitations on sensitivity and specificity. False-negative results yielded by TST can be due to anergy associated with progressive disease, compromised immune system, and poor disease during early development of disease (Field, 2001) whereas false-positive results are attributed to antigenic cross reactivity (Goodwin et al., 1988). Interferon-gamma (IFN-γ) release assay (IGRA) may be a better substitute to TST for detection of active TB in NHPs if MOT is not obtainable because results can be obtained in less than 36 hours, only one-time blood sampling is required and it is quantitative which can prevent subjectivity during post intradermal TST scoring. Despite having higher specificity than TST (97% vs 87%), sensitivity of IGRA is significantly lower than TST (68% vs 84%), resulting in more false-negative cases (Garcia et al., 2004b). The blood sample for IGRA must be available for testing not later than 8-10 hours after sampling, thus makes it inconvenient for remote sampling. When TB develops into later stage, the transient cell-mediated immune (CMI) response wanes and will be replaced by humoral immune response.

Enzyme-linked immunosorbent assays (ELISA) is important to diagnose TB in later stage by detecting TB-specific antibodies in NHPs. In recent research, early secreted antigenic target 6kDa (ESAT-6) was identified as the most seroactive antigen with 100% infected NHPs reacted against ESAT-6, followed by 90% produced antibodies against culture filtrate protein (CFP-10) and α-crystallin (Brusasca et al., 2003). Gold standard in diagnosing TB relies on bacterial culture in Lowenstein-Jensen but the incubation period is too long with minimum 6 weeks and may extend up to 16 weeks. The success of the culture also highly depends on the number of mycobacteria present in the sample. It becomes difficult especially during the early stage of disease when there are low numbers of mycobacteria (Bernacky et al., 2002). Ziehl-Neelsen staining can be performed to detect acid-fast bacilli microscopically after successful growth of colonies on culture (Ong et al., 2013). Identification of MTBC strain using specific mycobacterial primers in PCR for mycobacterial DNA provide a more rapid and accurate diagnosis of TB.

2.4 Diagnostic approaches in bovine TB

Due to the diversity of animal hosts involved, different diagnostic methods are preferred depends on the animal species and advanced technologies available. In cattle, the routine screening test is TST by injecting bovine PPD tuberculin into the caudal fold of tail to detect a delayed hypersensitivity reaction after 72 hours. According to OIE Terrestrial Manual 2018, a positive reaction is characterised by 4mm or more skin fold thickness but it can also be a false-positive due to sensitisation attributed to MAC.

Hence, cattle that show a positive reaction will be subjected to a comparative cervical test (CCT) whereby bovine PPD and avian PPD are injected on the same side of mid neck with 12cm to 15cm apart (Jajere et al., 2018). A positive case is identified when there is a 4mm or greater increase in skin thickness at bovine injection site than avium injection site. IGRA is the ancillary test to quantify the cytokine released in the cell-mediated immune response when whole blood is incubated overnight with bovine or avian tuberculin to trigger the production of IFN-γ from lymphocytes (Gormley et al., 2006). The commercially available product is called BOVIGAM™ TB Kit which requires heparinised whole blood.

2.5 Diagnostic approaches for TB in selected wildlife species – NHP

For NHPs, similar tests are used for TB screening. Intradermal TST is done by injecting 0.1 mL mammalian old tuberculin (MOT) or bovine PPD into eyelids or abdomen and the skin test reaction will be observed at 24, 48, 72 hours post-injection. There are studies reporting that abdominal TST is of limited use in NHPs TB surveillance programme due to the lower sensitivity of the test (Capuano et al., 2003; Motzel et al., 2003). Often, eyelid is used for intradermal TST as it is easier to be observed (Bushmitz et al., 2008). The eyelid reactions post-intradermal TST should be graded with a standardized scoring system with reaction scores 0, 1, or 2 as negative, score 3 as inconclusive , and scores 4 and 5 as positive (OIE Terrestrial Manual, 2022). A standard screening test consists of three TST with two-week interval are recommended (Bushmitz et al., 2008). For newly-arrived NHPs, one week of short

acclimation period should be allowed before conducting the first intradermal TST and a minimal quarantine period of 42 days is required (Bushmitz et al., 2008). IGRA is also available commercially for NHPs which is known as PRIMAGAM™ but the test kits are costly. Additional testing for more definitive diagnosis may include chest radiographs, ELISA, culture or PCR of pharyngeal swabs or bronchioalveolar lavage (BAL), Ziehl-Neelsen staining and et cetera (Rock et al., 1995). Bacterial culture and identification of MTBC is still known as the gold standard in NHPs (Lerche et al., 2008)

3.0 MATERIALS AND METHODS

3.1 Study animals and sample collection

This cross-sectional study complied with relevant wildlife authority, the Department of Wildlife and National Parks (PERHILITAN) and followed animal ethics guidelines (UPM/ IACUC/AUP-U035/2023). A total of 16 lar gibbons (*Hylobates lar*) were included in the study, selected through opportunistic sampling from three facilities; Malaya Gibbon Rehabilitation Project (GReP), Raub Pahang (n= 11), Malacca Zoo (n=3) and Taiping Zoo (n=2). The lar gibbons were immobilised by attending veterinarians of the facilities by using tiletamine-zolazepam (Zoletil®, Virbac) with a dose ranging from 4 to 5 mg/kg given intramuscularly through hand-injection or blow dart. The anaesthetised gibbons were placed at dorsal recumbency for the whole sampling procedures and closely monitored until recovery.

3.2 Blood sampling

A 23G indwelling catheter was inserted to the cephalic vein to collect a 3mL of blood sample and stored in the red plain tube. The blood was transported in the icebox and samples were sent to Clinical Pathology Laboratory, Faculty of Veterinary Medicine on the same day to centrifuge at 50000 rpm speed for 5 minutes. Aliquoted serum samples were then stored in -20°C until further analysis.

3.3 Intradermal tuberculin skin test (TST)

Both eyelids of the lar gibbons were cleaned with a cotton soaked with distilled water. An insulin syringe was used to inject 0.1mL of mammalian old tuberculin (MOT) into the edge of upper right palpebral. The mammalian old tuberculin (MOT) with concentration of 40% was manufactured by Colorado Serum Company, 4950 York St., Denver, CO. 80216 U.S.A. A skin bleb was seen on the eyelid. The same step was repeated on upper left palpebral margin with 0.1 mL of bovine tuberculin purified protein derivatives (bPPD) with concentration of 3000 IU and manufactured by Prionics Lelystad B.V. Platinastraat 33, 8211 AR Lelystad, Netherlands. Reaction of intradermal TST was observed at 24, 48 and 72 hours post-injection by direct observation of the animal handlers. Photos of the eyelids were taken by animal handlers and were graded by veterinarian using a standardized scoring system as shown in Table 3.1.

