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FULL PAPER

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Detection of *Mycobacterium tuberculosis* complex antibodies in free-ranged wild boar and wild macaques in selected districts in Selangor and reevaluation of tuberculosis serodetection in captive Asian elephants in Pahang, Peninsular Malaysia

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ABSTRACT. Tuberculosis (TB) is a chronic inflammatory and zoonotic disease caused by *Mycobacterium tuberculosis* complex (MTBC) members, affecting several domestic animals, wildlife species and humans. The preliminary investigation was aimed to detect antibody against MTBC among indigenous wildlife which are free-ranged wild boar, free-ranged wild macaques and captive Asian elephants in selected areas of Selangor and elephant conservation centre in Pahang, respectively. The results indicate that MTBC serodetection rate in wild boar was 16.7% (7.3–33.5 at 95% confidence interval (CI)) using an in-house ELISA bPPD IgG and 10% (3.5–25.6 at 95% CI) by DPP®VetTB assay, while the wild macaques and Asian elephant were seronegative. The univariate analysis indicates no statistically significant difference in risk factors for sex and age of wild boar but there was a significant positive correlation ($P<0.05$) between bovine TB in dairy cattle and wild boar seropositivity in the Sepang district.

KEY WORDS: antibody, ELISA, *Mycobacterium tuberculosis* complex, wild boar, wildlife

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Tuberculosis (TB) is a contagious and a zoonotic bacterial disease that infects domestic, wildlife animals and humans. It is caused by a group of *Mycobacterium tuberculosis* complex (MTBC) which include *M. bovis, M. tuberculosis*, *M. africanum, M. canetti, M. microti M. caprae,* and *M. pinnipedii* [13]. Most developed countries have been making efforts for a complete eradication of TB in the last decades, but this objective is yet to be achieved [19]. Huge sums of money have been allocated for TB eradication, but the disease is still endemic in some countries both developing and developed nations, whereas in others it is an emerging disease. This is because the activities of domestic and wildlife host in the transmission of animal TB differs from one particular region to another [9].

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Wild boars are known to contribute to the epidemiology of animal TB in some regions, acting as the maintenance or spill over host for instance wild boar [9]. Serology has been adopted for screening and diagnosis in wild boar and feral pigs due to easy procedure and faster diagnostic results. Serological diagnosis has better efficiency with regards to logistics, due to the normality of antibodies during transportation, storage and handling, which reduces its cost and increase simplicity of application [24, 25]. The 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 [4].

Mycobacterium that is associated with TB in non-human primates (NHPs) has not yet been identified which may reflect a poor research on the full NHPs pathogen spectrum [2]. It is implicated that TB in both captive and free-ranging NHPs is mainly caused by *M. tuberculosis*, *M. bovis* and *M. africanum* and thus lead to a presumption that NHP is susceptible to mycobacterial infection [27]. Tuberculosis has been reported in free-ranging macaques (*Macaca fascicularis*) and capuchin monkeys (*Cebus sapajus*) in Asia [6, 22, 26] but there is scarcity of such research studies on macaques and other NHPs in Malaysia [4, 12]. For Asian elephants, *M. tuberculosis* have been reported in captive elephants [14, 17] and wild Asian elephants that has close contact with humans [29]. In Malaysia, MTBC seroprevalence of 20.4−23.3% in semi-captive Asian elephant herd has been reported in 2013 and 2016 respectively in the National Elephant Conservation Centre (NECC), Pahang [16, 28]. The seroprevalence of wild boar and macaques remain unknown in Malaysia [4].

