UNIVERSITI PUTRA MALAYSIA

PREVALENCE OF INCLUSION BODY DISEASE (IBD) IN WILD CAUGHT RETICULATED PYTHON (Malayopython reticulatus))

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PREVALENCE OF INCLUSION BODY DISEASE (IBD) IN WILD CAUGHT RETICULATED PYTHON *(Malayopython reticulatus)*

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Prevalence of Inclusion Body Disease (IBD) in Wild Reticulated Pythons *(Malayopython reticulatus)*" by Mira Farhana Razlan 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 - Final Year Project.

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"Do not give up and do not be downhearted. You shall be uppermost if you are believers"

(Qur'an, 3:139)

Mira Raz

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Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Ilmiah Tahun Akhir.

PREVALEN UNTUK PENYAKIT INCLUSION BODY DISEASE DALAM ULAR SAWA BATIK *(Malayopython reticulatus)* LIAR

Oleh

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2022

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Inclusion body disease (IBD) ialah penyakit virus yang membawa maut dan senang berjangkit yang dicirikan oleh intrasitoplasma inklusi badan eosinofilik terhadap pelbagai tisu dan sel darah. Penyakit ini terutamanya memberi kesan kepada ular dari keluarga Boidae dan Pythonidae. Sehingga kini, penyebab dan patogenesis masih tidak jelas, namun bukti menunjukkan bahawa arenavirus dalam genus reptarenavirus merupakan punca IBD. Kebanyakan kejadian yang didokumenkan berlaku ke atas ular di dalam kurungan, namun belum ada kajian dijalankan terhadap ular sawa batik liar *(Malayopython reticulatus)* di Malaysia. Kajian

prevalen IBD dalam ular sawa batik liar ini menggunakan diagnosis yang disyorkan iaitu melalui histopatologi dan calitan darah. Persampelan secara kebetulan berjumlah N=50 ular sawa batik yang liar diperoleh daripada kilang pemprosesan ular bagi industri kulit ular sawa. Darah dikumpulkan dalam tiub heparin dan dua calitan darah nipis dilakukan untuk setiap sampel. Tisu-tisu otak, buah pinggang dan hati telah diambil dan diproses untuk histopatologi. Calitan darah diwarnakan dengan Hematoxylin dan eosin (H&E) dan pewarnaan Wright. Tisu histopatologi menggunakan pewarnaan H&E. Intrasitoplasma inklusi badan dicirikan oleh eosinofilik dan bulat dengan saiz berbeza telah dikesan terhadap 17 sampel dengan prevalen keseluruhan 34.0% (17/50), berdasarkan pengesanan sama ada masing-masing oleh histopatologi atau calitan darah (26.0% (13/50) dan 32.0% (16/50). Semua inklusi badan daripada calitan darah ditemui dalam sel darah merah manakala histopatologi tisu buah pinggang adalah paling tinggi (12/13) daripada sebanyak 92%. Tiada hubung kait antara jantina dengan pengesanan IBD. Hipotesis kami untuk mengenal pasti IBD dalam ular sawa liar tanpa tanda klinikal disokong oleh penemuan ini. Prevalen yang tinggi mengesyorkan keperluan untuk kajian epidemiologi selanjutnya dengan menggabungkan lebih banyak teknik diagnostik.

Kata kunci: Ular sawa batik; ular; inclusion body disease; histopatologi; calitan darah

ABSTRACT

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

PREVALENCE OF INCLUSION BODY DISEASE (IBD) IN WILD CAUGHT RETICULATED PYTHON *(Malayopython reticulatus)*

By

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Inclusion body disease (IBD) is a fatal and contagious viral disease characterized by eosinophilic intracytoplasmic inclusion bodies in various tissue and blood cells. This disease mainly affects snakes from the Boidae and Pythonidae family. To date, the aetiology and pathogenesis still remains ambiguous, however evidence points to arenavirus in the genus reptarenavirus as the cause of IBD. Most documented occurrences were captive snakes, however there is no study conducted in the indigenous wild reticulated python (*Malayopython reticulatus*) in Malaysia. The prevalence of IBD in wild reticulated pythons are determined in this study using an established presumptive diagnosis of histopathology and blood smear. A

convenience sampling of N=50 wild caught reticulated pythons were procured from a snake processing plant for python skin industry. Blood was collected in a heparin tube and two thin blood smears were done for each sample. Tissues from the brain, kidney and liver were taken and processed for histopathology. The blood smears were stained with Hematoxylin and eosin (H&E) and Wright's stain. Histopathological tissues were stained with H&E stain. Intracytoplasmic inclusion bodies characterized by variably sized eosinophilic and round bodies were detected in 17 samples with an overall prevalence of 34.0% (17/50) based on inclusion bodies detected either by histopathology or blood smear (26.0% (13/50) and 32.0% (16/50) respectively. All inclusion bodies from the blood smear were found in the RBC while kidney was predominant (12/13) from the histopathological examination (92%). There is no association between sex with the detection of IBD. Our hypothesis for identifying IBD in an asymptomatic wild python was supported by the findings. The high prevalence highlights the need for further epidemiological study by incorporating more diagnostic techniques.

Keywords: Reticulated python; snake; inclusion body disease; histopathology; blood smear

CHAPTER 1

INTRODUCTION

1.1 Background of study

Reticulated python *(Malayopython reticulatus)* are snakes in the Pythonidae family, which is a group of non-venomous snakes. The snakes are known as the world's longest snake and can grow up to 10 meters long (Auliya, 2002). Reticulated pythons are geographically widespread all over South and Southeast Asia (Murray-Dickson *et. al.*, 2017) and are mainly harvested for their skin (Murray-Dickson *et. al.*, 2017). Indonesia and Malaysia are the top exporters of reticulated python skins for the global market with about 300,000 reticulated pythons culled annually (Natusch *et. al.*, 2016). Other products of reticulated pythons include meat, gallbladder, and export of live specimens (Khadiejah et. al., 2021).

Inclusion Body Disease (IBD) is a notorious viral disease that is fatal in snakes of the Boidae and Pythonidae family, mostly captive snakes are affected. The earliest reported case of IBD was in North America back in 1970's. Since then, more cases of IBD in captive snakes have been reported in various countries ranging from Australia, Africa, Europe and even in Malaysia (Chang and Jacobson, 2010). The first occurrence ever in Malaysia was reported in 2009, involving 30 captive snakes that was presented with nervous and respiratory signs at a snake

sanctuary in Ulu Bendul, Negeri Sembilan (Noordin *et. al.,* 2009; Malaysiakini, 2008). Retrovirus was once believed to be the cause of IBD, however, recent studies showed that there is association between reptarenavirus and snakes with IBD (Marschang *et. al.,* 2014).

Current gold standard to diagnose IBD is by the presence of eosinophilic intracytoplasmic inclusion body in either Hematoxylin & eosin stained blood smear or histopathology (Keller *et. al.,* 2017; Hetzel *et. al.,* 2013). Sensitivity and specificity of this staining method for IBD is uncertain (Chang and Jacobson, 2010), however a distinct protein (inclusion body disease protein, IBDP) forms the inclusions that we usually see in order to diagnose snakes infected with IBD (Marschang *et. al.,* 2014). Typically, IBD can spread quickly in a confined snake population, leading to eradication of the whole collection of snakes (Korzyukov, 2020; Chang and Jacobson, 2010). Hence, the need for a more specialized, clinically appropriate diagnostic tool to diagnose IBD (Keilwerth *et. al.,* 2012).

It is currently unclear how IBD gets introduced into captive snakes. Although IBD usually affects captive snakes, there is an increasing concern that this disease will spread to native wild populations in regions where boid snakes are being bred to be released into the wild (Chang and Jacobson, 2010). In Brazil, there have been reports of IBD in wild boa constrictors and a study proposed that wild boas may play a role in reptarenavirus' natural circulation (Alfaro-Alarcón *et. al.,* 2022). To date, there are no reports on the prevalence of IBD in wild snake

populations, hence it is still unclear where this disease emerged from (Chang and Jacobson, 2010). Henceforth, the nature of IBD in wild populations is still not well understood.