3.4 Serology TB-antibody detection

Serological detection was done using Chembio Dual Platform Pathway (DPP) VetTB assay which is based on immunochromatographic technology. Selected tests used in this study was based on the availability of test kits and cost issues. Despite of the fact that this test kits were subjected for elephants and cervids, it had been evaluated in many other species including pigs, alpaca, cheetah, and African lions (Beltran-Beck et al., 2014; Lyashchenko et al., 2011; Kerr et al., 2020; Miller et al., 2012). Data showed that there was 100% sensitivity and specificity in elephants (Greenwald et al.,

2009); 77.8% sensitivity and 100% specificity in pigs (Beltran-Beck et al., 2014); 74% sensitivity and 98% specificity on alpaca (Lyashchenko et al., 2011). This test utilises two purified protein of *M. tuberculosis* and *M. bovis* which are MPB83 on test (1) line and CFP10/ ESAT-6 on test (2) line. MPB83 is highly expressed by *M. bovis* (Wiker, 2009), whereas ESAT-6 is the important virulence factor of *M. tuberculosis* (Sreejit et al., 2014). The most seroreactive antigen recognised by antibodies in NHPs is ESAT-6 protein (Brusasca et al., 2003). Serum samples were thawed to a temperature of 18 to 30°C before testing. The test units were labelled with sample names. A pipette was used to drop 5 µL of serum, followed by two drops of buffer into 'well 1'. After 5 minutes, four drops of buffer were added into 'well 2' to release the recombinant Protein A/G conjugated to colloidal gold particles to dye the antibodies captured on nitrocellulose membrane. The results were read 15 minutes after the addition of buffer into 'well 2'. Refer Figure 1 of Appendix A for the test procedure. To ensure the result is valid, a pink line must be observed on the control (C) line. Presence of either both or one test (1) line and test (2) line indicates a positive result. A negative result is characterized by no detectable line on test (1) and test (2) line. Refer Figure 2 of Appendix A for the interpretation of results.

Table 3.1. Standardized tuberculin reaction scoring system in OIE Terrestrial Manual 2022

Score	Observation	Outcome
0	No reaction	Negative
1	Bruises, extravasation of blood in the eyelid associated with the injection of tuberculin.	Negative
2	Various degrees of erythema of the palpebrum	Negative
3	Moderate swelling of the eyelids with or without erythema	Inconclusive
4	Obvious swelling of palpebrum with drooping with or without erythema	Positive
5	Necrosis of the eyelid with varying degrees of swelling, including eyelid partially or completely closed.	Positive

4.0 RESULTS

The result showed that all the lar gibbons were negative in intradermal TST and 18.75% (3/16) were seropositive in DPP VetTB assay. Among all the negative reactions in intradermal TST, 14 lar gibbons exhibited score 0 on both eyelids while there were two lar gibbons (Rangga and Mek) displayed score 2 reaction on left eyelids after 24 hours up to 96 hours post inoculation with bPPD.

Table 4.1. Results from intradermal TST and DPP VetTB Assay applied on NHPs

No	Location	ID	Intradermal tuberculin skin test (TST)		DPP VetTB
			MOT (right eyelid)	bPPD (left eyelid)	
1	GReP	Bobo	Score 0 (-)	Score 0 (-)	-
2	GReP	Chinta	Score 0 (-)	Score 0 (-)	-
3	GReP	Coley	Score 0 (-)	Score 0 (-)	-
4	GReP	Darling	Score 0 (-)	Score 0 (-)	-
5	GReP	Ebony	Score 0 (-)	Score 0 (-)	-
6	GReP	Embun	Score 0 (-)	Score 0 (-)	-
7	GReP	Mojo	Score 0 (-)	Score 0 (-)	-
8	GReP	Ud	Score 0 (-)	Score 0 (-)	-
9	GReP	Tony	Score 0 (-)	Score 0 (-)	-
10	GReP	Dexter	Score 0 (-)	Score 0 (-)	+
11	GReP	Rangga	Score 0 (-)	Score 2 (-)	+
12	Zoo Taiping	Mek	Score 0 (-)	Score 2 (-)	-
13	Zoo Taiping	Mat	Score 0 (-)	Score 0 (-)	-
14	Zoo Melaka	Maru	Score 0 (-)	Score 0 (-)	+

15	Zoo Melaka	Sandy	Score 0 (-)	Score 0 (-)	-
16	Zoo Melaka	Momo	Score 0 (-)	Score 0 (-)	-
Total			0/16		3/16 (18.75%)

-, negative result; +, positive result

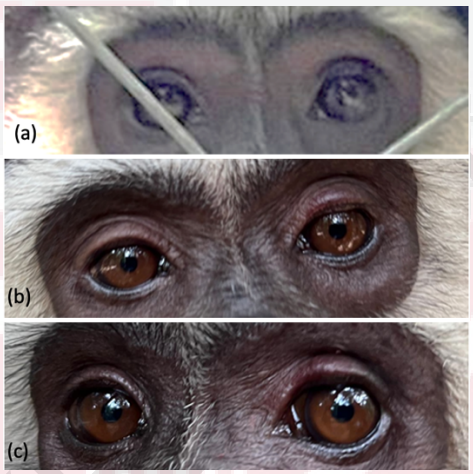


Figure 4.1. Eyelids of 'Rangga' post intradermal TST. (a) Score 0 reaction on both eyelids after 24 hours of TST; (b) Score 2 reaction on left eyelids post 48; (c) Score 2 post 72 hours injection with bPPD.

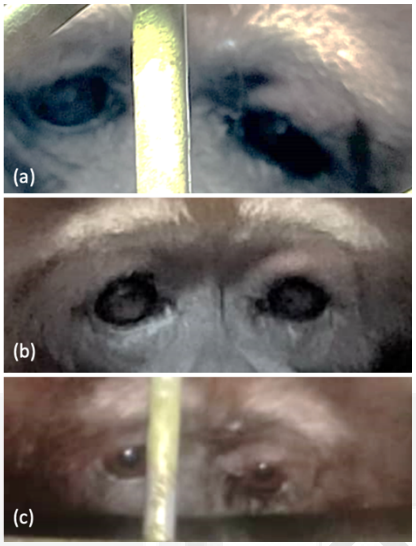


Figure 4.2. Eyelids of Mek post intradermal TST. Mek exhibited score 2 reaction on left eyelids post 24 (a), 48 (b) and 72 hours (c) injection with bPPD.

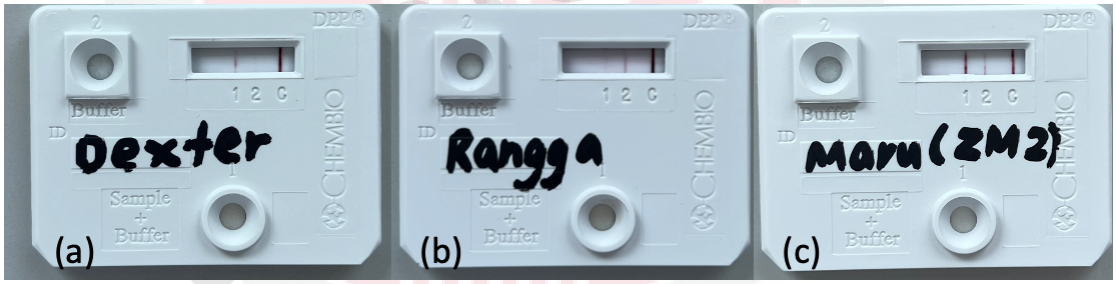


Figure 4.3. Interpretation of Chembio DPP® VetTB assay result. (a) Dexter was seropositive against test (1) line which was coated with MPB83. (b) Rangga was seropositive against test (2) line which was coated with CFP10/ ESAT-6. (c) Maru was seropositive against both test (1) and test (2) line which represented MPB83 and CFP10/ ESAT-6 respectively.

