

MOLECULAR DETECTION OF *BANDAVIRUS DABIEENSE* IN TICKS AND THEIR ANIMAL HOSTS COLLECTED FROM RECREATIONAL FORESTS IN SELANGOR

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ABSTRACT

Bandavirus dabieense (BD) is the causative agent of Severe Fever with Thrombocytopenia Syndrome (SFTS). The primary vector of BD is *Haemaphysalis longicornis*, a hard tick species within the family Ixodidae. Despite the public health significance of SFTS in endemic regions, data on BD prevalence in Malaysia remain unavailable. This study aimed to detect BD in ticks collected from Selangor, Malaysia. Field sampling was conducted in two active recreational forests, where small mammals were trapped. Both on-host and questing ticks were collected and morphologically identified alongside their animal hosts. Total nucleic acid was extracted from all tick and animal samples. Molecular species identification of animal hosts and ticks was performed using polymerase chain reaction (PCR) followed by DNA sequencing. Detection of BD was carried out using nested reverse transcription PCR (RT-PCR) targeting S-gene. A total of nine animals were captured, comprising *Sundamys muelleri* (n = 4), *Maxomys whiteheadi* (n = 2), *Rattus tiomanicus* (n = 2), and *Tupaia glis* (n = 1). Additionally, 50 on-host ticks, and 16 questing ticks were collected. Among the on-host ticks, *Ixodes granulatus* was the most abundant species (n = 34), followed by *Dermacentor tricuspidis* (n = 9), *Haemaphysalis hystricis* (n = 6), and *Amblyomma* spp. (n = 1). All questing ticks belonged to the *Dermacentor* genus. All tick and animal samples tested negative for BD. Although no evidence of BD circulation was detected, continuous surveillance with larger-scale sampling efforts is warranted to better assess the potential presence of BD in Malaysia.

KEYWORDS: Nested one-step reverse transcription-PCR, *Bandavirus dabieense*, ticks, small mammals

INTRODUCTION

Bandavirus dabiense (BD) is a tick-borne virus that causes the Severe Fever with Thrombocytopenia Syndrome (SFTS) infection, which was first identified in China in 2009 at rural areas of the Hubei Province and Henan Province (1). Following this, the disease spread to other East Asian countries, such as South Korea (2), Japan (3), Taiwan (4), as well as the Southeast Asian countries, such as Vietnam (5), Myanmar(6), and Thailand (7). The SFTS symptoms include fever, leukopenia, thrombocytopenia, and gastrointestinal complications such as diarrhea, nausea and vomiting (8). Currently, there is no prospective randomized studies on treatment strategies have proven to be effective against SFTS. The conservative treatment includes hydration, transfusion and administration of antipyretics and inotropic agents to alleviate symptoms. Others proposed the uses of antiviral agents, steroids, intravenous immunoglobulin and monoclonal antibodies to treat SFTS (8). BD is currently classified under the *Bandavirus* genus and *Phenuiviridae* family (9). The BD genome comprises three main segments: the L-, M- and S-segments (9). The L-gene segment functions to encode the RNA-dependent RNA polymerase, while the M-gene segment encodes the glycoprotein precursors. The smallest segment which is the S-gene, encodes the nucleoprotein and non-structural protein. The reported case fatality rate (CFR) of SFTS varies slightly across countries. The highest reported CFR was in Japan at 27.0% (10), followed by South Korea at 17.4% (11), and China at 8.2% (12).

This virus is transmitted to humans mainly through infected tick bites, with *Haemaphysalis longicornis* acting as the main vector (13). However, viral RNA has also been detected in other tick species, such as *Haemaphysalis flava*, *Rhipicephalus microplus*, *Amblyomma testudinarium*, *Dermacentor nuttalli*, and *Ixodes nipponensis* (14). Besides ticks, BD antibodies and RNA have also been detected in wild animals such as shrew, rodents, weasels, and hedgehogs (15). A study by Li et al. (16) revealed three modes of BD transmission: (1) tick bites, (2) direct contact with the blood and bodily fluids of a person infected with BD, and (3) direct blood contact or bites from infected animals. Currently, on the presence of this pathogen in local tick populations and their animal hosts in Malaysia are very limited. A seroprevalence study of BD in humans was conducted in neighboring Southeast Asian countries with Thailand reporting 1.1% (17) and Vietnam reporting 3.6% (18).