To our knowledge, there is no study on IBD in wild snakes in Malaysia, eventually leading to a scarcity of information known on the etiology agent, transmission, prevalence of IBD in wild snakes in Malaysia. In order to gain a better understanding on the epidemiology of IBD in wild snakes, this study was conducted to determine the prevalence of IBD in wild caught reticulated pythons in Malaysia.

1.2 Main objectives:

This study focuses on providing an insightful knowledge on the prevalence of Inclusion Body Disease (IBD) in wild caught reticulated python.

1.3 Specific objectives:

1. To detect IBD using microscopic examination of blood smear (H&E and Wright's stain) and histopathological slides of brain, kidney and liver (H&E stain).
2. To evaluate the correlation between sex with detection of IBD.

1.4 Justification

Inclusion body disease is a fatal and contagious disease known to cause acute or chronic disease in the Boidae and Pythonidae family. To date, there is no study conducted on IBD in wild caught reticulated python in Malaysia.

1.5 Hypothesis

Null hypothesis:

1. There is no prevalence of IBD in wild caught reticulated python.
2. There is no correlation between sex with detection of IBD.

Alternative hypothesis:

1. There is prevalence of IBD in wild caught reticulated python.
2. There is correlation between sex with detection of IBD.

CHAPTER 2

LITERATURE REVIEW

2.1 Reticulated python *(Malayopython reticulatus)*

The reticulated python *(Malayopython reticulatus)* or also known as "ular sawa batik" in Malay, is in the reptile Family Pythonidae, which consists of approximately 35 species of pythons. (Khadiejah *et. al.,* 2021). It is a non-venomous constrictor that can grow up to 8-10 m in length and weigh up to 270 kg. Females are generally larger than males in terms of size and weight (Auliya *et. al.,* 2002). They are nocturnal animals and are solitary, inhabit a wide range of habitats that include tropical rainforests, wetlands, and grassland forests, at elevations of 1200-2500 m (Ambariyanto *et. al.,* 1999). As for their diet, reticulated python prey on a broad range of animals and mammals being the most dominant prey (Shine *et. al.,* 1998). On the IUCN (International Union for Conservation of Nature) Red List assessed in 2011, this species is now categorized as Least Concern (LC), where this species is flourishing and is neither threatened nor near threatened (Stuart *et. al.*, 2018).

The reticulated python is native to Africa, Asia and Oceania (Khadiejah *et. al.,* 2021). The species is widely dispersed throughout mainland and island Southeast Asia (SEA) (Murray-Dickson *et. al.,* 2017). Reticulated pythons are a significant economic natural resource in SEA, and are mostly taken from the wild for their skins (Murray-Dickson *et. al.,* 2017). Besides being traded for their skins, pythons have also been traded domestically by the local people for food and medicine (Khadiejah *et. al.,* 2021). Reticulated pythons are heavily exploited by the leather industry and are regulated under Appendix II of the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Auliya *et. al.,* 2002). Malaysia is one among the main exporters of these skins, supplying to Europe and Asian markets since the 1970s (Nossal *et. al.,* 2016). Annually, over 300,000 reticulated pythons are culled in the wild in both Malaysia and Indonesia in order to supply skins for the global trade in exotic leathers (Natusch *et. al.,* 2016). In Malaysia, the number of skins exported have consistently been around 120,000 to 150,000 units per year ever since the year 2000 (Khadiejah *et. al.,* 2021).

2.2 Inclusion Body Disease (IBD) in Boid Snakes

2.2.1 Overview of Inclusion Body Disease (IBD)

Inclusion Body Disease (IBD) is one of the most infamous viral diseases that are seen worldwide in snakes that are members of the Boidae and Pythonidae families. The first case of IBD in Australia was only discovered as early as 1998 and involved a carpet python (*Morelia spilota)* as well as a diamond python *(Morelia spilota*

spilota) (Bush, 2000). A few other cases of IBD were also documented in 1998 in captive boa constrictors in the Canary Islands (Orós *et. al.,* 1998), Spain, and eventually in Belgium (Chang and Jacobson, 2010).

Between 1998 and 1999, there were also cases that were documented in captive palm vipers (*Bothriechis lateralis)* regarding a disease that was said to be similar to IBD in boid snakes (Raymond *et. al*, 2001). Like the name itself, IBD is defined by the development of eosinophilic or amphophilic intracytoplasmic inclusion bodies in cells in various organs that include neurons and glial cells from the central nervous system (CNS), epithelial cells from visceral organs and peripheral blood cells. Captive snakes are usually affected and in Australia, it is known that this disease mainly affects captive pythons. However, there is still a risk of local wild populations contracting this disease in other countries, since boid snakes are being bred to be released into the wild.

The causative agent of IBD still remains a debate. Different viruses have been isolated from snakes with IBD, including retrovirus, paramyxovirus and recently, reptarenavirus (Chang and Jacobson, 2010). Snakes affected with IBD show various clinical signs, primarily central nervous system syndromes such as opisthotonus, head tremor, anisocoria. Intermittent regurgitation, stomatitis and anorexia can also be seen in snakes with IBD (Hetzel *et. al.,* 2013).

Although the primary route of transmission of IBD still remains unknown, certain studies have shown that the bloodsucking snake mite (*Ophionyssus natricis)*

plays a role as a vector in the transmission of IBD (Oliveira *et. al.,* 2015). A study has proven that vertical transmission of the virus is also possible (Keller *et. al.,* 2017). Not just that, direct contact could also be a route of transmission for IBD (Chang and Jacobson, 2010). It has been studied that some snakes can develop subclinical infection as well as latent infection, especially in boas. However, there is no exact number as to how many percent of snakes with IBD show no clinical signs (Chang and Jacobson, 2010). This is a concern as these snakes which seem clinically healthy are being sold throughout the world (University of Florida, 2014).

At present, there are few diagnostic methods that can be used to make a tentative diagnosis of IBD. The most widely used diagnostic method is the identification by light microscopy of eosinophilic or amphophilic intracytoplasmic inclusion bodies in either peripheral blood cells or tissues (Chang and Jacobson, 2010). Studies have shown that a hematoxylin-eosin (H&E) stained blood smear is a quick and non-invasive method that is reliable enough to diagnose IBD (Keilwerth *et. al.,* 2012). Other advanced methods that can be used to diagnose IBD include viral isolation, molecular detection by the polymerase chain reaction (PCR), viewing the virus under a transmission electron microscope, western blot analysis and immunohistochemical (IHC) staining (Chang and Jacobson, 2010).

IBD in snakes is usually fatal, hence there is no definite therapy to treat snakes suffering from this disease. Euthanasia is usually recommended once the snakes are diagnosed with IBD since IBD is contagious (Carlisle-Nowak *et. al.,* 1998). It is rare to see only one snake in an entire collection infected with IBD (Chang and Jacobson, 2010). Once IBD is established in a confined snake

population, it is advised to euthanize all the snakes as IBD can spread rapidly within a population (Korzyukov *et. al.*, 2020). This justifies why efficient and extensive preventative measures are needed. It is imperative to detect inclusions in susceptible organs together with viral detection in quarantine stations to prevent spread of IBD from newly introduced snakes (Aqrawi *et. al.*, 2015).

2.2.2 Causative agent of IBD

In previous literature, retrovirus was taught to be the causative agent of IBD. It was not until 2012, where studies have shown that there is an association between snakes infected with IBD and reptarenavirus (Stenglein *et. al.,* 2017; Marschang, 2014). It is now believed that the causative agent of IBD is reptarenavirus. Reptarenavirus is a genus in the family Arenaviridae and is a negative sense RNA virus. A feature of reptarenavirus that can be seen under an electron microscope is sand-like particles (Abba *et. al.,* 2018). These reptarenaviruses are thought to be a sister group to mammalian arenaviruses, according to recent phylogenetic studies. It has been acclaimed that a way to differentiate reptarenavirus and mammalian arenavirus is that captive snakes are usually co-infected with various viruses. As of now, there has been no study done on reptarenavirus in wild snakes (Stenglein *et. al.*, 2017).

