SEROLOGICAL DETECTION OF EPIZOOTIC HAEMORRHAGIC DISEASE VIRUS (EHDV) IN DAIRY COWS FROM SELECTED FARMS IN MALAYSIA

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DOCTOR OF VETERINARY MEDICINE

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SEROLOGICAL DETECTION OF EPIZOOTIC HAEMORRHAGIC DISEASE VIRUS (EHDV) IN DAIRY COWS FROM SELECTED FARMS IN MALAYSIA

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ABSTRACT

Epizootic Haemorrhagic Disease Virus (EHDV) is a non-contagious viral infection with economic potential impacts, mainly affecting both wild and domestic ruminants. This study aimed to identify, expose and measure the antibody titre EHDV infection in dairy cow farms located in four states in Malaysia. One hundred twenty (n=120) bovine serum archive samples were obtained from the previous research project in four states, including Sabah, Selangor, Perak and Pahang. The samples were tested using a Competitive Enzyme-Linked Immunosorbent Assay (c-ELISA) kit to detect antibodies against EHDV. The steps were followed carefully and according to the manufacturer's instructions (IDVET). All ELISA results' optical densities (ODs) were then determined using an ELISA plate reader and read at 450 nm. The results were then calculated using the given formula and interpreted as positive, negative, and doubtful. The total prevalence of the 120 tested serum samples was 63%, 76 were positive against EHDV antibodies. Perak state showed the highest prevalence of 90%, with 18 positive samples out of 20. This was followed by Sabah, which had a prevalence of 80% with 16 positive samples out of 20. Meanwhile, Pahang was recorded with a prevalence of 53% with 21 positive samples out of 40, followed by Johor, which can be observed to record the lowest prevalence of 50% with 20 positive samples out of 40. Overall, the results indicate the presence and widespread distribution of EHDV infection across the selected four states with high-producing dairy cow farms in Malaysia.

ABSTRAK

Virus Epizootic Haemorrhagic Disease (EHDV) ialah jangkitan virus tidak berjangkit dengan potensi kesan ekonomi, terutamanya memberi kesan kepada ruminan liar dan domestik. Kajian ini bertujuan untuk mengenal pasti terdedah dan mengukur titer antibodi jangkitan EHDV di ladang lembu tenusu yang terletak di empat negeri di Malaysia. Seratus dua puluh (n=120) sampel arkib serum lembu telah diperoleh daripada projek penyelidikan terdahulu di empat negeri, termasuk Sabah, Selangor, Perak dan Pahang. Sampel telah diuji menggunakan kit Competitive Enzyme-Linked Immunosorbent Assay (c-ELISA) untuk pengesanan antibodi terhadap EHDV. Langkah-langkah telah diikuti dengan teliti dan mengikut arahan pengeluar (IDVET). Semua ketumpatan optik (OD) hasil ELISA kemudiannya ditentukan menggunakan pembaca plat ELISA dan dibaca pada 450 nm. Hasilnya kemudian dikira menggunakan formula yang diberikan dan ditafsirkan sebagai positif, negatif dan ragu berdasarkan garis panduan pengilang. Jumlah kelaziman 120 sampel serum yang diuji ialah 63%, 76 adalah positif terhadap antibodi EHDV. Dapatan seroprevalens menunjukkan Perak mempunyai prevalens tertinggi iaitu 90% dengan 18 sampel positif daripada 20. Ini diikuti oleh Sabah, yang mempunyai prevalens sebanyak 80% dengan 16 sampel positif daripada 20. Sementara itu, Pahang direkodkan dengan prevalens sebanyak 53% dengan 21 sampel positif daripada 40 diikuti Johor boleh diperhatikan mencatatkan prevalens terendah iaitu 50% dengan 20 sampel positif daripada 40 sampel. Secara keseluruhan, keputusan menunjukkan kehadiran dan pengedaran jangkitan EHDV secara meluas di empat negeri terpilih dengan ladang lembu tenusu dengan pengeluaran tinggi di Malaysia, dan Perak mempunyai prevalens negeri yang paling tinggi berbanding negeri lain.