Malaysia possesses vast tropical rainforests that provide favorable habitats for ticks and their animal hosts, with some of these forests designated as recreational forests for public use. Understory plants and leaf litter produce a humid microclimate, which prevents ticks from desiccating and allows them to survive (19). Thus, these humid and shaded conditions, together with an abundance of hosts that serve as blood-meal sources, create an ideal environment for tick survival in recreational forests. As recreational forests are actively visited by humans for outdoor activities, this increases human-tick contact and, consequently, the risk of zoonotic disease transmission. A study by Coutts and Hahn (20) reported a rising incidence of ticks and tick-borne diseases in recreational forests, posing an increasing risk to public health. For instance, a study by (21) detected BD in ticks collected from Deongyusan National Park, Republic of Korea. Therefore, the current study aims to screen for the presence of BD in local ticks and their animal host populations collected from recreational forests in Selangor to assess their potential risks to public health.

MATERIALS AND METHODS

Field Sampling

Field sampling was conducted at two recreational forests in Selangor, Malaysia: (1) Hutan Lipur Sungai Congkak, Hulu Langat (SC), and (2) Taman Eko Rimba Komanwel (EK), Rawang. Sampling was carried out at SC and EK during the end of May and in mid-June of 2025, respectively. Recreational forests were chosen for this study because these sites are popular with visitors for outdoor activities, making them suitable locations for assessing BD risk. A total of 80 wire traps were deployed at each sampling site along river stream-and hiking trails to capture rodents and scandentians over four consecutive days. The number of on-host ticks collected depended on the number of rodents and scandentians captured. In addition to on-host ticks, questing ticks were also actively searched for and collected from the surrounding vegetation.

Animal Processing and Tick Collection

All on-host ticks across various developmental stages (adult, nymph, and larva) were collected from rodents and scandentians using soft forceps. The feeding status of each tick (unfed, partially fed, or fully fed) at the time of collection was recorded. Each on-host tick was stored individually in a 1.5 mL microcentrifuge tube, labeled by locality, and assigned a unique identification code. In addition, questing ticks were collected from the surrounding vegetation and stored individually in 1.5 mL microcentrifuge tubes, labeled by locality and assigned a unique code. The developmental stage and feeding status of the questing ticks were also recorded. Following species identification, the animals were dissected to obtain blood and tissues, including the spleen, liver, and kidney. All animal handling and sampling procedures were conducted in accordance with the ethical guidelines approved by the Animal Care and Use Committee (ACUC), Ministry of Health Malaysia, under the approval number ACUC/KKM/02(06/2025).

Morphological Identification of Ticks and Animal Hosts

Each on-host and questing tick was morphologically identified to the species level, where possible, using relevant taxonomic keys under a stereomicroscope (22–25). Meanwhile, the euthanized animals were morphologically identified to the species level using relevant taxonomic keys by Francis (26) and recorded systematically. All animals were also given a unique identification code.

Nucleic Acid Extraction

Genomic DNA was extracted from the ticks and animal kidney tissues using commercially available extraction kits. For animal samples, genomic DNA was extracted from approximately 0.1 mg of kidney tissue using the DNeasy Blood & Tissue Kit (Qiagen, Germany), whereas DNA from ticks was extracted using the SPINeasy™ DNA/RNA/Protein All-In-One Kit (MP Biomedicals, USA). All extractions procedures were carried out in accordance with the manufacturers' protocols. Tick DNA extraction was performed according to species and developmental stage, with adult, nymph, and larval stages processed separately. Engorged ticks were extracted individually, while unfed ticks were pooled at a maximum of five individuals per tube prior to extraction. The extracted DNA was subsequently used to identify tick, rodent, and scandentia species using polymerase chain reaction (PCR).

