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RESEARCH ARTICLE

Surveillance and Detection of *Mycobacterium tuberculosis* Complex and *Mycobacterium avium* Complex in Captive Non-Human Primates in Zoological Parks

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A B S T R A C T

Tuberculosis (TB) is a globally important reemerging disease that is chronic, multi host infection caused by *Mycobacterium tuberculosis* complex (MTBC), which causes disease in livestock, wildlife and zoonotic TB in humans. Twelve (n=12) different species of NHPs were screened for MTBC and MAC in Zoo Melaka, Malaysia. The surveillance of MTBC and MAC was through the application of skin test, serology and PCR. The results showed two orangutan *Pongo* spp. (16.7%) were reactive to skin test with orangutan A showing obvious swelling of eyelids with drooping and varying degrees of erythema and orangutan B with moderate swelling of the eyelids with drooping and without erythema. For the antibody detection against MTBC, these two orangutans were positive by serology (16.7%), all other NHPs were antibody negative. Polymerase chain reaction from the blood of all NHPs was negative for the MTBC, however, all samples from blood and pharyngeal swab were PCR positive for MAC. In conclusion MAC was detected in captive NHPs population probably due to exposure to the environment without completely discriminating MTBC.

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INTRODUCTION

Tuberculosis (TB) is a globally important reemerging disease, that is chronic, multi host infection due to members of bacterial pathogens known as *Mycobacterium tuberculosis* complex (MTBC), which causes disease in livestock, wildlife and zoonotic TB in humans (Miller *et al.,*. 2019). The disease was discovered for over a century in both captive and free-ranging non-human primates (NHPs), and is caused by *M. tuberculosis,* and *M. bovis* (Mätz-Rensing *et al.,* 2015). The means of disease transmission include exposure to infected humans Lerche *et al.* (2008) and contact with infected domestic animals Sapolsky & Else (1987), zoo or wildlife animals (Parsons *et al.,* 2009; Stephens *et al.,* 2013; Michel *et al.,* 2013). Members of *Mycobacterium avium* complex (MAC) also infect NHPs in zoological gardens where various population of animal species are managed in a limited space. These animals are prone to diseases like that of livestock herds, when there is high density of animals that are exposed to various concentration of infectious agents in the population.

Mycobacterium avium complex infections in zoo animals is important in terms of animal welfare and conservation. Various outbreaks of MAC infection had been reported from zoos around the world (Burgess *et al.,* 2018; Roller *et al.,* 2020). Members of MAC are commonly diagnosed in humans and animals because they are not species specific, causing tuberculous lesions in lymph node and parenchymatous organs (Roller *et al.,* 2020). These mycobacteria pose a serious risk to zoos and wildlife sanctuaries Roller *et al.* (2020) as such, it has become an important part of disease prevention and control in many zoos and wildlife sanctuaries. (Roller *et al.,* 2020).

To diagnose tuberculosis in NHP, cell-mediated immune (CMI) response by means of skin test is used which require intradermal injection into the eyes (palpebral) followed by a 3-4 days' period of observation for evidence of immune response that is generally time consuming. The test detects delayed type hypersensitivity (DTH), which is a CMI response to tuberculin antigens (Vervenne *et al.,* 2004; Lerche *et al.,* 2008b; Engel *et al.,* 2013; Rosenbaum *et al.,* 2015). The application of molecular diagnostic methods has overcome these problems by improving the speed and accuracy of the identification of MTBC and MAC species. Used in combination with antibody detection, molecular diagnosis may help in differentiating active and latent tuberculous infections from animals (Maas *et al.,* 2013; Mikota *et al.,* 2015).

Existence and widespread of potential MTBC reservoirs such as wild boars, cervids and macaques in Southeast Asia, is an important factor to be considered for active TB disease surveillance (Che-Amat & Ong 2018). The occurrence in local wildlife species of potential reservoirs for TB such as wild boar, cervids, macaques and others are still unknown. To date, there is no screening and investigation performed in any local zoo in Malaysia including for NHPs. Thus, this study was conducted to get the first insight on the surveillance of mycobacterial organisms circulating among animals in local zoo with the objectives of detecting MTBC and MAC in different species of NHPs through the application of tuberculin skin test, serology and molecular biology techniques.