5.0 DISCUSSION

This study reported the preliminary data on the intradermal TST and antibodies detection against MTBC in lar gibbon in Malaysia. Although there was a score 2 reaction on left eyelids of 'Rangga' (GReP) and 'Mek' (Taiping Zoo), it was categorised as negative. It could be a false negative due to the waning effect of CMI response if the lar gibbon was infected long time ago (Field, 2001) or due to the early infection since CMI response in immunocompetent animals initiates approximately four weeks after exposure (Lekko et al., 2022). The erythema of palpebrum with minimal swelling could also be a reaction resulting from the cross reactivity with MAC or other environmental mycobacteria. As documented by Lekko et al. (2022), all tested captive NHPs from zoological settings were PCR positive for MAC. Hence, it is not uncommon to find MAC in NHPs. In this study, intradermal TST was complemented by antibodies detection assay due to the fact that intradermal TST always yield false positive and false negative result. Having said that, intradermal TST is the primary antemortem diagnosis to screen TB in NHPs by detecting delayed-type hypersensitivity (DTH). It is also the only ILAR/CDC approved screening method during quarantine period (Lerche et al., 2008). Alternative way to overcome the false positive yielded by single intradermal TST is to conduct comparative intradermal tuberculin skin test (TST) which utilises bovine tuberculin purified protein derivatives (bPPD) and avian tuberculin purified protein derivative (aPPD). Comparative intradermal TST can differentiate between animals infected with MTBC and those infected with MAC, increasing the specificity of TB detection (World Organisation for Animal Health, 2022). According to World Organisation for Animal Health

(WOAH), a standard intradermal TST involves three serial tests with a two-week interval. However, due to time constraints and restriction from the authorities on repeatedly tranquilizing and restraining the animals, only one intradermal TST was performed. Hence, the result was considered as presumptive diagnosis only.

We found low antibody detection (18.75%) with 'Dexter' (GReP), 'Rangga' (GReP) and 'Maru' (Malacca Zoo) exhibited seropositivity against MTBC, indicating a probable present or past infection. Since no clinical signs was shown by all the lar gibbons and no TST reaction, the development of humoral response was most likely a result of past exposure. Dexter was seropositive against MPB83 which is a primary virulence factor of *M. bovis* (Wiker, 2009) while Rangga was seropositive against CFP10/ ESAT-6 which is highly expressed by *M. tuberculosis* (Sreejit et al., 2014). On the other hands, Maru was tested seropositive against both antigens. These findings were consistent with what had been reported by Bernacky et al. (2002) where it stated that simian TB was caused primarily by *M. tuberculosis* and *M. bovis*. Through personal communication, 'Dexter' and 'Rangga' from GReP were surrendered by their owners who kept them as pets while Maru was born and raised by the care handlers in Malacca Zoo due to its mother's limb fractures from a fall. These three lar gibbons had a prolonged close contact with human. It had been reported that potential source of exposure in NHPs may be due to a close contact with an infected human (Montali et al., 2001; Garcia et al., 2004). However, the possibilities of MTBC exposure from other sources cannot be ruled out. In GReP, semi-captive enclosures located in the forest might expose the gibbons to wild animals like deer, macaques or boars as potential source of transmission. While for Maru, having stay on an island previously,

MTBC could be transmitted from the zoo visitors or from the wild macaques roaming around the zoo through respiratory droplets and/ or close contact.

The assumptions were made based on the fact that route of transmission of TB is through inhalation of infected respiratory droplets, ingestion and direct contact of infected material via mucus membrane or breaks in skin. Not only human could be a potential source of transmission, wild animals were reported to be reservoir hosts of TB and it had been well-described in wild boars, deer and badgers (Barasona et al., 2017; Corner et al., 2012; Palmer et al., 2004). Barasona et al. (2017) reported that MTBC could survive in harsh environment for months even in dry condition. This feature is likely due to the presence of the glycolipids known trehalose 6,6'-dimycolate (TDM) in cell wall, enabling MTBC to survive desiccation (Harland et al., 2008). Hence, indirect transmission of MTBC in the environment including soil, water, air and dust is possible but has always been neglected (Zhang et al., 2022).

6.0 CONCLUSION

There was no positive reaction in the lar gibbons post-intradermal TST, indicating no latent TB infection developed among the lar gibbons. Nevertheless, the possibility of false negative due to early infection or waning effect of cellular mediated immune response is still there unless it is confirmed by the completion of three times of serial TST. In this project, since there was only one time of intradermal TST conducted, presumption of exposure to *Mycobacterium* spp in lar gibbons with score 2 reaction was made. For antibody detection, three lar gibbons were tested seropositive against MTBC specifically *M. tuberculosis* and *M. bovis*, suggesting there was a past exposure of MTBC. The exact source of past exposure was unknown but it could be originated from infected human, spill-over from the wild hosts or contaminated of mycobacteria from the environment. This project highlighted the imperative of integrating diagnostic tests that evaluate both cellular and humoral immune response in TB surveillance for precise diagnosis because cellular immune response occurs in early stage but transient while humoral immune response manifests in later stage but persists. Stringent biosafety precautions must be taken when handling suspected TB cases in NHPs, irrespective of clinical signs. This is because infected NHPs with active TB may hide their clinical signs while shedding the etiological agent (Gibson, 1998). According to Centers for Disease Control and Prevention (1993), a newly imported NHPs must be quarantined for at least 31 days and must achieve three consecutive-negative TSTs with two-week intervals to be released. In fact, TST should be performed twice annually as a screening test for early detection to prevent a TB

outbreak. It is also highly recommended for zoo workers to perform TB screening test as humans can be the potential source of transmission to NHPs.

7.0 LIMITATION OF STUDY

The limitation of a single TST is the potential for cross reactivity with *Mycobacterium avium* complex. This means that individuals who have been sensitized to environmental mycobacteria may have a reaction to the tuberculin TST. Furthermore, it is important to note that a single TST is insufficient for definitively diagnosing TB due to the fact that it requires a minimum of four weeks to identify the TB-specific T cell response following infection with MTBC (Lekko et al., 2022). The Chembio DPP® VetTB assay has been utilised for off-label testing in various animal species, however its application in NHPs has not been documented thus far. Therefore, the sensitivity and specificity of this assay in NHPs were not known. The prevalence of TB in the local wildlife and the long-term survival of MTBC in the environment have not been well investigated. This complicates the efforts to ascertain the origin of the exposure.

8.0 RECOMMENDATIONS

In order to address the issue of cross reactivity with MAC, a potential solution would be to conduct a comparative tuberculin skin test in the future. This test would involve using both bPPD and aPPD to accurately determine if any false positive results are due to sensitization by MAC. It is recommended to conduct three consecutive TST

with a two-week interval between each test in order to reduce the number of false negative cases caused by the diminishing effect of the test or early stage of infection. In order to obtain a comprehensive understanding of the epidemiology of TB, it is possible to investigate the presence of TB in other free-ranged wild animals such as wild boar, deer and other wild ruminants. Additionally, samples can be collected from the environment, such as soil, water, and dust, to determine the source of MTBC. Future investigations using a variety of NHP species should be conducted, as the sensitivity of NHPs to MTBC varies. Prospective cohort studies can be undertaken on animals that have tested positive for antibodies to closely follow the development of clinical symptoms and tuberculosis-like lesions.