It was recently discovered that reptarenavirus have an ability to reassort and resemble, resulting in new and distinct genotypes (Abba *et. al.,* 2018). Although numerous researches have agreed that there is association between reptarenavirus infection and snakes infected with IBD, other studies have concluded that inclusion

bodies may not always be present in the blood of reptarenavirus-infected pythons, hence a python that may be infected with reptarenavirus might not have inclusion bodies in susceptible cells (Simard *et. al.*, 2020; Hyndman *et. al.*, 2019). Another known fact about reptarenavirus is that this virus may cause chronic infection in snakes (Hepojoki *et. al.*, 2015). Some researchers believe that there could be another virus acting synergistically with reptarenavirus in order to cause clinical infection in snakes (University of Florida, 2014).

2.2.3 Transmission of IBD

The primary route of transmission of IBD still remains ambiguous. There have been speculations that IBD is transmitted in various modes, ranging from horizontal, vertical and mechanical transmission (O'Rourke *et. al.*, 2015). Studies have unanimously agreed that mechanical transmission of IBD via snake mite (*Ophionyssus natricis)* is possible (Hetzel *et. al.*, 2013; Chang and Jacobson, 2010). Since this disease is highly contagious and can become established easily in a confined snake collection, it has been concurred that horizontal transmission (direct contact) is feasible (O'Rourke *et. al.*, 2015). Recent studies have also acclaimed that vertical transmission was demonstrated in pythons co-infected with reptarenavirus (Hetzel *et. al.*, 2017).

2.2.4 Clinical signs

Clinical signs of IBD are non-specific and variable (Marschang, 2014; Keilwerth *et. al.*, 2012). IBD-related clinical symptoms can vary from subclinical carriers to severe neurologic illness and even death (Marschang, 2014). Manifestations of

neurologic symptoms include torticollis, chronic regurgitation, disequilibrium, stargazing and inability to right itself when placed on dorsal recumbency (Chang and Jacobson, 2010). Additionally, snakes with IBD may exhibit anorexia, stomatitis and even pneumonia (Marschang *et. al.,* 2019). There have also been reports of lymphoproliferative disorders and round cell tumors in IBD-infected snakes (Marschang, 2014). Due to the non-specific clinical signs, a precise, clinically appropriate diagnostic technique is required to diagnose IBD (Keilwerth *et. al,* 2012).

2.2.5 Diagnostic tools

Currently, the gold standard for diagnosing IBD is by demonstrating the presence of characteristic eosinophilic, intracytoplasmic inclusion bodies in susceptible organs via cytological or histopathological examination (Keller *et. al.,* 2017; Hetzel *et. al.,* 2013). However, the specificity and sensitivity of this technique is still vague (Chang and Jacobson, 2010). Researchers have prompted a need for a more rapid and reliable diagnostic tool, as inclusion bodies may not be readily circulating and early stages of inclusion bodies may be overlooked (Chang *et. al.,* 2013; Keilwerth *et. al.,* 2012).

Current studies have claimed that inclusion bodies contain an antigenically distinct 68 KDa protein known as IBD protein (IBDP) (Marschang, 2014; Chang *et. al.,* 2013). With that, monoclonal antibodies (MAB) against IBDP were developed which can recognize IBDP band in western blots as well as the IBDP antigen in immunohistochemical (IHC) staining (Chang and Jacobson, 2010).

Researchers are leaning towards diagnosing IBD using immunohistochemical detection of IBDP. A study revealed that the anti-IBDP MAB was 100% specific and had an 83% sensitivity for identifying IBD (Chang *et. al*., 2013) and concluded this tested antibody can be used in the development of ante-mortem IBD immunodiagnostic testing for IBD.

Subsequently, molecular detection of the viral antigen can be performed to diagnose IBD such as RT-PCR and electron microscopy (Argenta *et. al.*, 2020; Chang and Jacobson, 2010). One of the special features of reptarenavirus that can be seen under electron microscope is sand-like particles and has a size about 50 to 300 nm (Abba *et. al.*, 2018).

2.2.6 Prevention

Inclusion body disease can spread quickly in a snake population, hence the need for an efficient and extensive prevention program (Korzyukov, 2020; Chang and Jacobson, 2010). We must be aware that the aim is to reduce the risk of IBD (Chang and Jacobson, 2010). Firstly, it is imperative to quarantine new snakes to prevent spread of the disease from newly-introduced snakes (Chang and Jacobson, 2010; Carlisle-Nowak *et. al.*, 1998). It is highly suggested that in zoos, the minimum quarantine period should be 90 days, however for boas the minimum quarantine period should be 6 months (Chand and Jacobson, 2010).

Furthermore, it has been concurred that many snakes infected with IBD may appear clinically healthy (Marschang, 2019; Chang *et. al.*, 2016). Thus the need for detection of viral antigen or inclusion bodies during the quarantine period (Aqrawi

et. al., 2015). Since it has been suggested that snake mites may be a vector for IBD, it is crucial that snakes must be free from mites to reduce the risk of IBD (Chang and Jacobson, 2010).

2.2.7 Prevalence of IBD in snakes

To date, the study done on prevalence of IBD is mostly on captive snakes. There are currently no reports on the prevalence of IBD in wild snakes (Chang and Jacobson, 2010). Based on former studies conducted, the prevalence of IBD in captive snakes in Belgium and US were 16.5% (48/292) and 19% (25/131) respectively (Simard *et. al.*, 2020; Chang *et. al.*, 2016). In Southeast Asia, there is scarcity on the reports of IBD in captive or wild snakes. The first study done on IBD in captive snakes in Malaysia was back in 2018 where a group of researchers revealed that 37.5% (15/40) samples of captive snakes were positive for IBD (Abba *et. al.*, 2016).

CHAPTER 3

MATERIALS AND METHOD

3.1 Animal and sample collection

Fifty carcasses (N=50) of wild-caught reticulated pythons were included in this cross-sectional study using a convenience sampling technique at a snake processing farm located in Chaah, Segamat, Johor, Malaysia. The snakes were originally caught from the oil palm plantation in Perak, Malaysia. Out of 50 snakes, 24 snakes were female and 26 snakes were male. A general examination was done on each reticulated python prior to sampling and recorded on a form. The study was authorized under University Putra Malaysia Animal Care and Use Committee Protocol U043/2022 and was approved by PERHILITAN JPHLTN.600/1/4 JLD2 (92).

Firstly, the body weight of each snake was taken using an electronic weighing scale, body condition score and sex were recorded. Then the snakes were euthanized humanely by the processing plant operator using a pneumatic stun gun according to the pressure specific (100+-10psi). Next, the gun was applied in the proper zone of the brain for reptiles. After hitting on the head of reptiles, the eyes were checked to confirm the animal is unconscious. Then, a cleaver is used to decapitate the animals.

Immediately following euthanasia, the snake was examined for the presence of ticks and mites. Blood was collected by draining out the jugular vein and into a heparin tube. Fresh two thin blood smears were done for each snake and let dry at room temperature. Then the blood smear is fixed by absolute methanol for 5 minutes. Morphometrics measurement included the head length, snout to vent length and vent to tail length. After the deskinning process, all liver, kidney and brain tissue samples were collected and placed in 10% formalin and cryotubes for histopathological analysis and archive for further study, respectively. The blood tubes and cryotubes were stored in an ice box and were transported back to UPM, Serdang.

3.2 Sample processing

3.2.1 Preparation of blood smear

The thin blood smears were stained with H&E and Wright's stains. Briefly, the slides were immersed into Harrishematoxline for 5 minutes. Then, distilled water is used to wash the Harrishematoxline, followed by immersion into an eosin stain for 10 seconds. Finally, distilled water is used to wash the eosin solution. After the staining process, the coverslip was mounted to the glass slide using Distyrene Plasticizer Xylene (DPX). The slides could now be examined using a light microscope.

For Wright's stain protocol, the slides should then be stain-flooded for two minutes with Wright's stain while being placed on a staining rack. Be sure to count the number of drops of Wright's stain used. Without pouring the stain off, buffered water is added onto the slides (use the same amounts of drops as Wright's stain). Gently blow on the slides to mix the buffered water and Wright's stain and let sit for 6-8 minutes. The slides were run under running tap water for not more than 20 seconds. Following the staining procedure, the coverslip was mounted to the glass slide using DPX, was let dry and ready to be viewed under a light microscope.