The evidence of indigenous wildlife animals such as wild boar, cervids and macaques as potential reservoir for TB is still unknown. In order to develop an initial data on the status of TB on indigenous wildlife species, we targeted mainly free-ranged wild boar, wild macaques, and captive Asian elephant. This study was conducted to detect antibody against MTBC using a serological assay focusing only on a small epidemiological range in selected districts in Selangor (free-ranged wild boar and wild macaques) and to analyze the possible risk factors. For captive Asian elephants, we reevaluate the antibody detection where the initial surveillance was conducted among elephants in the same conservation centre in Pahang previously [16, 28]. The study area was assumed to have a number of high-risk for a potential zoonotic TB in domestic cattle and human due to the diversity of wildlife and encroachment of wildlife into human settlement. The incidence of bovine TB among dairy farms particularly in the state of Selangor (west-central of Peninsular Malaysia) is constantly detected despite of test and cull policy using Bovigam® (Prionics AG, Schlieren, ZRH, Switzerland) and comparartive cervical skin test. According to the local state veterinary authority in Selangor, almost 30% of the cattle dairy farms had been closed due to the consistent TB reactors [3].

MATERIALS AND METHODS

Study area and animals

This research complied with relevant wildlife authority, the Department of Wildlife and National Parks (PERHILITAN) with a research permit issued number B-00156-15-19 (wild boar), W-00396-15-19 (wild macaques), B-00859-16-19 (Asian elephant) and UPM/AICUC/AUP-U040/2019 for animal ethics (Asian elephant). A total of N=93 serum samples were collected by a cross sectional sampling method from 2019 to 2020 from all three species. Thirty (n=30) serum samples were collected from free-ranged wild boar (*Sus scrofa*), sex (23 males and 7 females), age (4 yearling, 9 subadult, and 17 adults) that were harvested from five districts in Selangor, southern districts, Sepang (n=13), Kuala Langat (n=3), Hulu langat (n=2), western districts Kuala Selangor (n=8) and eastern districts Hulu Selangor (n=4) by a permission of local hunters and butchers. The hunting day was not fixed and conducted during the weekend with a very low regularity. About n=42 of wild macaques (*Macaca fascicularis*) sex (16 males and 26 females),age (5 yearling, 11 subadult, and 26 adults) from five districts in Selangor were collected from the opportunistic sampling from the PERHILITAN (State of Selangor), while n=21 samples of captive Asian elephants (*Elephas maximus*), sex (5 males and 16 females), age (7 young, 6 subadult, and 8 adults) were collected from the NECC in Pahang (east coast Peninsular Malaysia) where all captive elephants were originated from wild captured. The elephants tested in this study were the same individuals that were tested positive in the past (2013 and 2016). For each sample, gender, age, hunting site, and date of collection were recorded. The age of the wild boar was established using a tooth eruption pattern [23], and the animals were divided into three age categories: yearling (0–12 months); sub-adults (13–36 months); and adults (>36 months). The age of wild macaques was estimated by weight as described by the Institutional Animal Care and Use Committee (IACUC) policies and guidance, Division of Research, University of Houston (https://www.uh.edu/research/compliance/iacuc/iacuc-pol-guide/Associated%20growth%20chart.pdf). For captive Asian elephant, the age was determined based on the individual data provided by the NECC.

Blood collection

Blood sampling of the wild boars and wild macaques were performed in the field. About 5 to 10 ml of blood were collected via intracavernous venipuncture for wild boar [1] while vena cava venipuncture and ear vein venipuncture performed in dead wild macaques and Asian elephant respectively. The collected blood samples were kept in labelled plain tubes and centrifuged at the Clinical Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia for 1:5,000 rpm for 15 min. The serum was extracted into Citadel Deep-Well Plates and Cluster Tube (SSIbio, Lodi, CA, USA) and serum were aliquot, stored at −20°C, to be used for the antibody detection.