MATERIALS AND METHODS

Study animals and sample collection: A cross-sectional study was conducted by using tuberculin palpebral skin (intradermal) test, serology and molecular biology techniques. Blood samples were collected from twelve (N=12) different species of primates including gibbon (n=5), capuchins (n=2), siamang (n=2), mandrill (n=1) and orangutan (n=2), in Zoo Melaka and A'Famosa Safari Wonderland (central-south of Peninsular Malaysia). Pharyngeal swab was taken by sterile swab product. All animals were sedated by a zoo veterinarian before the procedures and sampling were carried out and animals were monitored until recovery. Blood samples were collected using 23-gauge syringe and needle and kept in labelled plain tubes and centrifuged at the Clinical Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia for 15000 *rpm* for 15 minutes within 3 hours of collection. The serum was extracted into Citadel Deep-Well Plates and Cluster Tube (SSIbio, USA) and the serum were aliquoted, stored at -20°C, to be used for the determination of antibodies detection.

Comparative tuberculin palpebral skin test: The tuberculin PPD kit is composed of Bovine tuberculin PPD 3000 and Avian tuberculin PPD 2500. It is manufactured by Prionics Lelystad B.V. Platinastraat 33, 8211 AR Lelystad, Netherlands. The bovine tuberculin purified protein derivatives is made from culture of *Mycobacterium bovis*, strain AN5 and avian tuberculin purified protein derivatives from culture of *Mycobacterium avium* subspecies *avium* strain D4ER. A comparative tuberculin palpebral skin test was conducted by administration of 0.1 mL of bovine purified protein derivative (bPPD) injected intradermally on the right palpebral and avium PPD (aPPD) on the left palpebral. They were injected at the edge of the upper eyelid of each NHPs using a sterile syringe and 23-gauge needle. The reading of the skin test involved observing the reactions site at 24, 48, 72 and 96 hours post injection (PI). The animals were graded as a reactive (Table 1) based on the tuberculin reaction scoring system (Bushmitz *et al.,* 2009; Hablolvarid *et al.,* 2019).

Serology TB antibody detection (Assure TB Ecotest® kit): Serology was done using a rapid test kit which is a visual immunoassay for qualitative presumptive detection of anti-TB antibodies as the protocol specified by the manufacturer. Briefly, 2 drops of serum (75 µl) were dropped into the center of the specimen well by using the disposable pipette. As the test run, sample will migrate across the membrane and the result is read after 10 minutes. Positive results will be indicated by two colored bands appearing on the membrane while, a negative result is indicated by only one colored band at the control region (C). If the control band fails to appear, the test is invalid, thus, it must be discarded.

DNA extraction and PCR assays: DNA was extracted from the blood and pharyngeal swab, using the Qiagen DNeasy Blood and tissue extraction kits based on the protocol specified by the manufacturer. Oligonucleotide primers are manufactured by Integrated DNA Technologies, Inc. USA, distributed by Apical Scientific Sdn Bhd Malaysia. Table 2 listed the primers used for detection of MTBC and MAC (Wilton & Cousins, 1992).

The first set of oligonucleotide primers, MYCGEN-F and MYCGEN-R generate 1030 bp is for genus detection common to all *Mycobacterium* species were used. MYCGEN-F and MYCAV-R generate 180 bp for detection of *M. avium*. (Wilton & Cousins, 1992). The primers were aliquot base on specified protocol by producers. The PCR was carried out as performed initially by (Wilton & Cousins, 1992). Briefly, a 25 µL reaction mixture was prepared containing 12.5 µLTopTaq Master Mix 2x (Qiagen), 5.5 µl RNase free water, 5µl DNA template and 1 µM of each primer: MYCGEN-F and MYCGEN-R amplifying 1030bp fragment targeting16S rRNA gene. MYCGEN-F and MYCAV-R amplifying 180bp fragment targeting 16S rRNA gene. Positive control used was