3.2.2 Preparation of histopathological slides

Tissue samples of liver, kidney and brain tissue samples were fixed in 10% formalin for 24-48 hours to prevent autolysis and maintain the tissue integrity. Next, the tissues were trimmed thin enough to be able to fit into the tissue embedding cassettes. All 3 samples (liver, kidney and brain) were pooled into one cassette.

Processing of liver, kidney and brain were performed using an automated tissue processor Leica® TP1020, Leica Biosystems, Weltzar, Germany). There were 3 main steps in tissue processing; dehydration, clearing and infiltration. The tissue samples were firstly dehydrated through a series of increasing ethanol solutions until it reaches 100%. This process is done to remove the water from the tissue samples. Next, the alcohol is then cleared with several baths of xylene. For the step of tissue processing, the tissue samples were infiltrated with paraffin wax at 71°C using a Leica® EG1160 paraffin-embedded station.

After the processing stage, the tissues are then embedded in molten paraffin. Metal block molds were warmed in the warm storage area of Tissues Embedding Station (TES). Next, the paraffin infiltrated tissues were removed from the cassette. The tissue samples (liver, kidney, and brain) should be placed on top of a mould that has been filled with a small amount of hot paraffin wax from the TES reservoir. The tissues were then oriented and gently pressed at the bottom of the mold using heated forceps. Place the labeled empty tissue cassette on top of the mold and hot paraffin wax is poured on as to cover the cassette. The mold is removed from the hot plate and placed onto the cold plate (-15°C) of the tissue cooling station. Once the wax hardened, the paraffin block is removed from the mold.

The next step was tissue sectioning. Using a Leica® RM2235 microtome, the tissue blocks were carefully trimmed at the thickness 15 μm to expose the tissues and then followed by tissue sectioning with the thickness of 5 μm. Prior to staining, the tissue sections were let out to dry for about 12 hours.

For the staining stage, the samples were stained using H&E staining. First, the slides were immersed in xylene solutions for 5 minutes to remove the paraffin wax. Next, the slides were immersed in 100% ethanol for 5 minutes. Then, the slides were immersed in 70% ethanol for 5 minutes to remove the xylene. After that the slides were rinsed under running tap water for 5 minutes to remove the alcohol. Next, the slides were immersed into a hematoxylin for 5 minutes. Furthermore, the slides were rinsed under running tap water until the water ran clear. The slides are dipped in 1% acid alcohol in 3 seconds and rinsed under running tap water again.

Then, the slides were immersed in eosin stain for 1 minute. The slides were sprayed with 95% alcohol. The slides were then rinsed again under running tap water until the water ran clear. Following that, the slides were sprayed with 95% alcohol and cleaned. The slides were then let out to dry.

Lastly, the coverslip was mounted onto the glass slide using DPX. The slides were then ready to be viewed under a light microscope.

3.3 Classification of IBD positive (IBD +ve) snakes

All the slides were viewed under a light microscope under 10x, 40x and 100x magnification. For the samples to be considered positive for IBD, there must be presence of characteristic eosinophilic, intracytoplasmic inclusion bodies in either blood smear and/or histopathology. Smears were categorized as IBD +ve if there is presence of characteristic inclusion bodies (IB) in either H&E and/or Wright's stained blood smear. Histopathology was categorized as IBD +ve if there is presence of characteristic IB in either liver and/or kidney and/or brain. The samples that are positive are identified and recorded.

3.4 Statistical analysis

A descriptive analysis was carried out to calculate the prevalence of IBD in wild-caught reticulated pythons. The percentage of prevalence is calculated based on the number of positive cases of IBD with the total number of samples. To evaluate the significance of the association between presence of ticks and IBD positive snakes,

Fisher's exact test was carried out. Differences at $P \leq 0.05$ were considered statistically significant. Moreover, the agreements between IB detection via H&E stained blood smear and histopathology as well as IB detection via Wright's stained blood smear and histopathology were assessed by calculating Cohen's Kappa agreement. Kappa values 0, <0.4, 0.4 - 0.75, >0.75, and 1 were considered as no agreement, poor agreement, good agreement, very good agreement, and perfect agreement, respectively. All statistical data analysis was performed using commercially available software (GraphPad Software by Dotmatics 2022 and IBM SPSS statistics software).

CHAPTER 4

RESULTS

4.1 Prevalence of inclusion body disease

The prevalence of IBD based on H&E stained blood smear and Wright's stained blood smear is (16/50) 32% and (13/50) 26% respectively. All inclusion bodies from the blood smear were found in the RBC. The prevalence of IBD based on histopathology is (13/50) 26%. Inclusion bodies were predominantly found in the kidney (12/13) and the other was found in the liver (1/13). Overall, 17/50 snakes were positive for IBD. It can be concluded that the prevalence of inclusion body disease in wild-caught reticulated pythons is 34%.

Table 4.1: Prevalence of IBD in wild reticulated pythons based on blood smear and histopathology examinations.

	Blood smear		**Histopathology**		
	H&E	Wright's	Brain	Liver	Kidney
Positive	16 (32%)	13 (26%)	0 (0%)	1 (2%)	12 (24%)
Negative	34	37	50	49	38
Total	50	50	50	50	50

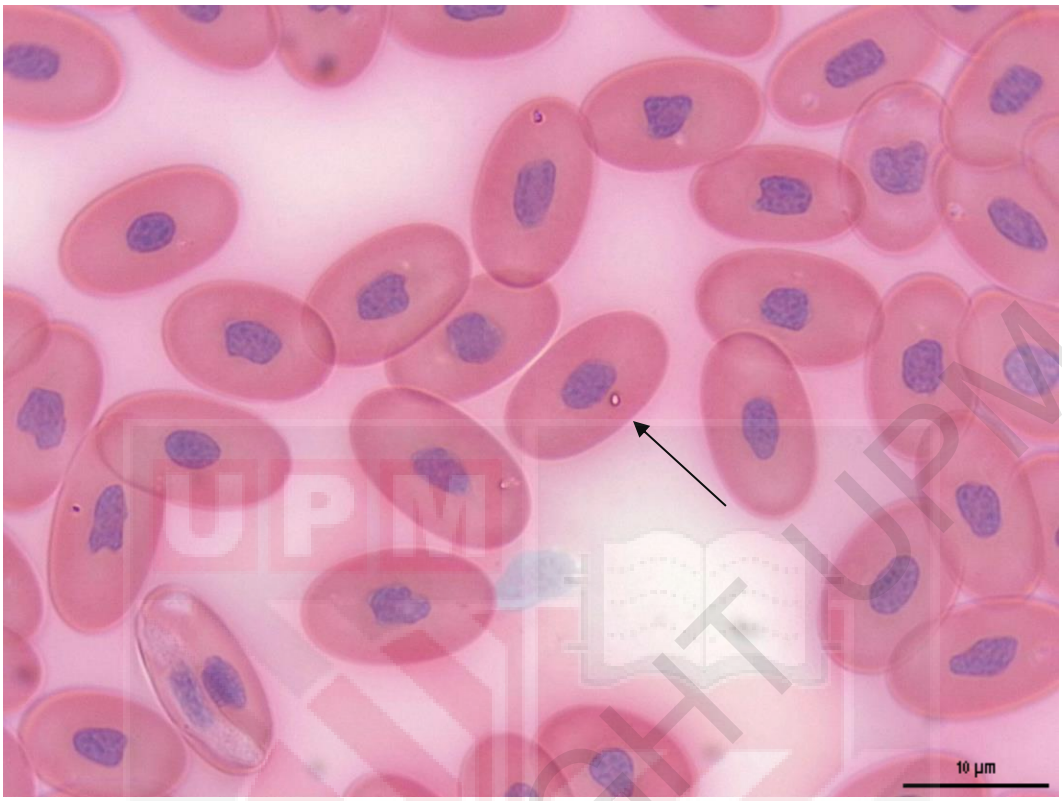

Figure 4.1 Blood smear: Arrow shows presence of eosinophilic, intracytoplasmic inclusion body in an erythrocyte (H&E stain, 100x)