For molecular detection of BD, RNA was extracted from on-host and questing ticks, as well as from the blood and tissue samples (spleen, liver, and kidney) of animal hosts using commercially available extraction kits. All extraction procedures were performed in accordance with the manufacturers'

protocols. RNA from animal samples was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Germany), while RNA from tick samples was extracted using the SPINeasy™ DNA/RNA/Protein All-In-One Kit (MP Biomedicals, USA). For animal blood samples, only serum was used for RNA extraction. Whole blood samples were centrifuged at 8,000 rpm for 10 minutes, after which the serum was collected. For tissue samples, approximately 0.1 mg of each tissue type was used for RNA extraction. Tick RNA extraction was performed according to species and developmental stage, with adult, nymph, and larval stages processed separately. Engorged ticks were extracted individually, whereas unfed ticks were pooled at a maximum of five individuals per tube prior to extraction. All extracted RNA samples were then stored at –80 °C until further analysis.

Molecular Identification of Ticks and Animal Hosts

The morphological status of ticks and animal hosts was verified using PCR and DNA sequencing. The PCR assay was performed using a commercial PCR kit, 2X PCR BIO Taq Mix Red (PCR Biosystems, UK), with previously published primer sequences (Table 1). For ticks, the mitochondrial 16S rDNA (*mt-16S rDNA*) sequence was targeted, while the Cytochrome c oxidase subunit I (*CO1*) gene was used for rodents and treeshrews. The PCR conditions for *mt-16S rDNA* were conducted as follows: initial denaturation at 95°C for one minute, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 50°C for 15 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for five minutes, before extending at 4°C for ten minutes and finally hold at 20°C.

Meanwhile, the PCR condition for the *CO1* gene followed (27) with slight modifications: initial denaturation at 94°C for two minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for one minute, and a final extension at 72°C for five minutes, before holding at 12°C. The PCR products for both organisms were run on 1.5% (v/v) agarose gel electrophoresis for the expected sizes (Table 1). Positive amplicons were outsourced for sequencing at Apical Scientific Sdn. Bhd, Selangor, Malaysia. The sequenced data were then aligned using BioEdit Sequence Alignment Editor software (28) and compared against reference sequences in the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) for species identification and confirmation.

Table 1. Primers used for species identification of ticks and their animal hosts

Organisms	Targeted Gene	Primer Sequence (5'-3')	Expected Size (bp)	References
Tick	<i>mt-16S rDNA</i>	Forward (CTGCTCAATGATTTTTTAAATTGCTGTGG)	460	(29)
		Reverse (CCGGTCTGAACTCAGATCAAGTA)		
Rodent & Shrew	<i>CO1</i>	Forward (CCTACTCRGCCATTTTACCTATG) Reverse (ACTTCTGGGTGTCCAAAGAATCA)	750	(27)

Molecular Detection of BD from Ticks and Animal Hosts

One-step reverse transcription-PCR (RT-PCR) was performed using a commercial kit, TransScript One Step RT-PCR SuperMix (TransGen Biotech, China), according to the manufacturer's instructions. The degenerate primer sequences were adopted from previous studies as shown in Table 2. The first round of PCR was initiated with a reverse transcription step at 45 °C for 30 minutes, followed by an initial denaturation at 94 °C for five minutes. This was followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 52 °C for 30 seconds, and extension at 72 °C for 30 seconds, with a final extension at 72 °C for five minutes and hold at 12 °C prior to the second round of PCR.

The second round of nested PCR was conducted using the first-round PCR product as the template, and a commercial PCR kit, 2X PCRBIO Taq Mix Red (PCR Biosystems, UK), was used following the manufacturer's protocol. The cycling conditions consisted of an initial denaturation at 94 °C for five minutes, followed by 25 cycles of denaturation at 94 °C for 20 seconds, annealing at 54 °C for 40 seconds, and extension at 72 °C for 30 seconds, with a final extension at 72 °C for five minutes and a hold at 12 °C. Synthetic RNA corresponding to the S-segment gene of BD was used as a positive control, while a blank reaction without any nucleic acid templates served as the negative control. PCR products from both the first and second rounds were subjected to 1.5% (w/v) agarose gel electrophoresis to confirm the expected amplicon sizes (Table 2). When present, the second-round PCR products were subjected to DNA sequencing. The sequenced DNA was then aligned using BioEdit Sequence Alignment Editor software (28). The sequencing data were subsequently align against reference sequences in the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) for species identification and confirmation. Finally, a phylogenetic tree was constructed using MEGA11 software (30).