Table I: Recommended tuberculin reaction scoring system

Score	Observation	outcome
0	No reaction	Negative
1	Bruises, extravasation of blood in to the eyelid associated with the injection of tuberculin skin test.	Negative
2	Various degrees of erythema of the palpebrum with minimal swelling	Negative
3	Moderate swelling of the eyelids with or without erythema	Positive
4	Obvious swelling of eyelids with drooping and varying degrees of erythema	Positive
5	Marked swelling with necrosis and with closed eyelids.	Positive

Table 2: Nucleotide sequence and expected product size of primers used in the PCR reaction

Gene	Primer name	Nucleotide sequence	bp	Target
16S rRNA	MYCGEN-F	AGAGTTTGATCCTGGCTCAG	1030	Genus MTBC
	MYCGEN-R	TGCACACAGGCCACAAGGGA		
	MYCGEN-F	AGAGTTTGATCCTGGCTCAG	180	*M. avium*
	MYCAV-R	ACCAGAAGACATGCGTCTTG		
	MYCINT-F	CCTTTAGGCGCATGTCTTTA	850	*M. intracellulare*
	MYCGEN-R	TGCACACAGGCCACAAGGGA		
Rv2073c (RD9)	Rv2073cF	5'TCG CCG CTG CCA GAT GAG TC 3'	600	*M. tb*
	Rv2073cR	5'TTT GGG AGC CGC CGG TGG TGA 3'		
hsp65[631]	hsp65F	5'ACC AAC GAT GGT GTG TCC AT 3'	441	Genus MTBC
	hsp65R	5'CTT GTC GAA CCG CAT ACC CT 3'_		

Table 3: Results from different diagnostic techniques applied on NHPs for MTBC and MAC detection

NHPs species	Skin test	Tuberculin reaction scoring	Serology	PCR MAC blood	PCR MAC bucal swab
Orangutan	Reactive	4	Positive	Positive	Positive
Orangutan	Reactive	3	Positive	Positive	Positive
Gibbon	Non-reactive	0	Negative	Positive	Positive
Gibbon	Non-reactive	0	Negative	Positive	Positive
Gibbon	Non-reactive	0	Negative	Positive	Positive
Gibbon	Non-reactive	0	Negative	Positive	Positive
Gibbon	Non-reactive	0	Negative	Positive	Positive
Capuchin	Non-reactive	0	Negative	Positive	Positive
Capuchin	Non-reactive	0	Negative	Positive	Positive
Siamang	Non-reactive	0	Negative	Positive	Positive
Siamang	Non-reactive	0	Negative	Positive	Positive
Mandrill	Non-reactive	0	Negative	Positive	Positive

Mycobacterium avium subspecies *avium Chester* (ATCC 15769) and negative control is RNase free water (Qiagen®). (Qiagen®). The amplification reaction was carried out in Mastercycler (Eppendorf, Germany). The amplification conditions were initially denaturation at 94ºC temperature, 5min for 1st cycle while final denaturation at 94ºC, 30 secs for 39 cycles. Annealing 62ºC, Annealing temperature for 3min. Initial extension at 75ºC, temperature for 3min while final extension at 75ºC, temperature for 5min (Wilton & Cousins 1992).