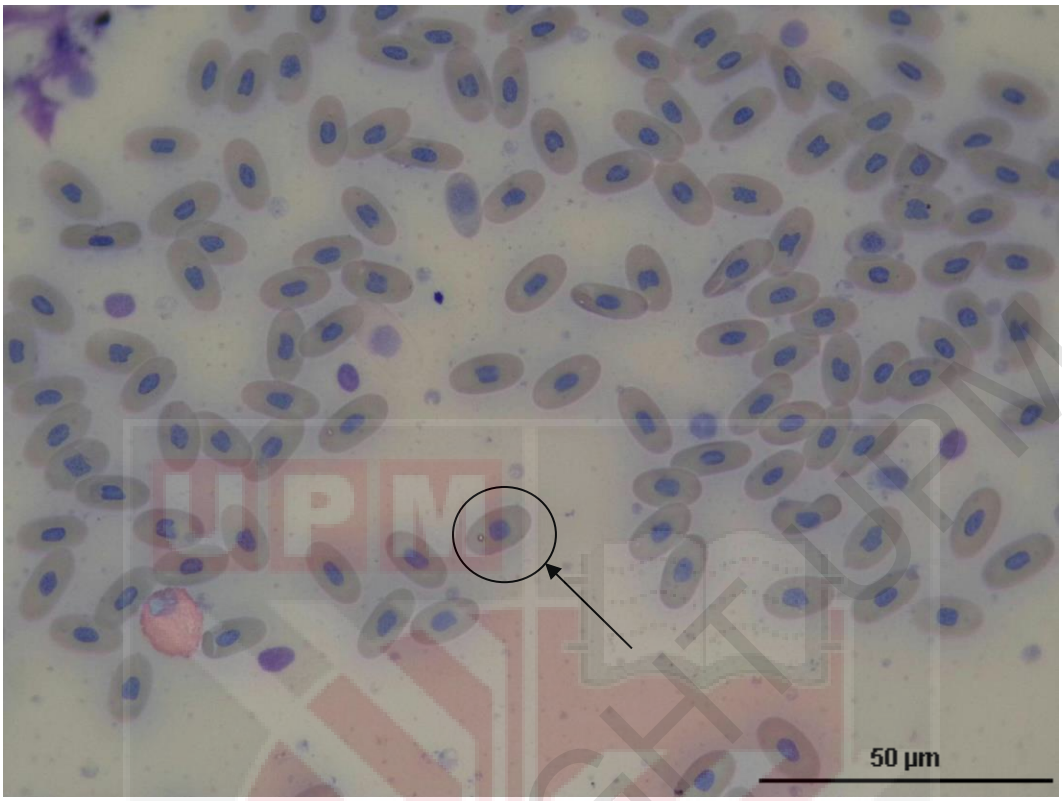

Figure 4.2 Blood smear: Arrow and circle shows presence of eosinophilic, intracytoplasmic inclusion body in an erythrocyte (Wright's stain, 40x)

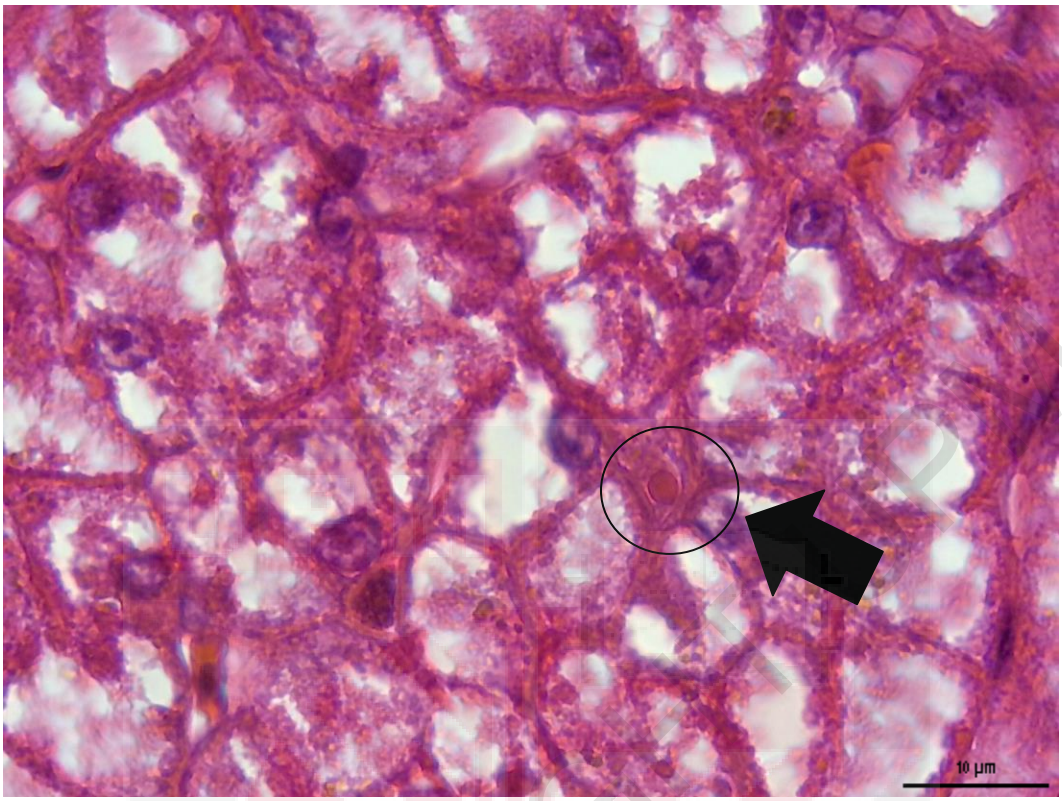

Figure 4.3 Histopathology: Arrow and circle shows presence of eosinophilic, intracytoplasmic inclusion body in hepatocyte (H&E stain, 100x)

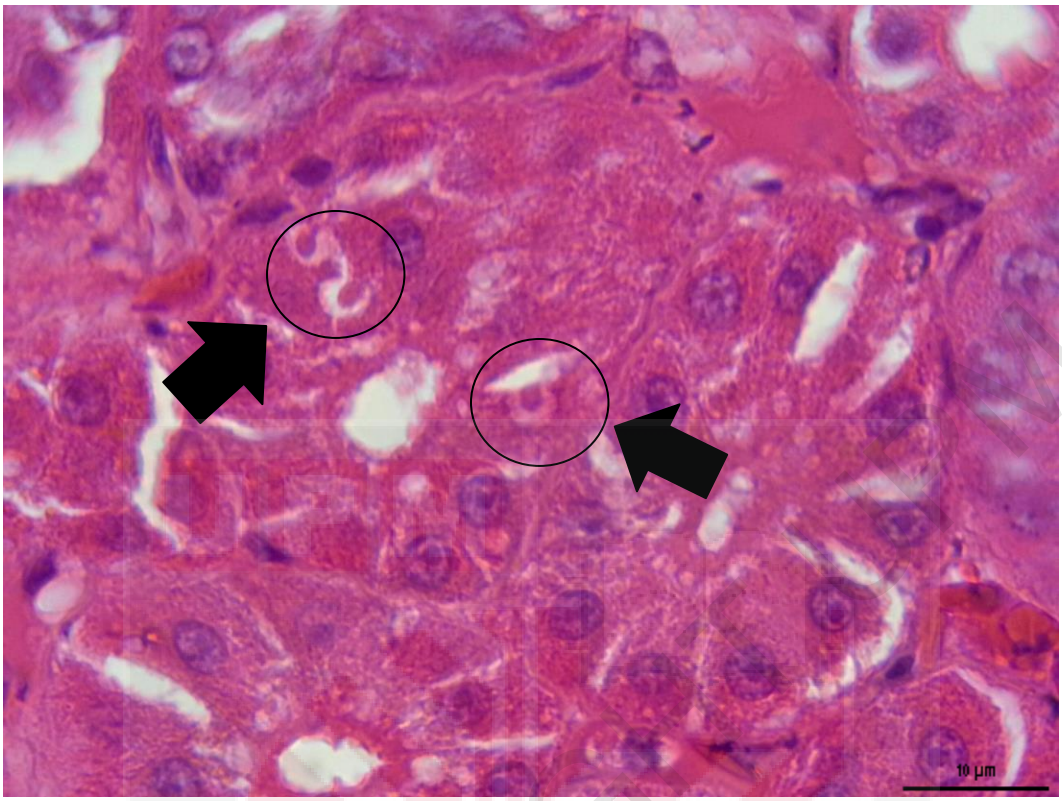

Figure 4.4 Histopathology: Arrows and circles show presence of multiple eosinophilic, intracytoplasmic in renal tubules (H&E, 100x)

4.2 Correlation between sex with detection of IBD

Based on the Fisher's exact test done to evaluate the significance of correlation between sex and IBD, the P=0.1359 which means it is not statistically significant. Hence, there is no correlation between sex with detection of IBD.