Table 2. Primers used for the detection of BD in ticks and animals' blood and tissues

Pathogen	Targeted Gene	Primer sequence (5'-3')	Expected Size (bp)	References
BD	S-gene (First round PCR)	Forward (CATCATTGTCTTTGCCCTGA)	461	(31)
		Reverse (AGAAGACAGAGTTCACAGCA)		
	S-gene (Second round PCR)	Forward (AAYAAGATCGTCAAGGCATCA)	346	(32)
		Reverse (TAGTCTTGGTGAAGGCATCTT)		

RESULTS

Species identification of ticks and animal hosts

During the four-day sampling period conducted at SC and EK, a total of nine small mammals were caught, and 66 ticks were collected (Table 3). Of the nine animals caught, only six were infested with ticks, resulting in an infestation rate of 67.0%. These small mammals were represented by four species: four *Sundamys muelleri* (44.5%), two *Maxomys whiteheadi* (22.2%), two *Rattus tiomanicus* (22.2%) and one *Tupaia glis* (11.1%). The molecular species identification was consistent with the morphological data, except for one animal, which was initially identified as *Maxomys rajah* but was later confirmed as *Maxomys whiteheadii* by PCR analysis.

A total of 50 on-host ticks were collected from the six infested animals. Among these, 34 were identified as *Ixodes granulatus* (68.0%) (Supplementary 1), nine as *Dermacentor tricusps* (*atrosignatus*) (18.0%) (Supplementary 2), six as *Haemaphysalis hystricis* (12.0%) (Supplementary 3), and one as *Amblyomma* spp. (2.0%) (Supplementary 4). Meanwhile, a total of 16 questing ticks were collected, all of which were found at SC. Of these, 10 were identified as *Dermacentor compactus* (62.5%) (Supplementary 5), four as *Dermacentor tricusps* (previously known as *D. atrosignatus*) (25.0%) (Supplementary 6), and two as *Dermacentor steini* (12.5%) (Supplementary 7). The morphological species identification results were consistent with the molecular identification results, except for eight on-host ticks. Initially, two were identified as *I. granulatus* larvae, two as *Amblyomma* larvae, and four as *Amblyomma* nymphs. However, molecular identification showed that all eight were *D. tricusps* (*atrosignatus*) ticks.

Table 3. The summary of ticks and their small mammal hosts collected in this study

Animal Species	No. of animals	collected	No. of animals	tick-infested	Tick infestation Rate (%)	No. of on-host tick	No. of Questing tick
SC							
Rodentia							
<i>Sundamys muelleri</i>	3 (33.3%)		3 (50.0%)		100	43 (86.0%)	0 (0.0%)
<i>Rattus tiomanicus</i>	1 (11.1%)		1 (16.7%)		100	2 (4.0%)	0 (0.0%)
<i>Maxomys whiteheadi</i>	1 (11.1%)		0 (0.0%)		0	0 (0.0%)	0 (0.0%)
Scandentia							
<i>Tupaia glis</i>	1 (11.1%)		0 (0.0%)		0	0 (0.0%)	0 (0.0%)
EK							
Rodentia							
<i>Sundamys muelleri</i>	1 (11.1%)		1 (16.7%)		100	3 (6.0%)	0 (0.0%)
<i>Rattus tiomanicus</i>	1 (11.1%)		0 (0.0%)		0	0 (0.0%)	0 (0.0%)
<i>Maxomys whiteheadi</i>	1 (11.1%)		1 (16.7%)		100	2 (4.0%)	0 (0.0%)
Questing Tick	0 (0.0%)		0 (0.0%)		0	0 (0.0%)	16 (100.0%)
Total	9		6		66.7	50	16

Screening of BD in ticks and their animal hosts

All questing ticks, on-host ticks, and their animal hosts were screened for the presence of BD. A total of 50 tick pools, comprising both on-host and questing ticks were tested for BD detection. For the animal hosts, serum, spleen, liver, and kidney tissues were used for BD screening. In total, 36 samples, nine each of serum, spleen, liver, and kidney, were tested from the nine captured animals. Following the screening of all tick pools and animals, no BD was detected in any of the tested samples (Table 4 and Table 5). Representative gel electrophoresis images of the nested RT-PCR products are shown in Supplementary 8 and Supplementary 9 for on-hosts and questing ticks, respectively. Meanwhile, Supplementary 10-13 display gel images of the animal sera, spleens, livers, and kidneys, respectively. No DNA bands of the expected amplicon size were observed in any of the tested samples. For both questing and on-host ticks, only selected gel images are shown to represent the overall results.