Serology (ELISA bPPD IgG, DPP® VetTB, Rapid Ecotest® TB kit)

The bPPD ELISA IgG test was adapted from the report by Che 'Amat *et al*. [5]. The antigen bPPD (AG Prionics Lelystad BV, Lelystad, FL, Holland) coated plates were incubated in a fridge overnight. The wild boar sera were added at 1:100 dilutions and

incubated at 37°C for 30 min. Detection of IgG antibodies was done with the addition of 100 µl of HRP-conjugated goat anti-pig IgG antibodies (Bethyl Laboratories, Inc., Montgomery, TX, USA) at 1:5,000 dilutions. After the addition of the conjugate, the plate was incubated at room temperature for 1 hr. After it had been washed 3 times with PBST, change in color occurred when added with, 50 µl 3,3′,5,5′ tetramethylbenzidine (TMB). The plate was protected from light for 15 min at room temperature before adding the 50 µl H_2SO_4 (2M) to stop the reaction. The optical densities (OD) were measured by ELISA plate reader at 450 nm. Sample to positive (S/P) ratio=S/P ≥3.0 while negative is S/P <2.9. Dual-path platform (DPP®) VetTB (Chembio Diagnostics, Systems, Inc., Medford, NY, USA) is a single use immunochromatographic test for antibody detection against *M. bovis and M. tuberculosis*. Serum samples were tested for specific antibodies as previously described by Che 'Amat *et al*. [5]. Both ELISA bPPD IgG and DPP®VetTB have moderate-good sensitivity and good specificity [5]. ELISA bPPD IgG tests were used for antibody detection in free-ranged wild boar while DPP® VetTB was used for captive Asian elephants. Rapid Ecotest® TB (Assure Tech. Hangzhou, China) kit is a rapid test kit which is a visual immunoassay for qualitative presumptive detection of anti-TB antibodies (*M. tuberculosis*) and the protocols followed the manufacturer's instructions. The kits have moderate sensitivity and good specificity as established by manufacturer.

Statistical analyses

Epitools epidemiological calculators were used to calculate the apparent prevalence. All risk factors (sex and age) were analyzed using the univariable logistic regression to calculate, the odds ratios, at a confidence interval of 95% (CI 95%) and $P>0.05$ was considered significant, Pearson correlation and all calculations were determined using software SPSS version 20.0 (IBM Corp., Armonk, NY, USA). Brief data of individual dairy cattle with a history of positive TB was provided by the Department of Veterinary Services (DVS) state of Selangor based on the year 2010 to 2020 which was used to reflect that TB was prevalent or had a history of occurrence in the area. A gross analysis was observed by tabulating and comparing between the two occurrences of bovine TB in dairy cattle and serodetection in wild boar in those related districts.

RESULTS

The result showed that MTBC antibody was detected with 16.7% at 95% CI (7.34–33.56) using ELISA bPPD IgG and 10% at 95% CI (3.5–25.6) using a DPP® VetTB assay in free-ranged wild boars (Table 1). The serodetection for male was 17.3% at 95% CI (6.98–37.14) and female was 14.2% at 95% CI for apparent prevalence respectively while serodetection status for yearling was 25% at 95% CI (4.56–69.94), subadult was 22.2% at 95% CI (6.32–54.74) and adult was 11.8% at 95% CI (3.29–34.34) for apparent prevalence respectively. The univariate analysis indicates no statistically significant difference $P>0.05$ in risk factors for sex and age (Table 3). As presented in Fig. 1, four out of nine districts in Selangor were seropositive for antibodies against TB among free-ranged wild boars. Based on the geographical separation, 4 seropositive (80%) wild boars were detected within the southern part of Selangor (Hulu Langat, Kuala Langat, Sepang) with a history of 17.6% (47/267) occurrence of bovine TB tested among dairy cattle from 2010–2020. There was a positive correlation between bovine TB cattle and wild boar seropositivity in the Sepang district which was statistically significant $P<0.05$ (Table 2). One seropositive antibody (20%) wild boar was detected in the eastern part of Selangor (Gombak, Hulu Selangor) with a history of 0.8% (1/121) occurrence of bovine TB from dairy cattle tested within the same period.