For a second set of oligonucleotide primers, Rv2073cF, Rv2073cR, for *M. tuberculosis* for a detection in macaques were used while hsp65F, hsp65R is for the detection of genus common to all *Mycobacterium* species (Huard *et al.,* 2003; Cedeño *et al.,* 2005). The primers were aliquot based on specified protocol by producers. The PCR was carried out as performed previously (Huard *et al.,* 2003; Cedeño *et al.,* 2005) refer (Table 2). Briefly, a 25 µL reaction mixture was prepared containg12.5 µL TopTaq Master Mix 2x (Qiagen), 5.5 µl RNase free water, 5µl DNA template and 1 µM of each primer. Primers Rv2073c'and Rv2073c amplifying 600bp, and hsp65F and hsp65R amplifying 441bp fragment targeting Rv2073c and hsp6[631]gene. Positive control used was *M. tuberculosis* H37R3 (ATCC 25177) and negative control was RNase free water (Qiagen®). Amplifications conditions started with initial denaturation for 5min at 94ºC temperature. It was followed by 25 cycles of 1 min at 94ºC, annealing at 1 min for 60ºC and initial extension at 1 min for 72ºC and final extension at 10 min for 72ºC. Meanwhile for L1 and L2, 30 cycles for amplification, initial denaturation 95ºC for 5 min, final denaturation at 95ºC for 1 min, annealing at 65ºC for 1 min and initial extension at 1 min for 72ºC and final extension at 10 min for 72ºC (Huard *et al.,* 2003; Cedeño *et al.,* 2005). For gel electrophoresis, 5µL of amplified DNA with an equal volume of 2 µL loading dye and subjected to electrophoresis at 80v for 2 hours on a 2% agarose gel. The agarose gel was viewed using Alphamager (Alpha Innotech) immediately after electrophoresis.

RESULTS

Responds of NHPs to tuberculin test: Results from this study showed response of two orangutans to tuberculin skin test. The orangutan A responses was observed after 24 hours up to 96 hours post inoculation with bPPD and aPPD. After careful monitoring and observation, there was an obvious swelling of both eyelids with drooping and varying degrees of erythema and based on tuberculin reaction scoring system it was scored as 4. The orangutan B responses was observed after 24 hours up to 96 hours post inoculation. From observation, there was a moderate swelling of the right eyelid with drooping and without erythema and based on the tuberculin reaction scoring system it was scored as 3 (Fig. 1).

Serological responds of NHPs to MTBC: For the antibody detection, those two orangutans were positive by serology 2/12 (16.7%), while all other NHPs were antibody negative (Table 3).

Polymerase chain reaction from blood and pharyngeal swab: Nucleic acids detection from the whole blood of all NHPs were negative for MTBC but all samples from blood (100%) and pharyngeal swab (100%) were PCR positive for MAC (Fig. 2 and 3).

DISCUSSION

This is the first identification and surveillance for MTBC and MAC among NHPs in selected zoological parks in Malaysia. Although TB screening in NHPs is recommended to be performed in zoological parks, however it is not a usual practice and to our concern, this is the first surveillance study in local zoo. Major issues for the under-performed TB screening in local zoos could be due to available test, inconvenient test procedure as the animal need to be sedated and immobilized using a chemical agent that might pose least risk. Ante mortem diagnosis in NHPs

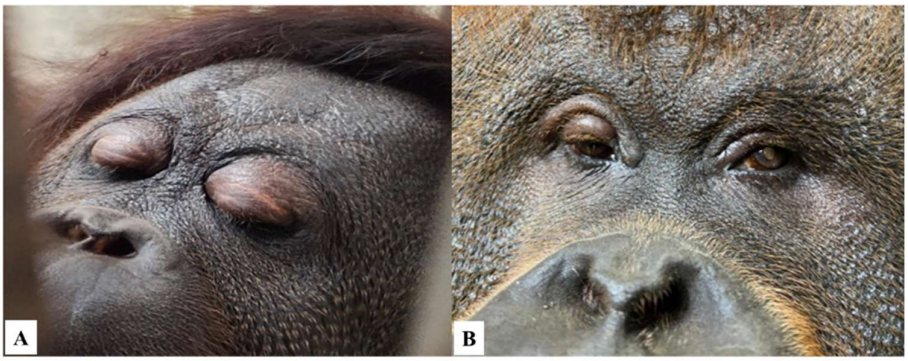

Fig. 1: Tuberculin palpebral skin reaction from orangutan **(A)** swelling of both eyelids with drooping and varying degrees of erythema and base on tuberculin reaction scoring system it was scored as 4. Orangutan **(B)** Moderate swelling of the right eyelid with drooping and without erythema and base on tuberculin reaction scoring system it was scored as 3.