Table 4. Detection of BD in ticks of different species and life stage collected in this study

Tick Species	Life Stages	BD Positivity Rate (%)
<i>Amblyomma</i> spp. (on-host) (n=1)	Adult (n=1)	0
<i>D. tricuspis</i> (on-host) (n=9)	Nymph (n=4) Larvae (n=5)	0
<i>H. hystricis</i> (on-host) (n=6)	Adult (n=1) Nymph (n=3) Larvae (n=2)	0
<i>I. granulatus</i> (on-host) (n=34)	Adult (n=24) Nymph (n=9) Larvae (n=1)	0
<i>D. compactus</i> (Questing) (n=10)	Adult (n=10)	0
<i>D. tricuspis</i> (Questing) (n=4)	Adult (n=4)	0
<i>D. steini</i> (Questing)	Adult (n=2)	0

(n=2)		
Total	66	0

Table 5. Detection of BD from animals using different tissues type

Animal Host Species	Type of Tissues	BD Positivity Rate (%)
<i>S. muelleri</i> (n=4)	Blood (n=4) Spleen (n=4) Liver (n=4) Kidney (n=4)	0
<i>M. whiteheadi</i> (n=2)	Blood (n=2) Spleen (n=2) Liver (n=2) Kidney (n=2)	0
<i>R. tiomanicus</i> (n=2)	Blood (n=2) Spleen (n=2) Liver (n=2) Kidney (n=2)	0
<i>T. glis</i> (n=1)	Blood (n=1) Spleen (n=1) Liver (n=1) Kidney (n=1)	0
Total	36	0

DISCUSSION

In our study, two recreational forests in Selangor were chosen as study sites. This is because forest ecosystems provide favourable conditions for tick survival and development during host-seeking periods, largely due to higher humidity and a greater availability of potential hosts (33). Small mammals found in recreational forests have been shown to carry multiple tick species on a single host (34). Studies across various recreational forest types—ranging from tropical sanctuaries to urban temperate forests show that the commonly found tick genera are *Haemaphysalis*, *Dermacentor*, and *Ixodes* (35–37). The tick and animal species obtained in this study were also consistent with those collected in a previous study conducted in recreational forests in Hulu Langat, Selangor (38). The presence of suitable small mammalian hosts (rodents and treeshrews) at the two sampling sites likely contributed to the high number of *I. granulatus*, as this tick species is known to infest small rodents and treeshrews (38) for its blood meals. Few of the ticks' morphological identification is not consistent with the molecular identification by PCR. This is due to the tick life stage at the time of morphology identification are still at nymph stage where few essential features are still underdeveloped and thus reduce the accuracy of the conventional identification.

Prior to nucleic acid extraction, unfed ticks were pooled while engorged ticks were not. Unfed ticks carry very small amounts of nucleic acid and thus by pooling, it will increase the total nucleic acid yield. Meanwhile, nucleic acid from engorged ticks were extracted individually as their size was big and can yield high total nucleic acid concentration. The *mt-16S rDNA* and *CO1* gene have been widely used for the molecular species identification of ticks (29) and animals (39). In this study, S-gene was used as the PCR target for BD detection from ticks and animals. The S-gene segment is the most conserved gene in the BD genome and thus minimizes the risk of false negatives caused by viral mutations (40). Few studies have reported the presence of BD in ticks collected from recreational forests. For example, BD has been detected in *Haemaphysalis longicornis*, *Ixodes persulcatus*, and *Dermacentor variabilis* ticks collected from recreational forests in China (41). In addition, BD was also detected in ticks collected from Deogyusan National Park, Republic of Korea (21). However, in the current study, no BD was detected in the ticks or animal hosts. Generally, the Minimum Infection Rate (MIR) of BD in ticks is very low. A previous study by Yang et al. (42) showed that the BD MIR in *Haemaphysalis longicornis* ticks collected from Hubei Province was only 0.07%. Furthermore, the reported BD MIR in questing ticks is also notably low. Another study by Xu et al. (43) showed that the BD MIR from questing ticks of *Amblyomma* spp., *Dermacentor* spp., *Haemaphysalis* spp. and *Ixodes* spp. had a very low pooled prevalence value of only 0.68% (95% CI 0.34-1.10%). Consequently, the negative results from the current study could be attributed to the very small sample size, which significantly lowered the probability of detecting the virus (44). Besides the small sample size, other contributing factors could include a low prevalence of BD in local ticks, the absence of confirmed SFTS cases in Malaysia to date, and the use of only a single target gene (the S-segment) in the RT-PCR.