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Thank You

DEDICATIONS

I dedicate my dissertation work to both my family and friends. I extend special thanks to my affectionate parents and siblings, who always encourage and support me.

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LIST OF ABBREVIATIONS

EHDV	-	Epizootic Haemorrhagic Disease Virus
BTV	-	Bluetongue Virus
dsRNA	-	Double Stranded Ribonucleic Acid
VP	-	Viral Protein
NS	-	Non-Structural
c-ELISA	-	Competitive Enzyme Linked Immunosorbent Assay
SST	-	Serum Separator Tube
HRP	-	Horseradish Peroxidase
TMB	-	Tetramethybenzidine

LIST OF SYMBOLS

%	Percentage
µL	Microliter
mL	Mililiter
°C	Degree Celsius

CHAPTER 1

INTRODUCTION

1.0 Introduction

The Epizootic Hemorrhagic Disease Virus, or EHDV, comes from the genus *Orbivirus* of the family *Reoviridae* (Noronha *et al*., 2021). This virus is a double-stranded RNA virus capsid and does not have an outer layer but also has a distinctive structure and icosahedral symmetry characteristic (Jiménez-Cabello *et al*., 2023).

This viral infection is non-contagious and is an arthropod-borne disease transmitted by a type of insect, *Culicoides* spp biting midges (Stevens *et al*., 2015). EHDV infection mainly infects wild and domestic ruminants, primarily white-tailed deer (*Odocoileus virginianus*) and cattle. EHDV has been identified and reported in the Americas, Africa, Asia, Australia, the Middle East and various parts of the islands of the Indian Ocean (Kamomae *et al*., 2018). The OIE Terrestrial Manual discusses that EHDV is recognized as a notifiable emerging disease with the potential to induce high mortality and morbidity, reaching approximately 90% in white-tailed deer. However, the severity of the disease varies based on the year and geographical region.

Clinical manifestations of EHDV infection in cattle do not exhibit severe clinical signs compared to those observed in white-tailed deer. White-tailed deer infected with EHDV in the peracute stage exhibit sudden death, while those in the chronic stage display pyrexia, respiratory distress, edema of the head and neck, oral erosion, and lameness. However, cattle affected by EHDV infection manifest pyrexia, decreased milk production, swollen conjunctiva, nasal and ocular discharge, stomatitis and dyspnea (Stevens *et al*., 2015).

Currently, researchers have identified and recognized at least seven EHDV serotypes in the world (EHDV-1, -2, -4, -5, -6, -7, and -8), and two new serotypes (EHDV-10 and another unnamed serotype) were introduced recently (Duan *et al*., 2022). These EHDV serotypes contribute to widespread disease dissemination, leading to significant economic production losses to farmers and nations.

Consequently, this study investigated the presence of EHDV infection in four states known for high-producing dairy cow farms in Malaysia. This includes Sabah, Selangor, Perak, and Pahang. This study aims to detect the presence of EHDV and determine the seroprevalence of the antibody titre against the EHDV in different states of dairy cow farms in Malaysia using the serological detection method.

1.1 **Research Problem Statement**

Various research and studies have been carried out on EHDV detection in America and Mediterranean countries and obtained serotypes EHDV-1, EHDV-2 and EHDV-7, resulting in significant economic production loss. However, in Malaysia, there is no reported case of EHDV infection by veterinary authorities in dairy cow farms in Malaysia.