9.0 REFERENCES

Barasona, J. A., Vicente, J., Díez-Delgado, I., Aznar, J., Gortázar, C., & Torres, M. J. (2017). Environmental presence of *Mycobacterium tuberculosis* complex in aggregation points at the wildlife/livestock interface. Transboundary and Emerging Diseases, 64(4): 1148-1158.

Beltrán-Beck, B., Romero, B., Boadella, M., Casal, C., Bezos, J., Mazariegos, M., Martín, M., Galindo, R. C., Pérez de la Lastra, J. M., Villar, M., Garrido, J. M., Sevilla, I. A., Asensio, F., Sicilia, J., Lyashchenko, K. P., Domínguez, L., Juste, R. A., de la Fuente, J., & Gortázar, C. (2014). Tonsils of the soft palate do not mediate the response of pigs to oral vaccination with heat-inactivated *Mycobacterium bovis*. Clinical and Vaccine Immunology, 21(8): 1128-1136.

Bernacky, B. J., Gibson, S. V., Keeling, M. E. & Abee, C. R. (2002). Non-human primates. Laboratory Animal Medicine, 2nd Ed (pp 676-777). Academic Press.

Biet, F., Boschiroli, M., Thorel, M., & Guilloteau, L. L. (2005). Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium-intracellulare* complex (MAC). Veterinary Research, 36(3): 411-436.

Brockelman, W., & Geissmann, T. (2020). Hylobates lar. The IUCN Red List of Threatened Species 2020: e.T10548A17967253. Retrieved on 19 August from https://dx.doi. org/10.2305/IUCN.UK.2020-2.RLTS. T10548A17967253.en.

Brusasca, P. N., Peters, R. L., Motzel, S. L., Klein, H. J., & Gennaro, M. L. (2003). Antigen recognition by serum antibodies in non-human primates

experimentally infected with *Mycobacterium tuberculosis*. Comparative Medicine, 53(2): 165-172.

Bushmitz, M., Lecu, A., Verreck, F., Preussing, E., Rensing, S. & Mätz-Rensing, K. (2009). Guidelines for the prevention and control of tuberculosis in non-human primates: Recommendations of the European Primate Veterinary Association Working Group on tuberculosis. Journal of Medical Primatology, 38(1): 59-69.

Capuano, S.V., Croix, D.A., Pawar, S., Zinovik, A., Myers, A., Lin, P.L., Bissel, S., Fuhrman, C., Klein, E., Flynn, J.L. (2003). Experimental *Mycobacterium tuberculosis* infection of cynomolgus macaques closely resembles the various manifestations of human *M. tuberculosis* infection. Infection and Immunity, 71(10): 5831-5844.

Centers for Disease Control and Prevention (1993). Tuberculosis in imported nonhuman primates--United States, June 1990-May 1993. Morbidity and Mortality Weekly Report, 42(29): 572-576.

Corner, L. A., O'meara, D., Costello, E., Lesellier, S., & Gormley, E. (2012). The distribution of *Mycobacterium bovis* infection in naturally infected badgers. The Veterinary Journal, 194(2): 166-172.

Fan, P. (2016). The past, present, and future of gibbons in China. Biological Conservation, 210: 29-39.

Field, M. J. (Ed.). (2001). Tuberculosis in the workplace. National Academies Press.

Fox, J. G., Campbell, L. H., Snyder, S. B., Reed, C., & Soave, O. A. (1974). Tuberculous spondylitis and Pott's paraplegia in a rhesus monkey (*Macaca mulatta*). Laboratory Animal Science, 24(2): 335-339.

Garcia, M. A., Bouley, D. M., Larson, M. J., Lifland, B., Moorhead, R., Simkins, M. D., Borie, D. C., Tolwani, R., & Otto, G. (2004a). Outbreak of *Mycobacterium bovis* in a conditioned colony of rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) macaques. Comparative Medicine, 54(5): 578-584.

Garcia, M. A., Yee, J., Bouley, D. M., Moorhead, R., & Lerche, N. W. (2004b). Diagnosis of tuberculosis in macaques, using whole-blood in vitro interferon-gamma (PRIMAGAM) testing. Comparative Medicine, 54(1): 86-92.

Geissmann, T. (2003). Symposium on gibbon diversity and conservation: concluding resolution. Asian Primates, 8(3-4): 28-29.

Geissmann, T. (2007). Status reassessment of the gibbons: results of the Asian primate red list workshop 2006. Gibbon Journal, 3(2007): 5-15.

Gibson, S. V. (1998). Bacterial and mycotic diseases. Nonhuman Primates in Biomedical Research (pp. 59-110). Academic Press.

Goodwin, B. T., Jerome, C. P., & Bullock, B. C. (1988). Unusual lesion morphology and skin test reaction for *Mycobacterium avium* complex in macaques. Laboratory Animal Science, 38(1), 20-24.

Gormley, E., Doyle, M. B., Fitzsimons, T., McGill, K., & Collins, J. D. (2006). Diagnosis of *Mycobacterium bovis* infection in cattle by use of the gamma-interferon (Bovigam®) assay. Veterinary Microbiology, 112(2-4): 171-179.

Greenwald, R., Lyashchenko, O., Esfandiari, J., Miller, M., Mikota, S., Olsen, J. H., Ball, R., Dumonceaux, G., Schmitt, D., Moller, T., Payeur, J.B., Harris, B., Sofranko, D., Waters, W.R. & Lyashchenko, K. P. (2009). Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. Clinical and Vaccine Immunology, 16(5): 605-612.

Harland, C. W., Rabuka, D., Bertozzi, C. R., & Parthasarathy, R. (2008). The *Mycobacterium tuberculosis* virulence factor trehalose dimycolate imparts desiccation resistance to model mycobacterial membranes. Biophysical Journal, 94(12): 4718-4724.

Jajere, S. M., Atsanda, N. N., Bitrus, A. A., Hamisu, T. M., & Goni, M. D. (2018). Occurrence of bovine tuberculosis among cattle herds from nomadic peri-urban settlements and cattle slaughtered at the municipal abattoir of Bauchi, North-Eastern Nigeria. Journal of Advanced Veterinary and Animal Research, 5(1): 53-59.

Kaewamatawong, T., Banlunara, W., Rungsipipat, A., Pirarat, N., Puranaveja, S., & Sommanustweechai, A. (2010). Disseminated tuberculosis in captive Malayan tapir (*Tapirus indicus*). The Thai Journal of Veterinary Medicine, 40(4): 427-431.

Kerr, T.J., Gumbo, R., Goosen, W.J., Rogers, P., Last, R.D. & Miller, M.A. (2020). Novel techniques for detection of *Mycobacterium bovis* infection in a cheetah. Emerging Infectious Disease, 26(3): 630.

Lekko, Y. M., Ooi, P.T., Omar, S., Mazlan, M., Sivapalan, N., Ramanoon, S. Z., Jesse, F.F.A., Jasni, S. & Che-Amat, A. (2022). Surveillance and detection of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* complex in captive non-human primates in zoological parks. Pakistan Veterinary Journal, 42(3): 340-345.

Lerche, N.W., Yee, J.L., Capuano, S.V., & Flynn, J.L. (2008): New approaches to tuberculosis surveillance in nonhuman primates. ILAR Journal, 49(2): 170-178.

