Short Communication

Development of an In-house aPPD ELISA for *Mycobacterium avium* Complex (MAC) Antibodies Detection in Zoo Primates

Yusuf Madaki Lekko[1,2], Azlan Che-Amat[1]*, Peck Toung Ooi[1], Sharina Omar[3], Siti Zubaidah Ramanoon[4], Mazlina Mazlan[3] and Faez Firdaus Abdullah Jesse[1]

[1]*Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2]*Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Maiduguri, Maiduguri PMB 1069, Borno State, Nigeria*
[3]*Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[4]*Farm and Exotic Animal Medicine and Surgery, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

In non-human primates (NHPs), *Mycobacterium avium* complex (MAC) species are the major source of non-tuberculous mycobacteriosis, causing tuberculous-like lesions in lymph nodes and parenchymatous organs in zoo and wildlife animals. Poor species-specific detection by serological diagnosis has negatively impacted the surveillance of MAC on non-human primates. Serum was collected from suspected twelve (n = 12) NHPs with no record of health monitoring, including gibbon (n = 5), capuchins (n = 2), siamang (n = 2), mandrill (n = 1), and orangutan (n = 2). An in-house avian purified protein derivative (aPPD) enzyme-linked immunosorbent assays (ELISA) antibody detection was developed and modified based on the established protocols. The aPPD ELISA for MAC antibodies detection at serum and Protein-G dilutions of 1:200-0.5µg/ml, respectively, detected 3/12 (25%) positive serum. At both serum and Protein-G dilutions of 1:100-0.05 and 1:300-1 µg/ml, the aPPD ELISA detected 12/12 (100%), respectively. The antibody was not detected for an in-house aPPD ELISA with serum and anti-monkey immunoglobulin G (IgG) dilutions at 1:100-0.5 and 1:300-1 µg/ml. However, 2/12 (16%)

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was detected using serum and anti-monkey IgG dilutions at 1:200-0.05 µg/ml. An in-house aPPD ELISA procedure for MAC antibodies detection in primates, at serum and Protein-G dilutions of 1:100-0.05 and 1:300-1 µg/ml, both have shown sensitivity and specificity of 100%, positive predictive value and negative predictive value of 100%, respectively. The serum and anti-monkey IgG have shown extremely low sensitivity and specificity. In conclusion, the performance of an in-house aPPD ELISA using three different dilutions on serum and conjugates in detecting MAC in a primate has shown that Protein-G horseradish peroxidase, as secondary conjugates were able to detect MAC antibodies.

Keywords: Antibodies, ELISA, *Mycobacterium avium* complex, non-human primates, Protein-G

INTRODUCTION

Non-human primates (NHPs) are susceptible to non-tuberculous mycobacteriosis caused by *Mycobacterium avium* complex (MAC) organisms that are slow-growing mycobacterium that are found in different sources, such as water, soil, and foodstuffs. Members of MAC are known to cause various diseases, which include similar diseases like tuberculosis in human beings and birds, lymphadenitis in humans and mammals, and Johne's disease in ruminants (Biet et al., 2005). These mycobacteria pose a serious risk to zoos and wildlife sanctuaries (Roller, Hansen, Böhlken-Fascher et al., 2020). It has become an important part of disease prevention and control in zoos and wildlife sanctuaries (Roller, Hansen, Knauf-Witzens et al., 2020).

Different categories of tuberculosis (TB) antibody detection tests have been developed for humoral response in different species, such as lateral flow test and ELISA (Bezos et al., 2014). Antibody ELISA kits against MAC in some species are usually not permitted for use with sera from another animal (Manning, 2011). Some

tests could give different results even from animals of the same species (Pruvot et al., 2013). Species differences exist with respect to serological results because of the specificity of the reagents inside the different ELISA kits sold commercially. The secondary antibodies included in the test kits have different capabilities in binding to IgG from different species (Pruvot et al., 2013). Some research has reported disparities in binding levels to IgG by different conjugates on different wildlife species (Kramsky et al., 2003; Pruvot et al., 2013).