1.2 **Research Questions**

I. Which region dairy cow farms in Malaysia have the most exposure to EHDV?

II. What is the antibody titre against EHDV infection in dairy cow farms in Malaysia?

1.3 **Research Hypothesis**

I. Dairy cow farms located in Peninsular Malaysia shows the highest exposure of EHDV infections.

II. Antibody titre against EHDV infection in dairy cow farms is high in Malaysia.

1.4 **Research Objectives**

I. To identify the exposed EHDV infection in four states of dairy cow farms in Malaysia.

II. To measure the antibody titre against EHDV infection in dairy cow farms in Malaysia.

CHAPTER 2

LITERATURE REVIEW

2.0 Morphology and structure Epizootic haemorrhagic disease virus (EHDV)

EHDV is a dsRNA, non-enveloped virion exhibiting a structure like others of the *Orbivirus* genus. It comprises an icosahedral capsid comprising three protein layers: the inner layer, intermediate layer (core), and outer capsid. It encoded seven structural proteins (VP1–VP7) and at least four non-structural proteins (NS1, NS2, NS3/NS3A, NS4). The outer protein layer comprises 60 trimers of VP2, and the most exposed virion protein has 120 trimers of VP5. The inner capsid comprises the sub-core formed by VP3, while VP7 constitutes the intermediate layer. Additionally, three minor structural proteins with enzymatic activities are present, namely VP1 (RNA-dependent RNA polymerase), VP4 (capping enzyme), and VP6 (helicase (Jiménez-Cabello *et al*., 2023).

Furthermore, highly conserved proteins are involved in genome replication, specifically VP1 (segment 1), VP4 (segment 4), and VP6 (segment 9). Meanwhile, in the sequence of amino acids of VP7, encoded by segment 7 among EHDV serotypes, over 90% of identical sequences can be identified. The immunodominant serogroup-specific protein VP7 undergoes testing in an Enzyme-Linked Immunosorbent Assay (ELISA) specific to each serogroup for disease diagnosis. Non-structural proteins support essential viral activities, including genome packaging, intracellular transportation, capsid assembly, virus release, and immune response control. These proteins are present in infected cells but absent from virus particles (Jiménez-Cabello *et al.,* 2023).

2.1 Distribution

The distribution of EHDV depending on the abundance of the biting midge Culicoides spp, the level of existing immunity in deer, and the genetic variations in susceptibility. EHDV was first described in white-tailed deer of New Jersey (USA) in 1955. EHDV infection of wild and domestic ruminants was reported in the Americas, Africa, Asia, Australia, the Middle East, and some islands of the Indian Ocean (W. Niedbalski *et al*., 2020). The common serotypes of EHDV infections in deer and cattle, especially in the United States and Canada, were EHDV-1, EHDV-2, and EHDV-6. In Africa, EHDV-1 and EHDV-6 were identified. However, 7 serotypes of EHDV (EHDV-1, -2, -5, -6, -7, -8, -10) were identified in East Asia and Japan between 1959 and 1960, EHDV-2 was first recorded with the name of Ibaraki virus hence became epidemic (Duan *et al*., 2022).

2.3 Transmission

EHDV is transmitted by arthropod-borne biting midges of the genus *Culicoides* spp. This transmission occurs in temperate regions such as the United States, North America, and the Mediterranean. This infection is mostly transmitted in the late summer and autumn, coinciding with peak vector populations. Meanwhile, in tropical regions, this infection can occur throughout the year. It is important to note that EHDV infection is not a known cause of disease in humans, as indicated in the (OIE Terrestrial Manual of 2021).

2.4 Clinical Manifestation

The clinical manifestation of EHDV in susceptible mammal hosts, especially white-tailed deer and other cervid species, often shows pyrexia, weak, serosal haemorrhages, and mucosal edema. However, infected EHDV in domestic livestock shows subclinical or asymptomatic but often to be the reservoir host for the virus.

2.5 Serology detection of Epizootic Haemorrhagic Disease Virus (EHDV)

Serological assays are a common approach for diagnosing and surveilling EHDV. Breard (2020) identified the competitive enzyme-linked immunosorbent assay (c-ELISA) as the most effective serological test for precise EHDV detection, focusing on measuring antibody levels against viral protein 7 (VP7). A recently developed commercial c-ELISA designed for ruminant EHDV VP7 Abs demonstrates excellent diagnostic specificity and satisfactory sensitivity. It can effectively detect the presence of IgG anti-EHDV VP7 in domestic ruminants, zoo animals, and wild deer (Breard, 2020).

CHAPTER 3

RESEARCH METHODOLOGY

3.0 Ethics Statement

No ethics application is required for this study, as the serum is obtained from archive samples from a previous research project with an approval code from the FPV Animal Ethics Committee: UMK/FPV/ACUE/RES/002/2021.