Besides ticks acting as vectors, BD also has a broad host range and has been detected in various animal species with differing carriage rates. The highest prevalence has been reported in cattle (up to 26.31%), followed by cats (17.46%), goats (9.10%), and rodents (8.44%) (45). In the present study, BD screening was conducted in rodents and tree shrews (Sarcophiidae); however, all samples tested negative. Viral detection was performed using serum, spleen, liver, and kidney tissues, which is consistent with the tissue types used for BD detection in animals in previous studies (45). All sera, spleens, livers, and kidneys tested in this study were negative. Although no BD was detected in either the ticks or the animal hosts in this study, the observed prevalence may not reflect the true infection rate due to the limited sample size. Furthermore, recent studies by the Acarology Unit at the Institute for Medical Research (IMR) have indicated the presence of BD in ticks and their animal hosts collected from other recreational forest locations in Selangor (unpublished data).

Future studies should expand sampling efforts to encompass a wider range of ecosystems, such as primary forests, plantation areas, and human settlements, to improve ecological representation and increase sample sizes. In addition, targeting alternative viral genomic segments, such as the M and L segments, may enhance detection sensitivity and specificity. The L-gene segment is highly conserved among BD strains, providing high specificity for molecular detection, whereas the M-gene segment exhibits greater sequence variability and can be used to differentiate BD genotypes. Furthermore, the application of more sensitive molecular techniques, including real-time PCR assays, should be considered in future investigations to improve the reliability of viral detection.

CONCLUSION

In this study, BD was not detected in any tick or animal samples collected from Hutan Lipur Sungai Congkak, Hulu Langat, and Taman Eko Rimba Komanwel, Rawang, Selangor. The absence of BD detection may be attributed to the limited sample size and potentially low viral circulation in Malaysia, which represents a key limitation of the present study.

Supplementary Materials: **Figure S1:** [Dorsal and ventral view of the male *I. granulatus* collected in this study], **Figure S2:** [Dorsal and ventral view of the larval stage *D. tricusps* collected in this study], **Figure S3:** [Dorsal and ventral view of the nymphal stage *H. hystricis* collected in this study], **Figure S4:** [Dorsal and ventral view of the fully engorged *Amblyomma* spp. collected in this study], **Figure S5:** [Dorsal and ventral view of the male *D. compactus* collected in this study], **Figure S6:** [Dorsal and ventral view of the male *D. tricusps* collected in this study], **Figure S7:** [Dorsal and ventral view of the male *D. steini* collected in this study], **Figure S8:** [Gel picture showing the detection result of BD from on-host tick samples by nested RT-PCR], **Figure S9:** [Gel picture showing the detection result of BD from questing tick samples by nested RT-PCR], **Figure S10:** [Gel picture showing the detection result of BD from animal serum samples by nested RT-PCR], **Figure S11:** [Gel picture showing the detection result of BD from animal spleen samples by nested RT-PCR], **Figure S12:** [Gel picture showing the detection result of BD from animal liver samples by nested RT-PCR], **Figure S13:** [Gel picture showing the detection result of BD from animal kidney samples by nested RT-PCR].

Data Availability: All data have been presented in the manuscript.

Author Contributions: **CJ:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing-original draft, Writing-review and editing. **PCF:** Conceptualization, Methodology, Resources, Data curation and Formal analysis. **SZA:** Conceptualization, Methodology, Resources, Data curation, and Formal analysis. **NAANK:** Conceptualization, Methodology, Resources and Formal analysis. **EFCL:** Conceptualization, Methodology, Resources, Data curation, Formal analysis, Writing-review and editing.

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Institutional Review Board Statement: This study was registered with the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia under the National Medical Research Register (NMRR No: NMRR ID-25-02614-CAN [IIR]). The usage of animal in this study protocol was approved by the ACUC-MOH with registration number of ACUC/KKM/02(06/2025).

Informed Consent Statement: Not applicable

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