Lyashchenko, K. P., Greenwald, R., Esfandiari, J., Rhodes, S., Dean, G., de la Rua-Domenech, R., Meylan, M., Vordermeier, Hm., & Zanolari, P. (2011). Diagnostic value of animal-side antibody assays for rapid detection of *Mycobacterium bovis* or *Mycobacterium microti* Infection in South American Camelids. Clinical and Vaccine Immunology 18(12): 2143–2147.

Machotka, S. V., Chapple, F. E., & Stookey, J. L. (1975). Cerebral tuberculosis in a rhesus monkey. Journal of the American Veterinary Medical Association, 167(7): 648-650.

Mansfield, K.G. & Lackner, A.A. (1997). Simian immunodeficiency virus-inoculated macaques acquire *Mycobacterium avium* from potable water during AIDS. Journal of Infectious Diseases, 175(1): 184-187.

Mätz-Rensing, K., Hartmann, T., Wendel, G. M., Frick, J. S., Homolka, S., Richter, E., Munk, M. H., & Kaup, F.J. (2015). Outbreak of tuberculosis in a colony of rhesus monkeys (*Macaca mulatta*) after possible indirect contact with a human TB patient. Journal of Comparative Pathology, 153(2-3): 81-91.

Montali, R. J., Mikota, S. K., & Cheng, L. I. (2001). *Mycobacterium tuberculosis* in zoo and wildlife species. Scientific and Technical Review, International Office of Epizootics, 20(1): 291-303.

Motzel, S.L., Schachner, R.D., Kornegay, R.W., Fletcher, M.A., Kanaya, B., Gomez, J.A., Ngai, DT-W., Pouch, W.J., Washington, M.V., Handt, L.A., Wagner, J.L., Klein, H. (2003). Diagnosis of tuberculosis in nonhuman primates. International Perspectives: The Future of Nonhuman Primate Resources. National Academies Press, 156-159.

Miller, M.A., Joubert, J., Mathebula, N., De Klerk-Lorist, L.M., Lyashchenko, K.P., Bengis, R., Helden, P.V., Hofmeyr, M., Olea-Popelka, F., Greenwald, R., Esfandiari, J., & Buss, P. (2012). Detection of antibodies to tuberculosis antigens in free-ranging lions (*Panthera leo*) infected with *Mycobacterium bovis* in Kruger National Park, South Africa. Journal of Zoo and Wildlife Medicine, 43(2):317–323.

Ong, B. L., Ngeow, Y. F., Razak, M. A., Yakubu, Y., Zakaria, Z., Mutalib, A. R., Hassan, L., Ng, H.F. & Verasahib, K. (2013). Tuberculosis in captive Asian elephants (*Elephas maximus*) in Peninsular Malaysia. Epidemiology and Infection, 141(7): 1481-1487.

Palmer, M. V., Waters, W. R., & Whipple, D. L. (2004). Investigation of the transmission of *Mycobacterium bovis* from deer to cattle through indirect contact. American Journal of Veterinary Research, 65(11): 1483-1489.

Parrish, N. M., Dick, J. D., & Bishai, W. R. (1998). Mechanisms of latency in *Mycobacterium tuberculosis*. Trends in Microbiology, 6(3): 107-112.

Rock, F. M., Landi, M. S., Meunier, L. D., Morris, T. H., Rolf, L. L., Warnick, C. L., McCreedy, B.J. & Hughes, H. C. (1995). Diagnosis of a case of *Mycobacterium tuberculosis* in a cynomolgus (*Macaca fascicularis*) monkey colony by polymerase chain reaction and enzyme-linked immunosorbent assay. Laboratory Animal Science, 45(3): 315-319.

Rosenbaum, M., Mendoza, P., Ghersi, B. M., Wilbur, A. K., Perez-Brumer, A., Cavero Yong, N., Kasper, M. R., Montano, S., Zunt, J. R. & Jones- Engel, L. (2015). Detection of *Mycobacterium tuberculosis* complex in New World monkeys in Peru. EcoHealt*h,* **12**: 288–297.

Simmons, J., & Gibson, S. (2012). Bacterial and mycotic diseases of nonhuman primates. Nonhuman Primates in Biomedical Research, 2: 105-172.

Sreejit, G., Ahmed, A., Parveen, N., Jha, V., Valluri, V.L., Ghosh, S. & Mukhopadhyay, S. (2014). The ESAT-6 protein of *Mycobacterium tuberculosis* interacts with beta-2-microglobulin (β2M) affecting antigen presentation function of macrophage. PLoS Pathogens, 10(10): e1004446.

Thorel, M. F. (1980). Isolation of *Mycobacterium africanum* from monkeys. Tubercle, 61(2): 101-104.

Vervenne, R.A.W., Jones, S.L., van Soolingen, D., van der Laan, T., Andersen, P., Heidt, P.J., Thomas, A.W., & Langermans, J.A.M. (2004). TB diagnosis in non-human primates: comparison of two interferon-gamma assays and the skin test for identification of *Mycobacterium tuberculosis* infection. Veterinary Immunology and Immunopathology, 100(1-2): 61-71.

Wiker, H. G. (2009). MPB70 and MPB83--major antigens of *Mycobacterium bovis*. Scandinavian Journal of Immunology, 69(6): 492–499.

Wilbur, A. K., Engel, G. A., Rompis, A., A Putra, I. G., Lee, B. P., Aggimarangsee, N., Chalise, M., Shaw, E., Oh, G., Schillaci, M. A. & Jones-Engel, L. (2012). From the mouths of monkeys: detection of *Mycobacterium tuberculosis* complex DNA from buccal swabs of synanthropic macaques. American Journal of Primatology, 74(7): 676–686.

Une, Y., & Mori, T. (2007). Tuberculosis as a zoonosis from a veterinary perspective. Comparative Immunology, Microbiology and Infectious Diseases, 30(5-6): 415-425.

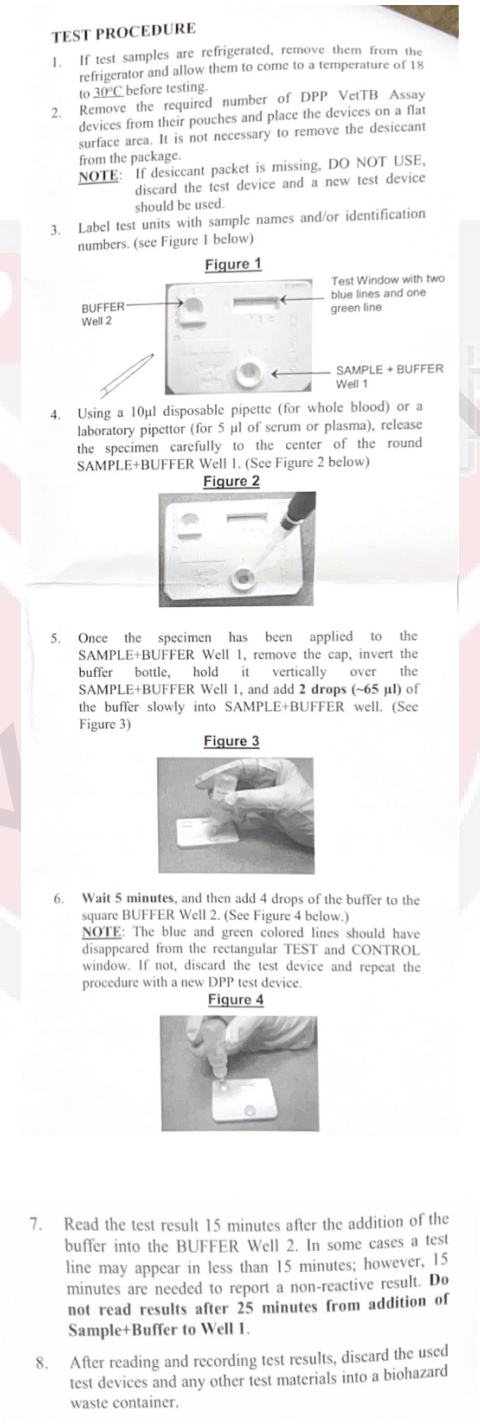
Vervenne, R.A.W., Jones, S.L., van Soolingen, D., van der Laan, T., Andersen, P., Heidt, P.J., Thomas, A.W., & Langermans, J.A.M. (2004). TB diagnosis in non-human primates: comparison of two interferon-gamma assays and the skin test for identification of *Mycobacterium tuberculosis* infection. Veterinary Immunology and Immunopathology, 100(1-2): 61-71.