Protein G is derived from the C and G groups of streptococci, an important immunoglobulin (Ig) for bacterial cell wall receptor binding. The Fc part of IgG and IgG subclasses is binding at different degrees. An example is the mouse IgG2, which is strongly attached to protein G, while mouse IgG1 is weakly bound (Kramsky et al., 2003). Protein G has recombinant forms that are used as laboratory reagents. These forms have removed the non-Ig binding part, which is the albumen, reducing its nonspecific

protein binding. The recombinant protein G has purification and Ig detection properties. As such, it is used as a laboratory reagent in various domestic and laboratory species. The use of recombinant protein G has not been well established beyond ruminant species of cattle, sheep, and goats (Kramsky et al., 2003). The development of a protein G ELISA for antibody detection was experimented with for use in different nondomestic artiodactylid species. Protein G exhibits a greater species specificity with little differentiation in isotype-binding cell wall proteins, e.g., protein A. Several research studies have reported a strong binding affinity between protein G and Ig from different species, as well as selective reactivity to IgG. This unique characteristic has led to our choice of protein G as the conjugate or secondary antibody for the use in this species in our study (Kramsky et al., 2000, 2003). The horseradish peroxidase (HRP)-conjugated anti-monkey IgG was produced by immunoelectrophoresis and reacts especially with monkey IgG and light chains common immunoglobulins of other monkeys. No antibody was detected against non-immunoglobulins serum proteins. It can cross-react with IgG from other species (Frost et al., 2014).

Antemortem diagnosis of tuberculosis in wildlife using serology is critical in monitoring the effects of *Mycobacterium tuberculosis* complex (MTBC) and MAC-associated infections for better surveillance and control measures. To our understanding, no ELISA kits exist for MAC detection in NHPs. Thus, an attempt to modify and develop an in-house ELISA protocol for MAC detection in NHPs by implementing a modified protocol as described by Boadella et al. (2011) and Pruvot et al. (2013), which had been evaluated in wild boar and wild ruminants in their study, respectively. Therefore, the study aims to develop an in-house aPPD ELISA protocol for detecting IgG antibodies to MAC in non-human primates.

MATERIALS AND METHODS

Ethical Approval

This research was approved following the review by the Department of Wildlife and National Parks (PERHILITAN, Malaysia) regulations and the university animal ethics committee approval (UPM/AICUC/AUP-U040/2019). Written informed consent was obtained from the zoological centres for the involvement of these animals in this study.

Study Animals

The study was carried out in Zoo Melaka and A'Famosa Safari Wonderland in central-south of Peninsular Malaysia after being requested by the resident veterinarians for clinical service and conducting TB and non-tuberculous mycobacteria screening on their captive primates. Animals were selected based on reduced activity and performance. There was no history of TB screening of the animals on the premises. Blood samples were collected from twelve (n = 12) NHPs, including gibbon (n = 5), capuchins (n = 2), siamang (n = 2), mandrill (n = 1), and orangutan (n = 2).

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Sample Collection and Processing

The zoo personnel managed all the animals. Zoo veterinarians sedated animals before conducting the sampling procedures, and the animals were monitored throughout the procedure until recovery. Blood samples were collected using a 23-gauge syringe and needle, stored in well-labelled plain tubes, and later centrifuged at the clinical studies laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia for 252 x *g* for 15 min within 3 hr of collection. The extracted serum was poured into Citadel Deep-well Plates and Cluster tube (SSIbio, USA), and the sera were stored at -20°C, which was used to determine antibodies. The skin test was conducted as described in the previous study (Lekko et al., 2022).

Development of An In-house ELISA aPPD Using HRP-conjugated Anti-monkey IgG for MAC Detection in Primates

The aPPD ELISA trials with modification were developed based on the established previous protocols (Boadella et al., 2011; Pruvot et al., 2013). Briefly, the 96-well ELISA plate was coated with antigen, aPPD at 5 μg/ml for at least 18 hr at 4ºC or overnight. Then, the wells were washed three times with 300 μl of phosphate-buffered saline (PBS) solution containing 0.05% Tween 20 (PBST, Abcam, United Kingdom) and blocked for 1 hr with 140 μl of 5% skim milk (Sunlac, Malaysia) in PBST at room temperature. After emptying the plate, the NHPs sera were added at 1:100, 1:200, and 1:300 dilutions and incubated at 37ºC for 30 min. IgG antibody detection was performed by adding 100 μl of HRP-conjugated anti-monkey IgG antibodies (Abcam, United Kingdom) at 0.5 μg/ml 1:500 dilutions and incubating the plate at room temperature for 1 hr. The plate was removed and washed three times with PBST, then tetramethylbenzidine (TMB, Promega Corp., USA) at 50 μl 3,3',5,5' was added to the plate and prevented from light for 15 min at room temperature. Fifty (50) μl 2M sulphuric acid (H_2SO_4, Sigma-Aldrich, USA) was used as a stop reaction. The optical densities (OD) were measured by an ELISA plate reader (Tecan Austria GmbH, Austria) at 450 nm.