DISCUSSION

This study reported the preliminary data on the antibody detection of MTBC among a selected samples of free-ranged wild boars in five districts in Selangor with 16.7% and 10% serodetection using ELISA bPPD IgG and DPP® VetTB assay respectively. The seropositive conversion to MTBC in wild boar observed in this study might be due to the fact that wild boars move a long distance scavenging for food and this may bring them into contact with domestic livestock who are possible TB reactors [20]. The incidence of bovine TB among dairy farms particularly in the state of Selangor is constantly detected despite of test and cull policy. However the validity of the wild boar presence needs to be supported with observational and ecological surveillance of the wild boar in or around the proximity of the farms and molecular typing of both MTBC isolates from cattle and wild boar for the identical strain matching. Exposure of free-ranged wild boar to MTBC is consistent with the distribution of bovine TB from cattle [20]. According

Table 1. Apparent seroprevalence of *Mycobacterium tuberculosis* complex in wild boars, wild macaques and captive Asian elephants

Animals	Total sample collected	Serological diagnostic test	No. of sample tested	Positive	Prevalence (95% CI)
Wild boar	30	ELISA bPPD IgG	30	5	16.7 (7.3–33.5)
		DPP® VetTB	30	3	10.0 (3.5–25.6)
Wild macaques	42	Ecotest® TB kit	42	0	0
Asian elephants	21	DPP® VetTB	21	0	0

CI, confidence interval. DPP® VetTB: Chembio Diagnostics Systems Inc., Medford, NY, USA; Ecotest® TB kit: Assure Tech (Hangzhou) Co., Ltd., Zhejiang, China.

Table 2. Correlation between positive bovine tuberculosis cattle and seropositive wild boar location

Location/District	Bovine TB cattle	No. of wild boar sample	Seropositive wild boar	Pearson correlation	Sig (2-tailed)
Sepang (south)	38	13	1	0.960**	0.002
Kuala Langat (south)	1	3	2	0.000	0.000
Hulu Langat (south)	8	2	1	−0.945	0.212
Hulu Selangor (east)*	121	4	1	−0.311	0.382
Kuala Selangor (west)*	99	8	0	0.000	0.000

**Correlation is significant at the 0.01 level (2-tailed). *Hulu Selangor (Gombak n=14 and Hulu Selangor n=107) and Kuala Selangor (Klang n=93, Kuala Selangor n=1 and Petaling n=5). TB, tuberculosis; Sig, significance.

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Table 3. Univariable association between *Mycobacterium tuberculosis* complex seropositive status and assumed risk factors for wild boar

Variables	No. of samples	Serology positive	% Serology positive	95% CI	S.E	OR	95% CI	*P*-value
Sex								
Male	23	4	17.3	6.98–37.14	1.212	1.263	0.117–13.591	0.847
Female	7	1	14.2	2.57–51.31		Ref		
Age								
Yearling	4	1	25.0	4.56–69.94	1.378	2.500	0.168–37.260	0.506
Subadult	9	2	22.2	6.32–54.74	1.100	2.143	0.248–18.498	0.488
Adult	17	2	11.8	3.29–34.34		Ref		

CI, confidence interval; Ref, reference category; S.E, standard error; OR, odds ratio.