World Organisation for Animal Health (2022). OIE Terrestrial Manual 2022 (12th ed.). OIE.

Zhang, H., Liu, M., Fan, W., Sun, S., & Fan, X. (2022). The impact of *Mycobacterium tuberculosis* complex in the environment on one health approach. Frontiers in Public Health, 10, 994745.

Zhao, J., Siddiqui, S., Shang, S., Bian, Y., Bagchi, S., He, Y., & Wang, C. R. (2015). Mycolic acid-specific T cells protect against *Mycobacterium tuberculosis* infection in a humanized transgenic mouse model. Elife, 4, e08525.

Appendix A- Chembio Dual-Path Platform (DPP®) VetTB assay test procedure and interpretation of results



TEST PROCEDURE

1. If test samples are refrigerated, remove them from the refrigerator and allow them to come to a temperature of 18 to 30°C before testing.
2. Remove the required number of DPP VetTB Assay devices from their pouches and place the devices on a flat surface area. It is not necessary to remove the desiccant from the package.
   NOTE: If desiccant packet is missing, DO NOT USE, discard the test device and a new test device should be used.
3. Label test units with sample names and/or identification numbers. (see Figure 1 below)

Figure 1

4. Using a 10µl disposable pipette (for whole blood) or a laboratory pipettor (for 5 µl of serum or plasma), release the specimen carefully to the center of the round SAMPLE+BUFFER Well 1. (See Figure 2 below)

Figure 2

5. Once the specimen has been applied to the SAMPLE+BUFFER Well 1, remove the cap, invert the buffer bottle, hold it vertically over the SAMPLE+BUFFER Well 1, and add **2 drops (~65 µl)** of the buffer slowly into SAMPLE+BUFFER well. (See Figure 3)

Figure 3

6. **Wait 5 minutes**, and then add 4 drops of the buffer to the square BUFFER Well 2. (See Figure 4 below.)
   NOTE: The blue and green colored lines should have disappeared from the rectangular TEST and CONTROL window. If not, discard the test device and repeat the procedure with a new DPP test device.

Figure 4

7. Read the test result 15 minutes after the addition of the buffer into the BUFFER Well 2. In some cases a test line may appear in less than 15 minutes; however, 15 minutes are needed to report a non-reactive result. **Do not read results after 25 minutes from addition of Sample+Buffer to Well 1**.
8. After reading and recording test results, discard the used test devices and any other test materials into a biohazard waste container.

Figure 1. Test procedure of Chembio DPP® VetTB assay.

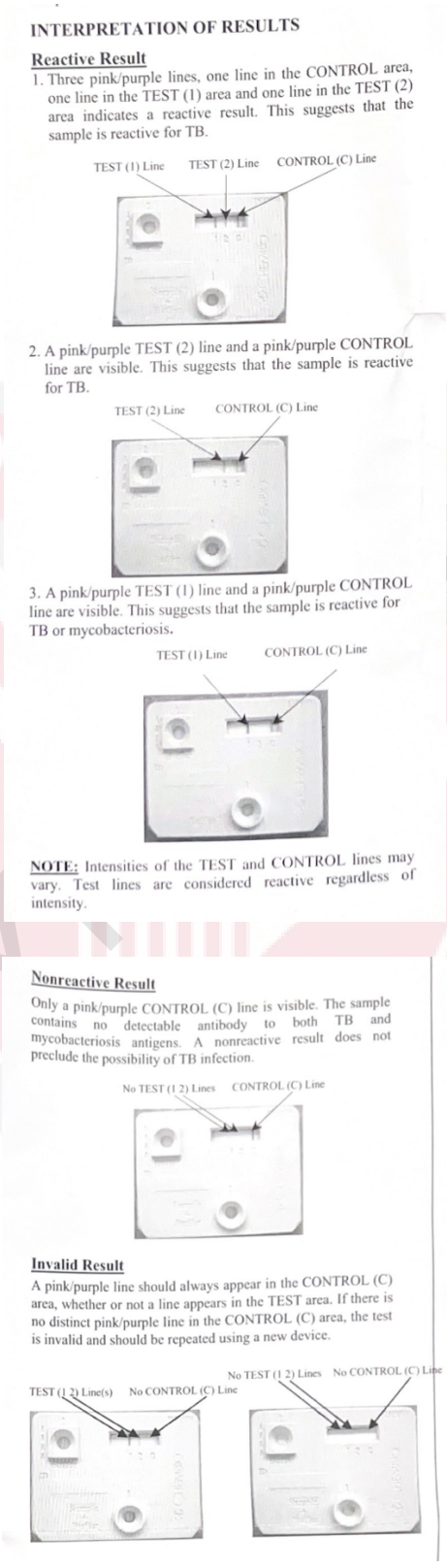


Figure 2. Interpretation of Chembio DPP® VetTB assay result.

Appendix B- IACUC approval letter

UNIVERSITI PUTRA MALAYSIA
BERILMU BERBAKTI

PEJABAT TIMBALAN NAIB CANSELOR (PENYELIDIKAN DAN INOVASI)
OFFICE OF THE DEPUTY VICE CHANCELLOR (RESEARCH AND INNOVATION)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Date:	01st August 2023
AUP No.:	UPM/IACUC/AUP-U035/2023
Project Title:	Cell mediated and humoral response detection against *Mycobacterium spp* in Captive Gibbons and Comparison of Clinicopathological Findings of Lar Gibbons (*Hylobates lar*) in Different Management Systems, Age and Sex.
Principal Investigator:	Dr. Azlan Che' Amat
Members:	Dr. Muhamad Ridhwan Affendi, Dr. Kavitha Jayaseelan, Dr. Zahidah Izzati Zeid, Mariani Binti Ramli, Ng Pooi Gin, Sathishwaran A/L Magis Paran.
Attending Veterinarian:	Dr. Azlan Che'Amat, Dr. Azalea Hani Othman.
Committee Decision:	The committee has reviewed and approved the proposed animal utilisation protocol, subject to relevant permit and/ or owner's consent.
Project Classification:	Chronic
Category of Invasiveness:	B
Source of Animals:	1. **Gibbon Conservation Society**, Raub, Pahang. 2. **Zoo Negara Malaysia**, Jalan Taman Zooview, Taman Zooview, 68000 Ampang, Selangor. 3. **Zoo Melaka**, Jalan Tun Abdul Razak, Hang Tuah Jaya, 75450 Ayer Keroh, Melaka. 4. **Zoo Taiping**, Jalan Taman Tasik Taiping, Taman Tasik Taiping, 34000 Taiping, Perak.
Number of Animals Approved:	20 Gibbon
Housing:	Gibbon Conservation Society, Zoo Negara Malaysia, Zoo Taiping, Zoo Melaka.
Duration	01st August 2023 – 30th December 2023

Ethical approval is required in the case of amendments to the approved animal utilisation protocol (AUP). Please apply using Form 105. Kindly submit a final/annual report (Form 106) upon study completion, or before expiry of approval.