Table 4.2: Correlation between sex with detection of IBD

	Positive	Negative	Total
Male	6	20	26
Female	11	13	24
Total	17	33	50

4.3 Agreement between blood smear and histopathology

Based on Cohen's Kappa Agreement, there is substantial agreement between H&E stained blood smear and histopathology for detection of IBD (Kappa agreement = 0.758, $P<0.05$; Table 4.3.1). There is moderate agreement between Wright's stained blood smear and histopathology for detection of IBD (Kappa agreement = 0.584, $P<0.05$; Table 4.3.2).

Table 4.3.1: The agreement between Hematoxylin & Eosin stained blood smear and histopathology for detection of IBD in reticulated pythons

	Histopath+	**Histopath-**	**Total**
H&E blood smear+	12	4	**16**
H&E blood smear-	1	33	**34**
Total	**13**	**37**	**50**

Observed agreement: (12+33)/50 = 0.9

Kappa agreement = 0.758, P<0.05

Table 4.3.2 The agreement between Wright's stained blood smear and histopathology for detection of IBD in reticulated pythons

	Histopath+	**Histopath-**	**Total**
Wright's stained blood smear+	9	4	**13**
Wright's stained blood smear-	4	33	**37**
Total	**13**	**37**	**50**

Observed agreement: (9+33)/50 = 0.84

Kappa agreement = 0.584, P<0.05

CHAPTER 5

DISCUSSION

The overall prevalence of IBD in wild-caught reticulated python in the present study is 34%. According to studies done previously, the prevalence of IBD in captive snakes in Belgium and the US were (16.5%) and (19%) respectively (Simard *et. al.,* 2020; Chang *et. al.,* 2016). We can conclude that the prevalence of IBD in wild snakes is higher than that in captive snakes. The prevalence could be lower in captive snakes because smaller or early stages of inclusion bodies may be missed in H&E stained sections and inclusion bodies may not be plentiful in visceral organs (Chang *et. al.,* 2013).

Furthermore, in this study, the pythons appear to be clinically healthy although positive for IBD. This is comparable to former studies that were done where snakes appearing clinically healthy can be positive for IBD (Marschang, 2019; Chang *et. al.,* 2016). It is widely known that boa constrictors primarily can have persistent subclinical infection (Hetzel *et. al.,* 2021; Simard *et. al.,* 2020). However, based on previous literature, pythons infected with IBD, usually a rapid progression of nervous symptoms can be seen (Ossiboff, 2018). Some nervous symptoms include torticollis, disequilibrium, stargazing and inability to to right

itself when placed in a dorsal recumbency (Chang and Jacobson, 2010). It is possible that maybe wild pythons infected with IBD can have subclinical infection and may develop clinical signs in the future.

Nonetheless, the progression of this disease in wild snakes must be further studied (Simard *et. al,* 2020). A previous study has reported that pythons with a rapid progression of nervous symptoms would have inclusions that are often restricted to the central nervous system (Ossiboff, 2018). Pythons showing central nervous system signs when examined histologically, a non-suppurative meningoencephalitis is frequently found (Schumacher, 2006). This could justify why in this study, no inclusions were found in the brain since the snakes did not manifest any CNS signs. Next, most inclusion bodies are found predominantly in the kidneys and this is supported by a former study done, where inclusion bodies are usually seen in kidneys compared to the liver (Abba *et. al.,* 2018).

Blood smear examination yields more positive samples (16/50) compared to histopathology examination (13/50). This could be justified by a previous study that concluded snakes that appear clinically healthy may have viraemia (Keller *et. al,* 2017). This suggests that the possible pathogenesis for IBD could start from having viraemia before the virus spreads to the visceral organs.

Blood smear and histopathology was used as a diagnostic tool in this study. To date, the current gold standard for the diagnosis of IBD is by the identification

of the IB in cells in cytological and/or histological tissues (Keller *et. al.,* 2017; Hetzel *et. al.,* 2013). Hematoxylin and eosin staining was chosen to stain both blood smear and histopathology. It is acclaimed that inclusion bodies are easier to be detected in H&E stained preparations (Chang *et. al.,* 2010). According to a study, it was concluded that blood smear can accurately identify IBD in boas. It was also noted that IBD can be swiftly and non-invasively detected using blood smears (Keilwerth *et. al.,* 2012). To add on, based on Cohen's Kappa agreement, there is substantial and moderate agreement between blood smear and histopathology. This can be deduced that blood smear and histopathology is a reliable tool to diagnose IBD.

CHAPTER 6

CONCLUSION

The overall prevalence of inclusion body disease in wild caught reticulated pythons in this study is 34%, which is higher than that of captive snakes. The prevalence of IBD based on H&E stained blood smear and Wright's stained blood smear is 32% and 26% respectively. It can be presumed that we could detect more inclusion bodies in blood smear as compared to histopathology. It is possible that these snakes might have viraemia and this finding could contribute to a better understanding of the possible pathogenesis of IBD in wild snakes. There is no correlation between sex with the detection of IBD, which we can deduce that sex is not a risk factor for IBD. Last but not least, both blood smear and histopathology is a reliable diagnostic tool for IBD, as supported by the results of Cohen's Kappa agreement.

CHAPTER 7

RECOMMENDATIONS

Cross-sectional study design was chosen in this study due to time constraint. For future studies, it is recommended to proceed with prospective study. By doing prospective study, this could provide a more comprehensive insight on the progression of IBD in wild snakes, hence contributing to an enhanced understanding of the epidemiology and pathogenesis of this disease. In this study, the sampling location was limited to only one location which was in Perak, Malaysia. The prevalence calculated in this study does not represent the overall prevalence of IBD in Malaysia as a whole. Molecular detection is definitely recommended for future studies. Molecular detection of the agent could help identify the causative agent of IBD. This could further strengthen the findings of this study. Lastly, in this study, only one section of each organ was examined. It is suggested to examine different sections for each organ as to not overlook the presence of IB in that particular organ.