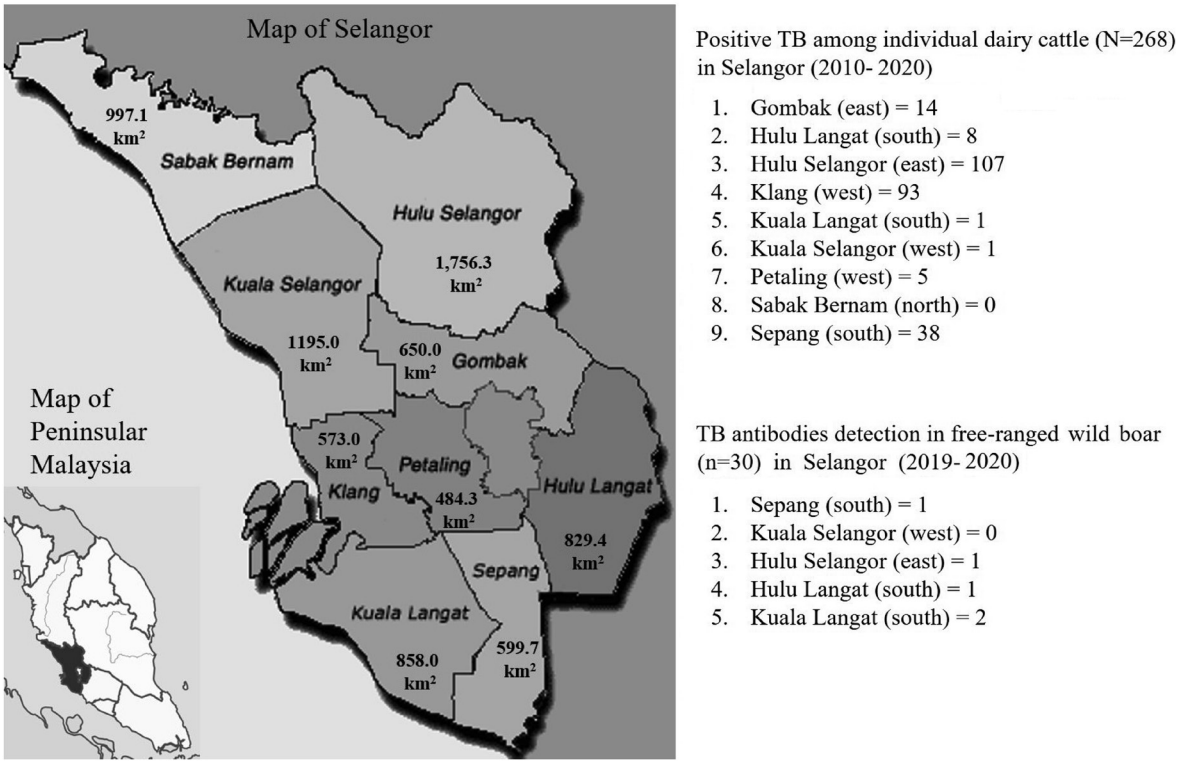

Fig. 1. The total number of dairy cattle in 2010–2020 (n=267) and the total number of wild boars positive serodetection (n=5) are shown. Sampling sites of free-ranged wild boar in 5 districts in Selangor (west of Peninsular Malaysia) with tuberculosis (TB) antibodies detection. Information on the individual dairy cattle with history of TB from the Department of Veterinary Services of Selangor in 9 districts in Selangor. (Map of Peninsular Malaysia and state of Selangor were adapted from the https://ms.wikipedia.org/wiki/Selangor and https://kualaselangor.selangor.gov.my/kualaselangor.php/pages/view/98?mid=209).

to the state veterinary authority, almost 30% of dairy farms have been closed due to the occurances of TB and this high prevalence among dairy farms in Selangor, could play a role in the spillover transmission from cattles to wildlife such as wild boar and vice versa. However this epidemiologic reason need further investigation as wild boar are known to be true TB reservoir.

In serological diagnosis, a combination of two or more diagnostics results in parallel or in series improve diagnostic accuracy of a test [24]. Several antibody detection assays had been produced for the screening of antibodies against predominant antigens of MTBC. These assays used multiple antigens in them which may aid in the improvement in diagnostic accuracy of a test. Antigens such as MPB70, MPB83, ESAT-6, CFP-10, Rv3615c and Rv3020c antigens increased the sensitivity of the assay when compared to the use of single antigens [24]. Some ELISA techniques had been used for evaluation of MTBC antibodies in pigs and wild boars with different levels of sensitivity and specificity [5, 7, 25]. Some factors such as species being tested, stage of disease, economic reasons, and simplicity of a test, influence our choice of diagnostic test. Selected tests used in this study were based on the availability of test kits, validated protocols in selected species and cost issues.