~~PROF. DATO'~~ DR. MOHD AZMI MOHD LILA
Chairman
Institutional Animal Care and Use Committee
Universiti Putra Malaysia

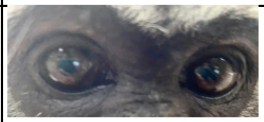
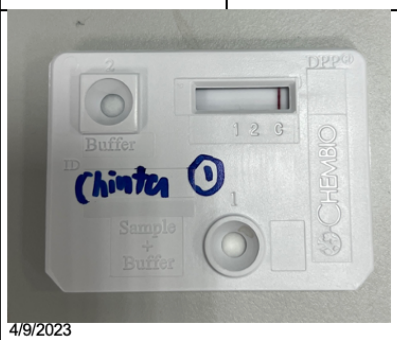
Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
Pejabat Timbalan Naib Canselor (P&I) 603-9769 1002, Pejabat Pentadbiran TNCPI 603-9769 1608, Pejabat Pengarah, Pusat Pengurusan Penyelidikan (RMC) 603-9769 1610, Pejabat Pengarah, Putra Science Park (PSP) 603-9769 1291
http://www.tncpi.upm.edu.my

Appendix C- Reaction scores post intradermal TST and Chembio DPP ® VetTB assay result

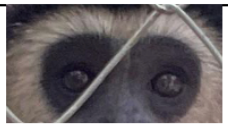
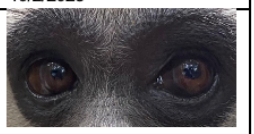
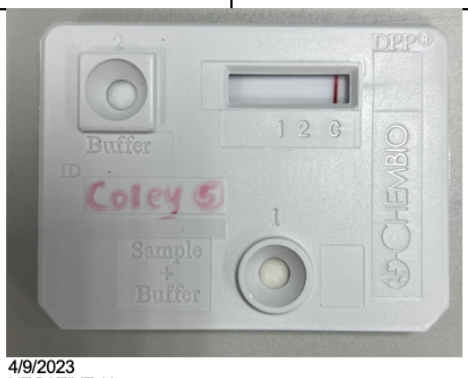
Date: 16/2/2023
Animal ID: Bobo
Sex: M
Age: 3 years old

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	17/2/2023	18/2/2023	19/2/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 4/9/2023 NEGATIVE (-ve)		

Date: 16/2/2023
Animal ID: Chinta
Sex: F
Age: 4 y/o

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	17/2/2023	18/2/2023	19/2/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 4/9/2023 NEGATIVE (-)		

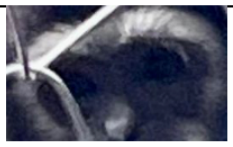
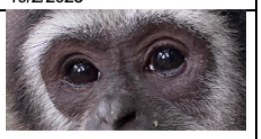
Date: 16/2/2023
Animal ID: Coley
Sex: M
Age: 5 y/o

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	17/2/2023	18/2/2023	19/2/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 4/9/2023 NEGATIVE (-)		

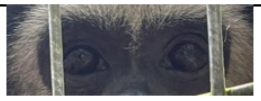
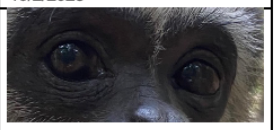
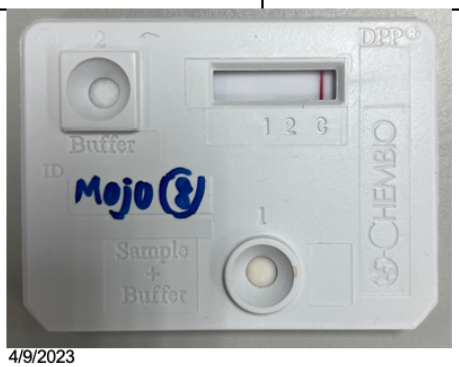
Date: 16/2/2023
Animal ID: Darling
Sex: F
Age: 7 y/o

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	17/2/2023	18/2/2023	19/2/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 29/9/2023 Very light band- NEGATIVE (-ve)		

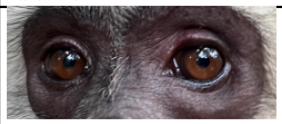
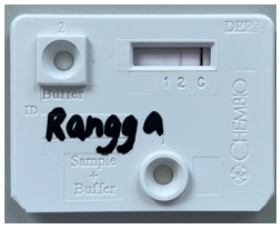
Date: 16/2/2023
Animal ID: Embun
Sex: F
Age:3 y/o

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	17/2/2023	18/2/2023	19/2/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 4/9/2023 NEGATIVE (-ve)		

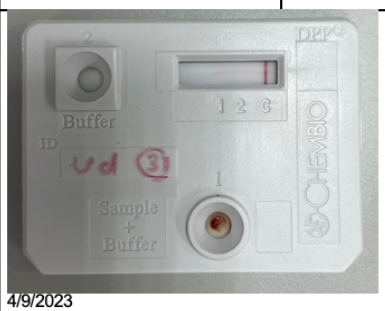
Date: 16/2/2023
Animal ID: Mojo
Sex: M
Age: 8 y/o

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	17/2/2023	18/2/2023	19/2/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 4/9/2023 NEGATIVE (-ve)		

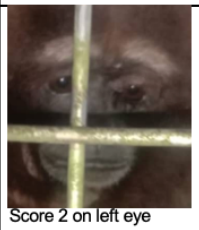
Date: 16/2/2023
Animal ID: Rangga
Sex: M
Age: 4 y/o

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	17/2/2023	18/2/2023	19/2/2023
Score	 Score 2 on left eye	 Score 2 on left eye	 Score 2 on left eye
Chembio DPP ® VetTB assay.	 29/9/2023 POSITIVE (+ve)		

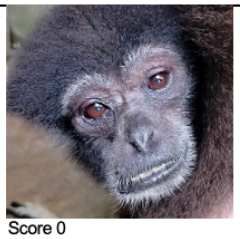
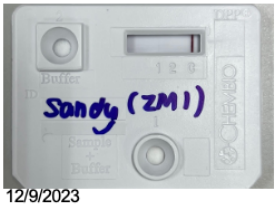
Date: 16/2/2023
Animal ID: Ud
Sex: F
Age: 11 y/o

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	17/2/2023	18/2/2023	19/2/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 4/9/2023 NEGATIVE (-ve)		