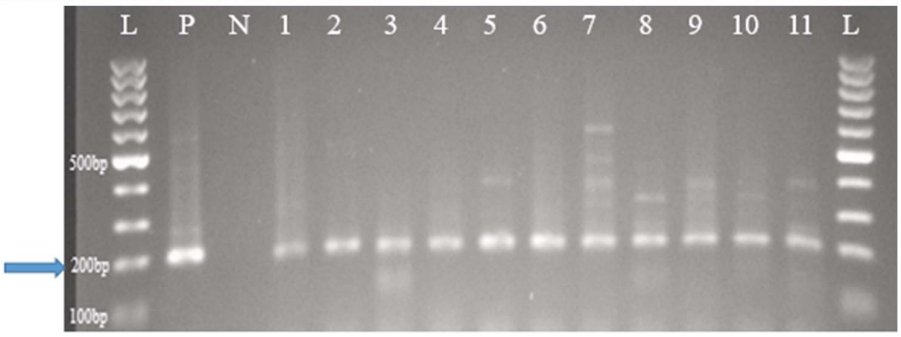

Fig. 2: Result MAC-specific PCR using pharyngeal swab on different species of monkeys. Lane L: 100bp ladder, lane P: positive control, lane N: negative control, lanes 1 to 11 are positive samples.

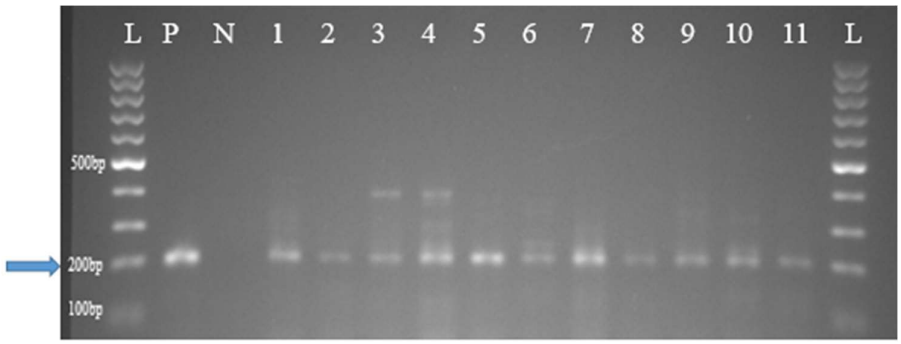

Fig. 3: Result MAC-specific PCR using Blood of different species of monkeys. Lane L: 100bp ladder, lane P: positive control, lane N: negative control, lanes 1 to 11 are positive samples.

rely on skin test for the development of delayed hypersensitivity response towards mycobacterial antigen. Although the two orangutan showed tuberculin skin reaction, the results were considered inconclusive, therefore repetition is required as recommended by many regulations for NHPs screening for TB. This is because cell-mediated immune response in competent animals occurs approximately 4 weeks after exposure. Unfortunately, due to the risk of tranquilization and demanding process involved in restraining these NHPs and management concern, the procedure was not repeated.

The response to either bPPD or aPPD was an indication that the animals were infected by *Mycobacterium* species either MTBC or MAC respectively (Lerche *et al.*, 2008b; Bushmitz *et al.*, 2009; Frost *et al*., 2014). Its stated that the degree of hypersensitivity response and the diagnostic accuracy of skin test corresponds with the number of tubercle bacilli, the amount of circulating, primed antigen–specific T cells and the quantity of specific antigen present in the tuberculin preparation (Lerche *et al.*, 2008b; Bushmitz *et al.*, 2009; Frost *et al*., 2014). Tuberculin skin testing in NHPs were considered poor in terms of the test performance because it might cause false positive and false negatives and thus, serial testing is required (Cousins & Florisson, 2005). Detection of skin test in experimentally infected TB in cynomolgus and rhesus monkey was reported to be low with 66.7% and 87.5% (Frost *et al.,* 2014). The orangutans have greater sensitivity than other NHPs to tuberculin test and they maybe sensitized to mycobacterial antigens after being exposed to MAC. In a study 12/20 orangutans responded positively to at least one tuberculin that was injected intradermally. Most of the animals reacted positively to *M. avium* PPD and *M. bovis* PPD (Frost *et al.,* 2014).