3.1 Sample Collection

A total of one hundred twenty (120) blood samples from the previous archive samples were collected via the coccygeal vein technique. The samples were taken from high-producing dairy cow farms in Malaysia, including Sabah, Perak, Pahang, and Johor. The obtained blood samples were transferred into serum separator tubes (SST) to allow serum separation without centrifugation. Subsequently, the samples were stored in a polystyrene box with ice packs during transportation. Upon arrival at the laboratory, the samples were stored at -20 °C in the chiller.

3.2 Serology Detection (ELISA kit)

The diagnostic kit was designed to detect antibodies against the EHDV VP7 protein. The method proved suitable for serum or plasma from bovine, deer and all susceptible species. The wells were coated with a VP7 recombinant protein. The tested samples and the controls were added to the 96-well microplates. If present, the anti-VP7 antibodies formed an antibody complex, masking the VP7 epitopes.

An anti-VP7 peroxidase (HRP) conjugate was added to the microplates, fixing the remaining free VP7 epitopes and forming an antigen-conjugate-HRP complex. After washing, the substrate solution (TMB) was added to eliminate the excess conjugate. The resulting colouration depended on the quality of specific antibodies in the tested sample. In the absence of antibodies, a

blue solution appears, which becomes yellow after the stop solution is added; in the presence of antibodies, no colouration appears. Lastly, the microplates are read at 450nm.

3.3 Sample Preparation

All the apparatuses and equipment were prepared before the procedure, including multichannel pipettes, disposal tips, 96-well microplate, distilled water, wash system, and 96-well microplate reader. Before starting the procedure, distilled water was prepared for the wash solution (1X) by diluting the wash concentrate (20X), as shown in Box 1.

300µL x 6 times wash x 96-well microplate x 2 = 345600µL,

Conversion from µL to mL = 345600 x 0.001

= 345.6mL of distilled water needed to dilute the wash solution

Assume 500mL of distilled water is needed to dilute the wash solution, so use the dilution formula M1V1=M2V2 to obtain the volume of wash concentrate needed to mix in distilled water.

$$M1V1 = M2V2$$

$$20(V1) = 1(500)$$

$$V1 = 25mL$$

Box 1: Wash Solution Preparation and Calculation

All the reagents were homogenized by inversion or vortex and placed at room temperature at 21 °C ± 5 °C. In order to avoid differences in incubation times between samples, a 96-well microplate containing the test and control samples was prepared beforehand and then only transferred into an ELISA microplate using a multi-channel pipette.

The commercially available EHDV ELISA kit was evaluated following the manufacturer's instructions. Pre-dilution microplates were added to the 96-well. 20 µl of positive control (Cpos) was added to wells A12 and B12. Then, 20 µl of negative controls (Cneg) was added into wells C12 and D12. Meanwhile, 50 µl of dilution buffer 2 was added to each well. At the same time, 20

µl of serum samples to be tested were added to the remaining wells. After that, 96-well containing the test and control samples were placed into an ELISA microplate using a multi-channel pipette and incubated at 21°C for 45 min. While waiting for the 96-well microplate finish incubate, a conjugate 1X was prepared by diluting the concentrated conjugate 10X to 1/10 in dilution buffer 3, as shown in Box 2.

Conjugate 10X : 1X

100 µl dilution buffer x 96 x 2= 19200 µl

Conversion from µL to mL = 19200 x 0.001= 19.2 mL (20mL dilution buffer)

Use dilution formula M1V1=M2V2 to obtain a volume of conjugate 1X needed to mix in a 20mL dilution buffer.

$$M1V1 = M2V2$$

$$10X(V1) = 1(20)$$

$$V1 = 2mL$$

Box 2: Conjugate 1X Preparation and Calculation

After incubation, the wells were emptied, and each well was washed three times with approximately 300 µl of the wash solution that had been prepared. 100 µl of the conjugate 1X was added to each well. The plate was covered, and incubation was carried out for 30 minutes ± 3 minutes at 21 °C ± 5 °C. After the second incubation, the wells were emptied, and each well was washed three times with approximately 300 µl of the wash solution. 100 µl of the substrate solution was added to each well. The plate was covered, and incubation was carried out for 15 minutes ± 2 minutes at 21 °C ± 5 °C in the dark room. Lastly, 100 µl of the stop solution was added to each well to stop the reaction. The optical density (O.D.) was then read and recorded at 450 nm.