Development of An In-house ELISA aPPD Using HRP-recombinant Protein G for MAC Detection in Primates

For aPPD ELISA (HRP-recombinant Protein G), the protocol followed as previously described by Boadella et al. (2011) and Che' Amat et al. (2015) with modification. Sera were added to the plate at a dilution rate of 1:100, 1:200, and 1:300 in PBS, and incubation was done for 1 hr at 37ºC, and then washing of plates was performed three times with PBST. Protein G conjugates (Abcam, United Kingdom) were added at a dilution of 0.5 μg/ml (1:10,000) in PBST and incubated for 80 min at 37ºC. The OD was measured using an ELISA plate reader at 450 nm.

Statistical Analyses

The sensitivity (Se) and specificity (Sp) were determined based on the polymerase chain reaction (PCR) positive results from

Table 2

Dilutions, sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV) of different enzyme-linked immunosorbent assays (ELISA) protocols in zoo primates

Test	Serum dilutions-conjugates dilutions (µg/ml)	Se (95% CI)	Sp (95% CI)	PPV (95% CI)	NPV (95% CI)
ELISA protein G HRP	1:200-0.5	25% (8.8-53.2)	57.1% (36.1-75.5)	25% (8.8-53.2)	57.1% (36.5-75.5)
ELISA anti-monkey IgG HRP	1:100-0.5	-	-	-	-
ELISA protein G HRP	1:100-0.05	100% (75.7-100)	100% (75.7-100)	100% (75.7-100)	100% (75.7-100)
ELISA anti-monkey IgG HRP	1:200-0.05	16.6% (29.6-44.8)	54.5% (34.6-73.0)	16.6% (29.6-44.8)	54.5% (34.6-73.0)
ELISA protein G HRP	1:300-1	100% (75.7-100)	100% (75.7-100)	100% (75.7-100)	100% (75.7-100)
ELISA anti-monkey IgG HRP	1:300-1	-	-	-	-

Note. CI = Confidence interval; IgG = Immunoglobulin G; HRP = Horseradish peroxidase

protein G dilutions of 1:200-0.5 µg/ml, showed a very low Se of 25% and Sp of 57.1%, positive predictive value (PPV) of 25% and negative predictive value (NPV) of 57.1%. While at 1:100-0.05 and 1:300-1 µg/ml dilutions, ELISA aPPD has both Se and Sp of 100% and PPV and NPV of 100%. The serum and anti-monkey IgG at 1:200-0.05 µg/ml dilutions, the ELISA aPPD have very low Se (16.6%) and 54.5% Sp, 16.6% PPV, and 54.5% NPV (Table 2).

DISCUSSION

This trial is among the several protocols developed for detecting serum antibodies against MAC in captive zoo primates. The performance of an in-house aPPD ELISA using three different dilutions on serum and conjugates in detecting MAC in primates showed that the protein G HRP, as secondary conjugates, was able to detect MAC antibodies better than anti-monkey IgG HRP conjugates. It showed that secondary antibodies have different capabilities in binding to IgG from different species (Kramsky et al., 2003; Pruvot et al., 2013). The aPPD ELISA at 1:100-0.05 and 1:300-1 µg/ml dilutions showed a good Se (100%) and 100% Sp. Other report using aPPD ELISA in wild ruminants showed 73% Se and 90% Sp; wild boar shows 79.2% Se and 100% Sp reported by Boadella et al. (2011), and 72.6% Se and 96.4% Sp were reported by Aurtenetxe et al. (2008). The OD obtained showed better discrimination of serum and conjugates dilutions at 1:100-0.05 and 1:300-1 µg/ml with protein G HRP. Decrease in OD values of serum and conjugates dilutions at 1:100-0.5 and 1:300-1 µg/ml was observed with anti-monkey IgG HRP conjugate. Aurtenetxe et al. (2008) reported that the serum dilutions above 1:500 yield decreased in OD values. This test's moderate sensitivity and specificity

could be used for comprehensive disease monitoring and surveillance of MAC in the host (Boadella et al., 2011). However, factors such as the cut-off values, sample types, status of infection or species examined and prevalence of cross-reacting environmental mycobacteria could affect the sensitivities of a test (Bezos et al., 2014; Downs et al., 2018).