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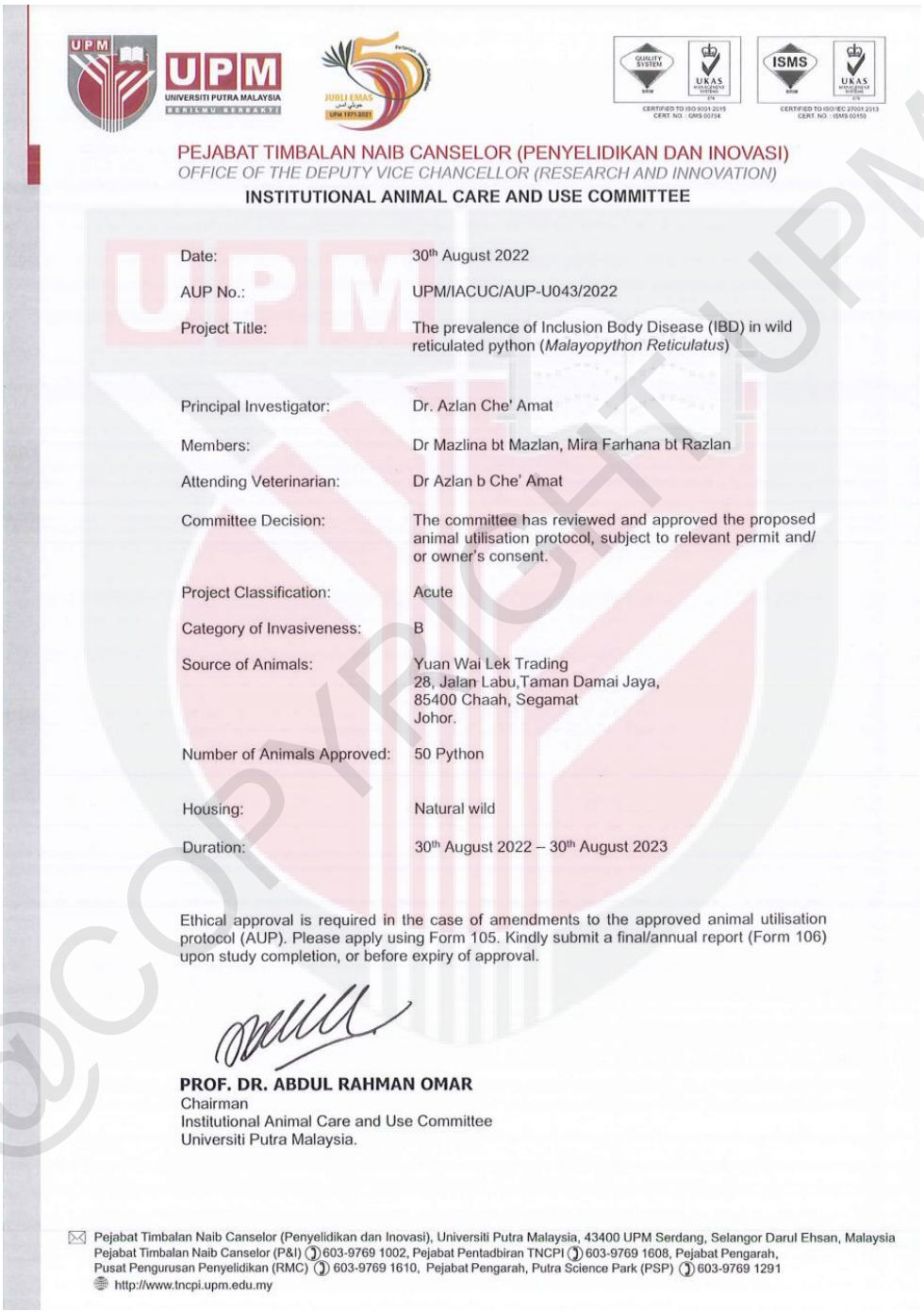
APPENDICES

APPENDIX A

Approval letter from IACUC UPM for final year project proposal – U043/2022



PEJABAT TIMBALAN NAIB CANSELOR (PENYELIDIKAN DAN INOVASI)
OFFICE OF THE DEPUTY VICE CHANCELLOR (RESEARCH AND INNOVATION)
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Date:	30th August 2022
AUP No.:	UPM/IACUC/AUP-U043/2022
Project Title:	The prevalence of Inclusion Body Disease (IBD) in wild reticulated python (*Malayopython Reticulatus*)
Principal Investigator:	Dr. Azlan Che' Amat
Members:	Dr Mazlina bt Mazlan, Mira Farhana bt Razlan
Attending Veterinarian:	Dr Azlan b Che' Amat
Committee Decision:	The committee has reviewed and approved the proposed animal utilisation protocol, subject to relevant permit and/ or owner's consent.
Project Classification:	Acute
Category of Invasiveness:	B
Source of Animals:	Yuan Wai Lek Trading 28, Jalan Labu,Taman Damai Jaya, 85400 Chaah, Segamat Johor.
Number of Animals Approved:	50 Python
Housing:	Natural wild
Duration:	30th August 2022 – 30th August 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. DR. ABDUL RAHMAN OMAR
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 B

Approval letter from PERHILITAN for final year project proposal - JPHLTN.600/1/4 JLD2 (92).

IBU PEJABAT
JABATAN PERLINDUNGAN HIDUPAN LIAR DAN
TAMAN NEGARA (PERHILITAN) SEMENANJUNG MALAYSIA
HEADQUARTERS
DEPARTMENT OF WILDLIFE AND NATIONAL PARKS (DWNP)
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Laman Web : www.wildlife.gov.my

Ruj. Kami: JPHLTN.600-6/1/4 JLD2 (92)
Tarikh: 29 September 2022

Dr. Azlan Bin Che' Amat
Fakulti Perubatan Veterinar
Universiti Putra Malaysia
43400 UPM Serdang
SELANGOR

YBrs Dr,

KEPUTUSAN PERMOHONAN MENJALANKAN PENYELIDIKAN

Dengan hormatnya saya diarah merujuk kepada keputusan Mesyuarat Jawatankuasa Penyelidikan Jabatan PERHILITAN Bil.10/2022 adalah berkaitan.

2. Sukacita dimaklumkan bahawa Jabatan **meluluskan** permohonan YBrs. Dr untuk menjalankan penyelidikan seperti butiran di bawah:

Nama Pemohon	:	**Dr. Azlan b. Che' Amat**
Rakan Saing	:	**Mira Farhana bt. Razlan (Pelajar Tahun Akhir)**
Institusi Pemohon	:	**Universiti Putra Malaysia (UPM)**
Tajuk	:	***The Prevalence of Inclusion Body Disease (IBD) in Wild Reticulated Python***
Lokasi	:	**Yuan Wai Lek Trading, Chaah Segamat Johor (penyelidik telah mendapat persetujuan daripada syarikat untuk menjalankan persampelan)**
Tempoh kajian	:	**September 2022 - Oktober 2022**
Spesies hidupan liar	:	**Ular sawa batik *(Python reticulatus)* diperolehi melalui syarikat Yuan Wail Lek Trading**

3. Sehubungan itu, YBrs Dr dipohon untuk melakukan beberapa perkara seperti berikut :

a) Kebenaran masuk ke kawasan kajian hendaklah diperoleh daripada pengurusan kawasan berkenaan;

b) Rakan Saing dan *Co-author* penyelidikan ini ialah Encik Mohd Lutfi bin Abdullah, Bahagian Konservasi Ex-Situ;

c) Sebarang sampel tidak boleh dibawa ke luar negara;

d) Berkongsi hasil penyelidikan seperti laporan, penerbitan kertas saintifik, tesis dan data melalui rakan saing Jabatan;

e) Menyumbang penulisan kepada"*Journal of Wildlife and Parks*" (JWP) Jabatan PERHILITAN;

f) Mengemukakan satu laporan hasil penyelidikan yang lengkap kepada Jabatan dalam tempoh dua (2) bulan selepas tamat penyelidikan;dan

g) Penyelidikan hendaklah diselesaikan dalam tempoh yang dinyatakan dalam permit.

4. Sebarang pertanyaan mengenai perkara ini, YBrs Dr boleh berhubung dengan Sekretariat Jawatankuasa Penyelidikan Jabatan PERHILITAN di talian 03-90866900 untuk maklumat lanjut. Segala perhatian dan kerjasama YBrs Dr dalam perkara ini didahului dengan ucapan terima kasih.

Sekian.

"WAWASAN KEMAKMURAN BERSAMA 2030"
"HIDUPAN LIAR UNTUK GENERASI AKAN DATANG"
"BERKHIDMAT UNTUK NEGARA"

Saya yang menjalankan amanah,

(DR. PAZIL BIN ABDUL PATAH)
Pengarah
Bahagian Konservasi Ex-Situ
b.p Ketua Pengarah
Jabatan Perlindungan Hidupan Liar dan
Taman Negara (PERHILITAN)