All the other ten NHPs (gibbon, capuchin, siamang and mandrill) showed negative results in the skin testing, but the concern was about the limited efficacy of skin test due to the production of false negatives reactions caused by poor detection during early disease and in latency period as the results of infection. Other factors are immune-suppression due to other diseases and drug interference. False negative reactions also might be due to improper injections, interpretation bias or suboptimal concentration of antigen during in preparation. (Lerche *et al.,* 2008; Bushmitz *et al.,* 2009; Frost *et al.,* 2014). An alternative to skin test, is the PRIMAGAM® for NHPs that was validated in rhesus and cynomolgus monkeys. Although the test showed a low sensitivity of 68% and specificity of 97% and not validated in other NHPs species. In addition, The PRIMAGAM® require technicalities and high cost Garcia *et al.* (2004), therefore it was not included in this study.

The combination of positive skin test with antibody detection and PCR can ruled out the possibility of false positive reaction. Serological detection of antibodies against members of MTBC specific antigens are important in the production of an immunodiagnostic methods for TB diagnosis (Kanaujia *et al.,* 2003). The choice of antibody kits was influence by species being tested, stage of disease, economic reasons, and simplicity of a test. Relationship exist amongst TB in NHPs and immunological response towards early secretory antigenic target (ESAT-6), culture filtrate protein (CFP-10), and MPB83. Purified antigens are known to increase diagnostic specificity and sensitivity. These proteins have been evaluated as antigen targets for detection of TB-specific antibodies in NHPs. Other studies had used Multiplex Microbead Immunoassay (MMIA), Multiantigen Print Immunoassay (MAPIA), and Prima-TB STAT-PAK (Brusasca *et al.,* 2003; Lyashchenko *et al.,* 2007). Several host and environmental factors can affect the performance of diagnostic test, such as age, sex, general health, disease severity, stress, climate and season. Most of these factors will interact with one another in serological test. The quality of blood samples used has an important impact on test results. This is the reason why it is important to validate diagnostic test, on not only the target species and populations, but also the situation in which the test will be carried out and where the samples are obtained. Haemolysis of blood sample was found to reduce the sensitivity of some ELISA kits in other wildlife animals. (Chambers, 2013).

Environmental mycobacteria such as the members of MAC constitute a very interesting group in terms of ecology. They are isolated in all types of water sources, and able to produce biofilms because they could survive for a long time in the environment. In addition, they are found in fresh or frozen fruits and vegetables, contaminated feed stuff, bedding materials, soil and saw dust, where they are identified as natural reservoir of MAC (Hulinova Stromerova & Faldyna, 2018). This study is similar to that of Roller *et al.* (2020) who reported many MAC positive samples by PCR from different NHPs and other zoo animal. PCR amplification has been applied to detect the presence of MTBC in buccal swabs from pig tailed-macaques and free- ranging macaques in Asia and capuchin monkeys (Wilbur *et al.,* 2012; Engel *et al.,* 2012; Rosenbaum *et al.,* 2015). Buccal swab sample collection may help in overcoming challenges associated with the use and interpretation of skin test, radiographs and physical examination in NHP. Recognizing the limitations of regularly used techniques, direct detection of mycobacterial organisms from samples has several advantages and is fast gaining acceptance and applications in zoological and wildlife setting (Rosenbaum *et al.,* 2015).

Conclusions: In conclusion, MTBC Reactive NHPs were detected through serology and skin test while MAC Nucleic acids were detected through PCR in NHPs population in the local zoo. The combination of both tuberculin skin test, serology and PCR can significantly improve the overall effectiveness of TB screening programs for NHPs and ruled out the possibility of false positive and false negative reaction encountered with only tuberculin skin test. Molecular detection of important mycobacteria using biological samples, hold promises in advancing the ability to detect and characterized the existence either of MTBC or MAC in NHPs populations.

Author contribution: YML and AC Conceived and designed Project. YML, AC and NS Data collection. YML, AC, SO Methodology. AC, PTO, SO, JFFA, JS Supervision. YML analyzed data. AC, PTO, SO, JFFA, JS review & editing. All authors read the manuscripts and approved the contents.

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