CHAPTER 4

RESULT AND DISCUSSION

4.0 Epizootic Haemorrhagic Disease Virus (EHDV) infection in dairy cows from selected farms in Malaysia

Based on the results obtained, Pahang state showed (21) positives followed by (16) negatives and (2) doubts. However, Johor showed (20) positives followed by (170 negatives and 3) doubts. Meanwhile, Perak showed (18) positives followed by (1) negative and (1) doubtful and lastly, Sabah showed (16) positives followed by (2) negatives and (2) doubtful. The data obtained are represented as shown in Figure 1.

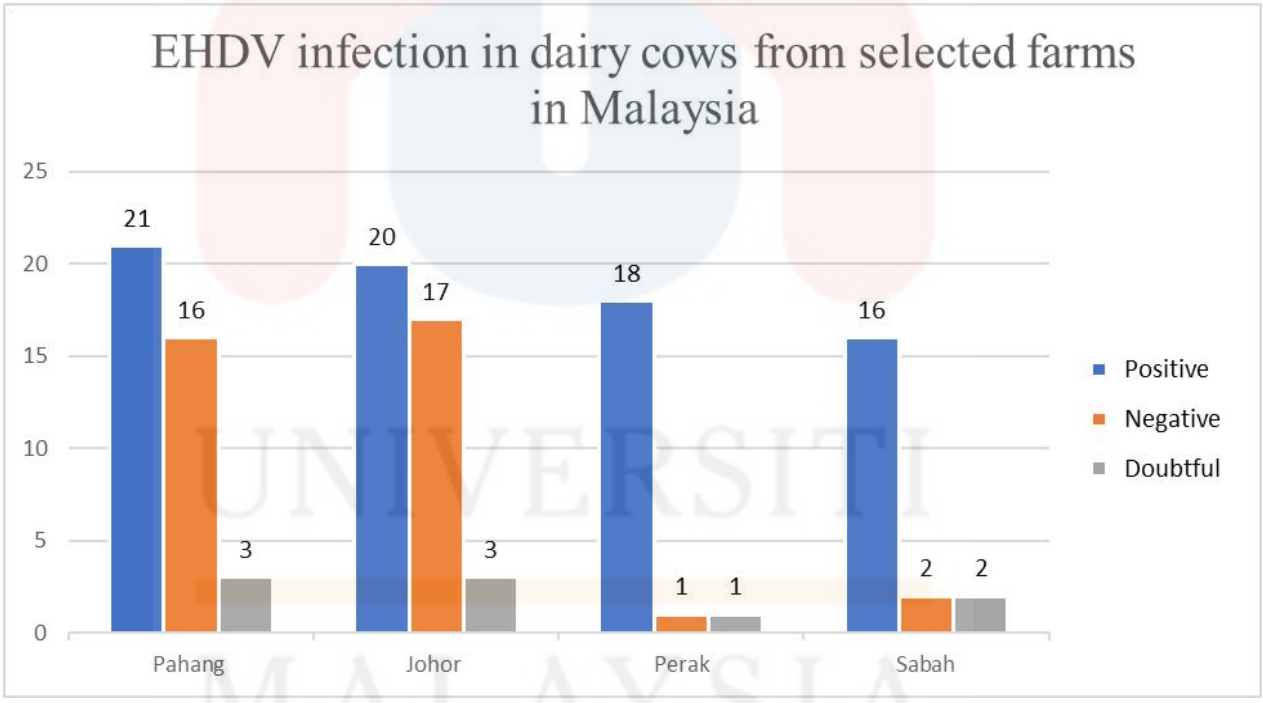

Figure 1: Status of EHDV infection in dairy cows from selected farms in Malaysia

4.1 Positive sample distribution across the Peninsular and East of Malaysia.

The distribution of positive samples of EHDV across the Peninsular Malaysia and East Malaysia was recorded. This shown in Figure 2. Pahang had the highest positive samples, with 28% of total positive samples, followed by Johor 26%, Perak 24% and Sabah 21%.