Poor serodetection of MTBC in wild boars in this study could be due to the antigens used, which was a bPPD that was reported to have low sensitivity [5], and its sensitivity usually increased markedly with the severity of the disease. Our constraints while conducting the study was the small sample numbers, this was due to the difficulty in obtaining samples from the hunters because they were social or pleasure hunters where the hunting was not regular. This differs from other arrangement of hunting events in other countries, here in Malaysia, a well organized or commercial hunting does not occur. In addition, the carcass was processed by illegal butcher and the meat was not inspected by the relevant authority, eventually causing them to feel unsecured and did not provide good cooperation. Nevertheless, the preliminary results were well justified within a small epidemiological setting and could serve as an important data to further explore in a wider scope.

The seropositive wild boars were detected within the southern part of Selangor (Hulu Langat, Kuala Langat, Sepang) where a bovine TB among dairy cattles had been reported from 2010 to 2020. There was a positive correlation between bovine TB cattles and wild boars seropositivity in the Sepang district. This confirmed the report that difference in prevalence values obtained from different parts of the world was due to wild boars exposure to MTBC which was regular with TB outbreaks from cattles locations and also wild boar movement. It was reported that the approximate distance covered by positive wild boar and the nearby TB outbreaks in cattle is only 7.38 km distance which was proportional to daily wild boar movement which ranges from 1 to 16 km [11].

The univariate statistics indicated that there was no significant difference between age and sex (P>0.05), the sample size may have some effect, as we had few subadults and yearling representing age categories, while we had more males and few females in the sex categories, also the overall sample size in this study was small and this had impacted on our statistics. The lack of significant difference between age and sex was in agreement with other studies [15] but other findings seemed to contrast [21], where adult wild boars had a higher significant seroprevalence status of 50% (70.4) when compared to yearling with 2% (11.1) and subadults with 16% (39.0). As for sex categories females had a high seroprevalence status of 22% (52.4) compared to males which had 9% (42.9) [21]. In other studies, it was reported that age categories had significance higher seroprevalence in wild boar yearling of 56%, when compared to adults 30% and sub adults 23%, while males 39% and females 29% with no statistically significant difference observed [18], while statistically significant differences were found between male 9.6% and female 12% [11]. In future studies, it is recommended increase in wild boar sample size and the use of systematic sampling for a better way to compare and less biased by sex, age and region.

Studies had reported TB in elephants using TB STAT-PAK® and DPP® VetTB assays for surveillance and diagnostic purpose in elephants [17]. These tests had proven to have a sensitivity of 100% and a specificity of 95% [10]. In a reported outbreak of TB, a commercially available interferon gamma release assay (IGRA) (PRIMAGAM Prionics AG, ZRH, Switzerland) for NHPs was validated in rhesus and cynomolgus monkeys. The test shows a sensitivity of 68% and specificity of 97% [8]. In this study, we are unable to detect MTBC serologically in macaques and elephants probably due to the absence of mycobacterial antibodies in these animals. Since the confirmation of TB positivity in captive elephants, an improved management and treatment of the elephants have been implemented at the NECC (personal communication). This might have contributed to the absence of TB antibody detection in elephants in this study. Confounding factors such as sample size, host and environmental factors can affect the performance of diagnostic tests. They are age, sex, general health disease severity, stress, climate and season [24].

In conclusion, this study reported the first antibody detection in free-ranged wild boar in selected districts in Selangor while for free-ranged macaques and Asian elephants were not detected. It indicated the exposure to MTBC in wild boars that might have important implications not only for conservation but also for TB control in livestock and public health concern on zoonosis. The preliminary evidence of the status of TB infection in wildlife will promote more epidemiological research in wildlife and improved livestock management in Malaysia. In spite of challenges on sample numbers and availability of sensitive and validated serological tests in wildlife, further investigation is needed to incorporate samples in many areas and use of other potential antibody test kits.

CONFLICTS OF INTEREST. The authors declare that they have no competing interests.

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