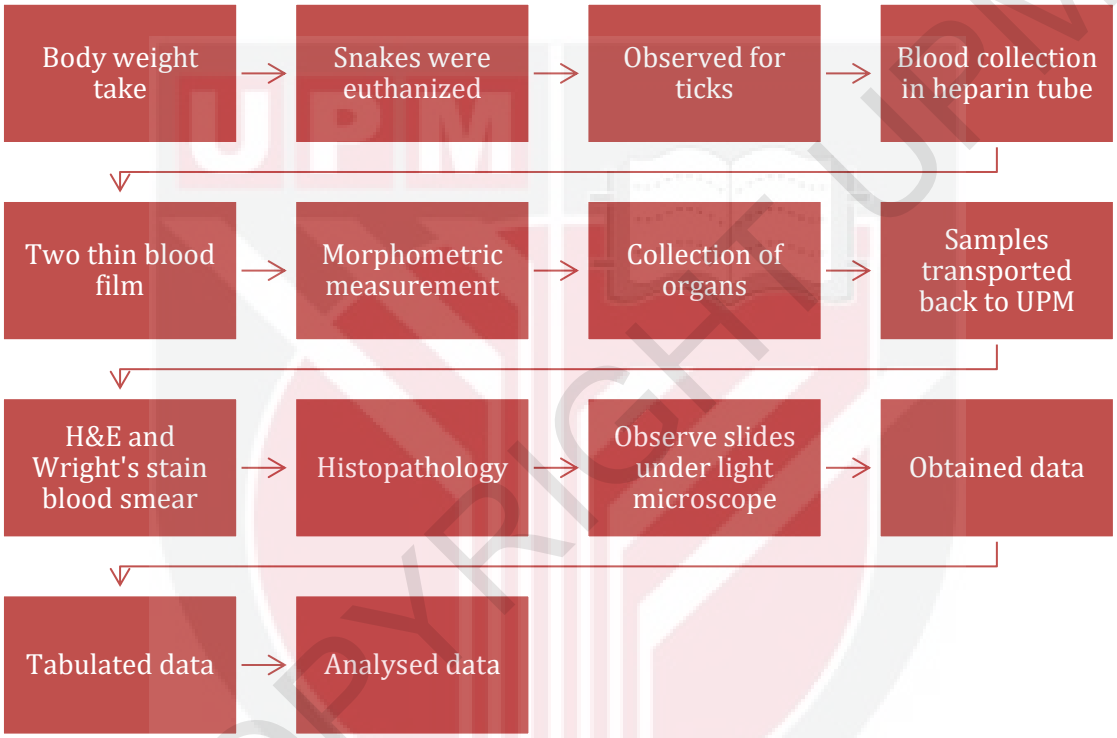
s.k.

Ketua Pengarah
Timbalan Ketua Pengarah (Konservasi)
Pengarah PERHILITAN Selangor
Pengarah PERHILITAN Johor

APPENDIX C

Flow chart of summarized work flow

N = 50



APPENDIX D

Data obtained during sampling

						BODY LENGTH				BLOOD			LIVER		KIDNEY			REMARKS
No.	Sample	Location	Sex	BWt	BCS (/5)	Head length	Snout vent length	Vent to tail	Mites/ticks	Plain	Heparin	Smear	Formalin	Fresh	Formalin	Fresh	Brain	
1	S01/2022	Perak	F	5.5	3.5	8.1	298	45		/	/	/	/	/	/	/	/	
2	S02/2022	Perak	F	8	4	8.9	301	48		/	/	/	/	/	/	/	/	
3	S03/2022	Perak	M	10.5	4	7.6	261	43		/	/	/	/	/	/	/	/	
4	S04/2022	Perak	F	6.3	4	9.6	296	43	T	/	/	/	/	/	/	/	/	
5	S05/2022	Perak	M	11.4	4	8.8	287	42		/	/	/	/	/	/	/	/	
6	S06/2022	Perak	F	11.2	4	9.6	102	44		/	/	/	/	/	/	/	/	
7	S07/2022	Perak	M	6.4	4	7.7	299	45	T	/	/	/	/	/	/	/	/	
8	S08/2022	Perak	M	8.3	4	8.5	103	39	T	/	/	/	/	/	/	/	/	
9	S09/2022	Perak	F	10.8	4	8.2	280	44	T	/	/	/	/	/	/	/	/	
10	S10/2022	Perak	F	12.5	4	9.8	97	45		/	/	/	/	/	/	/	/	
11	S11/2022	Perak	M	8.1	4	7.6	280	45	T	/	/	/	/	/	/	/	/	
12	S12/2022	Perak	M	8.9	4	7.3	320	51	T	/	/	/	/	/	/	/	/	
13	S13/2022	Perak	M	13.3	4	8.7	310	49	T	/	/	/	/	/	/	/	/	
14	S14/2022	Perak	F	8.4	4	8.4	319	45	T	/	/	/	/	/	/	/	/	
15	S15/2022	Perak	M	9.8	4	8.3	280	45	T	/	/	/	/	/	/	/	/	
16	S16/2022	Perak	F	7.8	4	7.9	297	49		/	/	/	/	/	/	/	/	
17	S17/2022	Perak	M	8.4	4	7.6	307	47		/	/	/	/	/	/	/	/	
18	S18/2022	Perak	M	9.1	4	8.3	105	47	T	/	/	/	/	/	/	/	/	
19	S19/2022	Perak	F	8.1	4	8.8	304	50	T	/	/	/	/	/	/	/	/	
20	S20/2022	Perak	F	9.6	4	8.6	108	46	T	/	/	/	/	/	/	/	/	
21	S21/2022	Perak	M	10.9	4	8.5	293	45		/	/	/	/	/	/	/	/	
22	S22/2022	Perak	F	9.4	4	8.3	302	45	T	/	/	/	/	/	/	/	/	
23	S23/2022	Perak	M	8.5	4	7.5	296	46		/	/	/	/	/	/	/	/	
24	S24/2022	Perak	F	9.5	4	7.4	295	47	T	/	/	/	/	/	/	/	/	
25	S25/2022	Perak	F	13	4	8.8	105	43	T	/	/	/	/	/	/	/	/	
26	S26/2022	Perak	F	11	4	8.7	298	45		/	/	/	/	/	/	/	/	
27	S27/2022	Perak	F	7.8	4	7.2	293	46		/	/	/	/	/	/	/	/	
28	S28/2022	Perak	F	10.2	4	8.6	298	47		/	/	/	/	/	/	/	/	
29	S29/2022	Perak	F	10.5	4	8.8	305	71		/	/	/	/	/	/	/	/	
30	S30/2022	Perak	F	12.7	4	8.6	305	51	T	/	/	/	/	/	/	/	/	
31	S31/2022	Perak	F	6.8	4	6.9	251	41	T	/	/	/	/	/	/	/	/	
32	S32/2022	Perak	M	11.7	4	9.5	313	46	T	/	/	/	/	/	/	/	/	
33	S33/2022	Perak	M	10.3	4	7.5	285	46	T	/	/	/	/	/	/	/	/	
34	S34/2022	Perak	M	8.9	4	8.6	265	46	T	/	/	/	/	/	/	/	/	
35	S35/2022	Perak	M	5.1	3.5	7.1	98	43	T	/	/	/	/	/	/	/	/	
36	S36/2022	Perak	M	10.3	4	9.1	312	49	T	/	/	/	/	/	/	/	/	
37	S37/2022	Perak	F	10.2	4	8	295	46	T	/	/	/	/	/	/	/	/	
38	S38/2022	Perak	M	11.7	4	8.7	304	48	T	/	/	/	/	/	/	/	/	
39	S39/2022	Perak	F	12.1	4	7.7	295	50	T	/	/	/	/	/	/	/	/	
40	S40/2022	Perak	M	12.4	4	8.7	306	51		/	/	/	/	/	/	/	/	
41	S41/2022	Perak	M	13.2	4	9.5	324	48	T	/	/	/	/	/	/	/	/	
42	S42/2022	Perak	M	11	4	9.5	299	53	T	/	/	/	/	/	/	/	/	
43	S43/2022	Perak	M	13.6	4	9.2	319	53	T	/	/	/	/	/	/	/	/	
44	S44/2022	Perak	M	10	4	9.2	304	49		/	/	/	/	/	/	/	/	
45	S45/2022	Perak	M	6	4	6.1	98	40		/	/	/	/	/	/	/	/	
46	S46/2022	Perak	F	8.3	4	9.2	302	49	T	/	/	/	/	/	/	/	/	
47	S47/2022	Perak	F	10	4	7.8	314	49	T	/	/	/	/	/	/	/	/	
48	S48/2022	Perak	M	8.8	4	9.1	288	45		/	/	/	/	/	/	/	/	
49	S49/2022	Perak	M	10.6	4	8.5	314	48	T	/	/	/	/	/	/	/	/	
50	S50/2022	Perak	F	7.9	4	7.6	315	46		/	/	/	/	/	/	/	/	