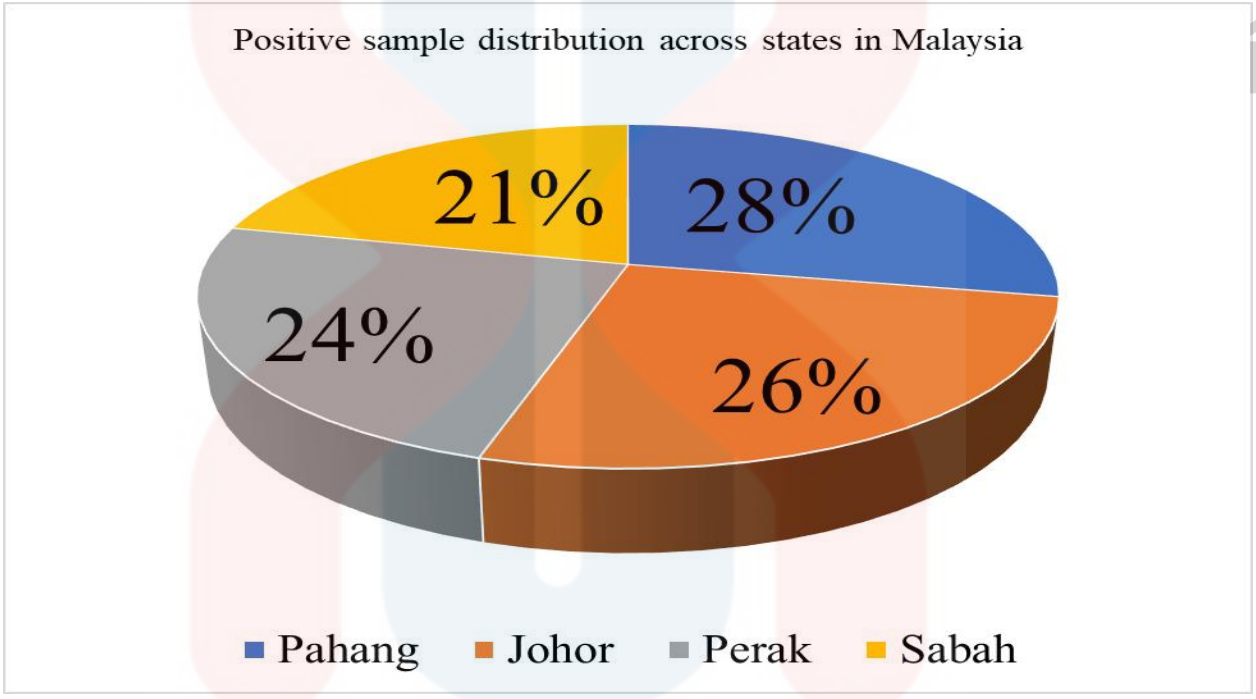

Figure 2: The percentage of positive sample distribution across states in Malaysia

4.2 Seroprevalence of antibody titre against EHDV

The seroprevalence of antibody titre against EHDV is shown in Table 3. The total seroprevalence was 63% in all four states tested. Perak had the highest prevalence (90%), followed by Sabah (80%). In contrast, Johor had the lowest prevalence at 50%, followed by Pahang, 53%.

Table 3: Seroprevalence of antibody titre against EHDV

State	Animals free from infection	Animals infected	Total animals	Prevalence (%)
Pahang	19	21	40	53
Johor	19	20	40	50
Perak	2	18	20	90
Sabah	4	16	20	80
Total	44	76	120	63

4.3 Discussion

This study was the first in Malaysia to successfully detect EHDV antibodies in serum samples using the serological diagnostic method Enzyme-linked Immunosorbent Assay (ELISA). This ELISA had been previously demonstrated to exhibit no cross-reaction with Bluetongue virus (BTV) and could detect exposure to all serotypes of EHDV using an EHDV VP7 monoclonal Ab (Breard *et al*., 2020). OIE approved this C-ELISA kit detecting antibodies against EHDV VP7 as an excellent selection for EHDV infection surveillance (OIE, 2021).