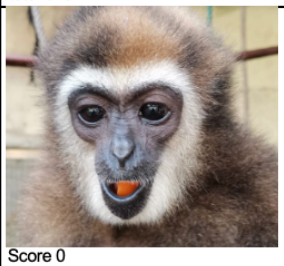
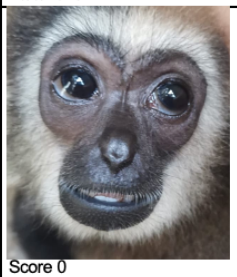
Date: 1/9/2023
Animal ID: Mek (LG 2)
Sex: Female
Age: 8 y/o (estimated)

Observation	**24 hours post injection**	**48 hours post injection**	**72 hours post injection**
Date	2/9/2023	3/9/2023	4/9/2023
Score	 Score 2 on left eye	 Score 2 on left eye	 Score 2 on left eye
Chembio DPP ® VetTB assay.	 12/9/2023 NEGATIVE (-ve)		

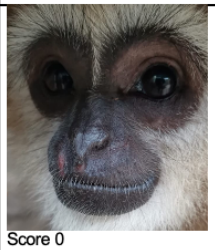
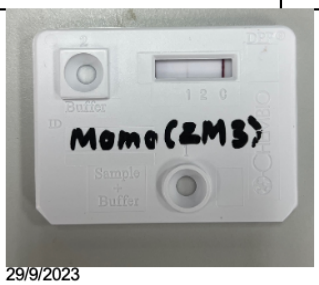
Date: 7/9/2023
Animal ID: Sandy
Sex: F
Age: ~ 10 y/o

Observation	**24 hours post injection**	**48 hours post injection**	**72 hours post injection**
Date	8/9/2023	9/9/2023	10/9/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 12/9/2023 NEGATIVE (-ve)		

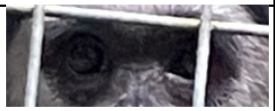
Date: 7/9/2023
Animal ID: Maru
Sex: M
Age: 2 y/o

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	8/9/2023	9/9/2023	10/9/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 29/9/2023 POSITIVE (+ve)		

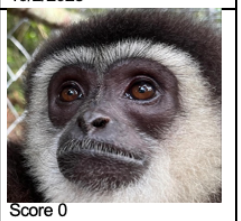
Date: 26/9/2023
Animal ID: Momo
Sex: Female
Age: -

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	27/9/2023	28/9/2023	29/9/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 29/9/2023 Light band- NEGATIVE (-ve)		

Date: 16/2/2023
Animal ID: Dexter
Sex: M
Age: 7 y/o

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	17/2/2023	18/2/2023	19/2/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 29/9/2023 POSITIVE (+ve)		

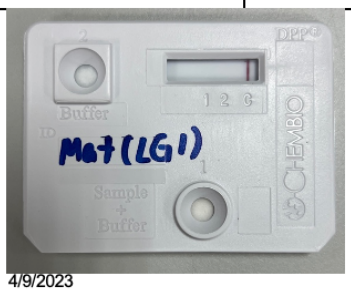
Date: 16/2/2023
Animal ID: Ebony
Sex: F
Age: 5 y/o

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	17/2/2023	18/2/2023	19/2/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 4/9/2023 NEGATIVE (-ve)		

Date: 16/2/2023
Animal ID: Tony
Sex: M
Age: 9 y/o

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	17/2/2023	18/2/2023	19/2/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 4/9/2023 NEGATIVE (-ve)		

Date: 1/9/2023
Animal ID: Mat (LG1)
Sex: Female
Age: 8 y/o (estimated)

Observation	24 hours post injection	48 hours post injection	72 hours post injection
Date	2/9/2023	3/9/2023	4/9/2023
Score	 Score 0	 Score 0	 Score 0
Chembio DPP ® VetTB assay.	 4/9/2023 NEGATIVE (-ve)		

Appendix D- Photos

Figure 1. Blood sample was collected via cephalic vein of the lar gibbons.

Figure 2. Mammalian old tuberculin (MOT) was injected into right eyelid while bovine PPD was injected into left eyelid.

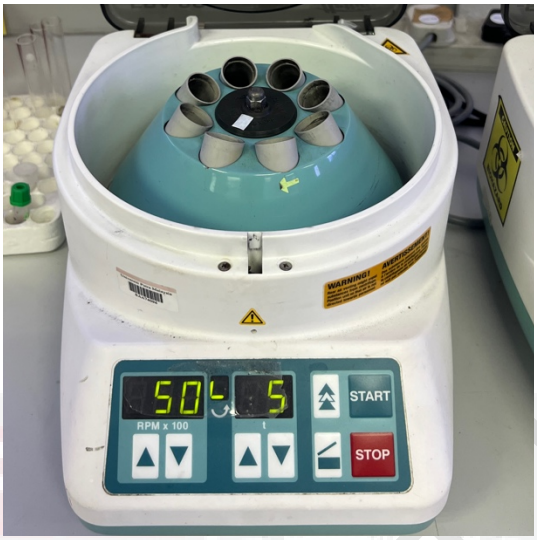


Figure 3. Blood samples stored in red plain tubes were centrifuged at 5000 rpm for 5 minutes.

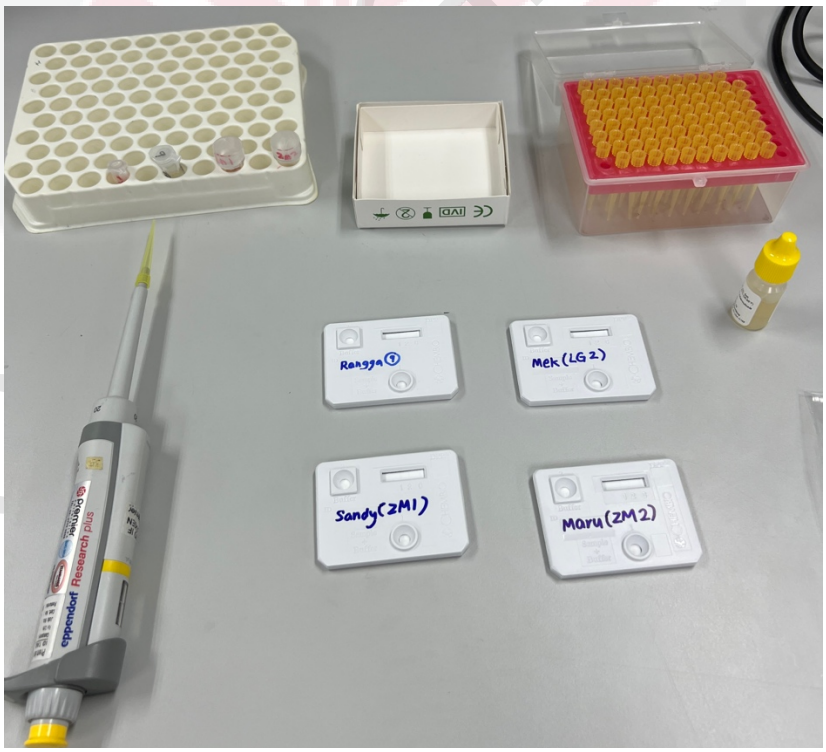


Figure 4. Serological detection was done using Chembio DPP ® VetTB assay.