The choice of secondary antibodies has an important effect on the result of an ELISA test due to species-associated differences in affinity. Species differences exist in the outcomes of serological results due to the specificity of the reagents inside the different ELISA kits. The secondary antibodies included in the test kits have different capabilities in binding to IgG from different species (Kramsky et al., 2003; Pruvot et al., 2013). A few research studies have reported disparity in binding levels to IgG by different conjugates in different wildlife species (Kramsky et al., 2003; Pruvot et al., 2013). The level of unspecific binding of serum immunoglobulins to the reagents of the ELISA kits remains unpredictable. It could affect the results by reducing the signal-to-noise ratio, the ratio of the OD of positive and negative control in each serum sample and conjugate concentration (Pruvot et al., 2013). Modifying these reagents is needed to help determine cut-off values when using an ELISA kit for wildlife animal sample testing.

Cross-reactions between MAC and MTBC could affect the diagnosis of TB, especially when there is lacked diagnostics on wildlife. For this reason, an in-house aPPD ELISA protocol for MAC antibody detection in NHPs was developed and evaluated. MAC infections in NHPs are important in terms of animal welfare, conservation, and disease monitoring. Various outbreaks of MAC infection have been reported from NHPs around the world (Roller, Hansen, Böhlken-Fascher et al., 2020; Roller, Hansen, Knauf-Witzens et al., 2020). Serological diagnosis of MAC infection, such as ELISA, would be useful in evaluating wildlife samples for MAC infection. More so, ELISA kits commercially available for MAC are mostly available for use in livestock ruminants and not available for wildlife species (Pruvot et al., 2013).

Similar studies were performed in wild ruminants following modification of commercial IDEXX ELISA kit for detecting MAP in elk (*Cervus elaphus*), bison (*Bison bison*), and caribou (*Rangifer tarandus*) by using protein G, anti-bovine, and anti-deer conjugates with Se of 73% and Sp of 90% (Kramsky et al., 2000, 2003; Pruvot et al., 2013) and in fallow deer (*Dama dama*)" with 72% Se and 100% Sp (Prieto et al., 2014). MAC antibodies were also detected using a commercial IDEXX kit for detecting MAP in Sumatran orangutan (*Pongo abelii*), bonobo (*Pan paniscus*), and gorillas (*Gorilla gorilla gorilla*) using protein G (Roller, Hansen, Böhlken-Fascher, et al., 2020). Although the conventional plate ELISA test is used, better detection of IgG was demonstrated by the use of protein G HRP as secondary conjugates for the development of in-house ELISA, and this is

similar to studies of Kramsky et al. (2003) and Pruvot et al. (2013) on wild animals. The major limitation of our research is the sample size used, as this was the number allowed to be evaluated by zoo authorities. The results of this study supported our hypothesis; despite the low sensitivity of the in-house ELISA, the use of protein G conjugates at some dilution could enhance the performance of the plate ELISA.

CONCLUSION

The performance of an in-house aPPD ELISA using three different dilutions on serum and conjugates in detecting MAC in primates showed that the protein G HRP, as secondary conjugates, was able to detect MAC antibodies better than anti-monkey IgG HRP conjugates. A developed protocol of an in-house aPPD ELISA for antibody detection against MAC in primates showed a moderate sensitivity on selected serum and conjugated dilutions. Thus, it may provide an option for better sensitivity for TB testing and diagnosis for MAC infection in primates. Therefore, to produce acceptable data results, it is necessary to make changes and modifications in ELISA kits, especially the secondary antibodies or conjugates specific to wildlife species. However, with the difficulty in producing wildlife diagnostics kits, it has been proven that modifications in plate ELISA could be used to improve confidence in test results.

ACKNOWLEDGMENTS

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