Based on the data obtained, positive results from the studies indicate exposure to EHDV infection because of the successful detection of the antibody against EHDV. However, an asymptomatic antibody prevalence could not be explained by poor detection. Several factors contributed to the observed EHDV stability, such as innate host resistance, maternal antibody transfer, vector species composition and seasonality (Niedbalski *et al*., 2019).

The total seroprevalence of EHDV antibodies recorded in Malaysian dairy cattle among the total samples collected was 63%, which was quite worrying as it was high as determined serologically by using the ELISA kits. An analysis of evidence proved that the values could increase when more study and specific investigation is performed. This was shown when a national serologic investigation conducted in China between 2014 and 2019 showed bovines are more susceptive to EHDV infection with a seroprevalence of 100 % compared to goats and sheep with a seroprevalence of 50% (Duan *et al*., 2022). However, another serological study was conducted in Guangdong province cattle farms in China, where the seroprevalence recorded a striking 57.87%, suggesting that EHDV infection in cattle is more widely spread than in other regions of China (Zhu *et al*., 2023). Furthermore, a similar study was done in French Guiana, France, after successfully isolating EHDV from blood samples using virological and serological analyses. The virological and serological prevalence rates for EHDV infection are 40% and 60%,

respectively. Thus, this indicates that frequent investigation and detection are carried out, raising concerns about whether we aim to prevent future epidemics before they arise. It is necessary to mention that although no outbreaks were reported in Malaysia, EHDV infection was recognized as a notifiable emerging disease and required strict regulations such as mandatory quarantine and control movement. This showed that other countries had taken precautionary steps to prevent future epidemics.

At the state level, the data obtained showed that EHDV was widely distributed throughout Malaysia. Perak was recorded to have the highest seroprevalence (90%), with 18 seropositive animals out of 20 total animals. Sabah followed this (80%) with 16 out of 20 total seropositive animals. Meanwhile, Pahang recorded seroprevalence of (53%) with 21 seropositive animals out of 40 total animals and Johor recorded the lowest seroprevalence (50%) with 20 seropositive animals out of 40. Despite having a similar climate environment and being geographically close, the seroprevalences of EHDV varied significantly from one state to the next. This variation is possibly affected by different factors such as distribution and abundance of the biting *Culicoides* spp, level of existing immunity and farm management.

Given that there is evidence of possible endemic EHDV infection in the state, this could result in significant economic losses in dairy cows, leading to reduced productivity characterized by reduced milk yield and high mortality (Kedmi *et al*., 2010). Therefore, alternative ways to overcome this EHDV infection in the state population are developing local vaccines and implementing insect control measures during high vector activity. Furthermore, a future study that revealed the economic impact of EHDV infection on cattle in Malaysia was required to ensure a further accurate evaluation of the effects of EHDV infection on food production in Malaysia.

CHAPTER 5

CONCLUSION

5.0 Conclusion

Conclusively, this study demonstrates that approximately two-thirds (63%) out of 120 serum samples EHDV infection was successfully detected using c-ELISA kit in dairy cows from selected farms in Malaysia. Despite its presence, this infection is spreading in domestic livestock in Malaysia but there are no apparent clinical symptoms observed in affected dairy cattle. In this study, antibodies against EHDV were detected in dairy cows with seropositive samples distributed across the Peninsular Malaysia and East Malaysia ranging from 21%, 24%, 26% and 28% in Sabah, Perak, Johor and Pahang, respectively. This shows that the seropositive rated of EHDV were higher in Peninsular Malaysia than in East Malaysia. However, seroprevalence of antibody titre against EHDV shows Perak had the highest prevalence (90%), Sabah (80%), Pahang, (53%), followed by Johor had the lowest prevalence at (50%), which required enhanced surveillance in the future.

5.1 Recommendations and Future Work

With the large number of imported cattle or dairy cows in Malaysia, the study can be improved by increasing the sample size across the states. Besides that, strategies in the epidemiological study of EHDV should be formulated. However, serological tests on imported dairy cows upon arrival in the country should be conducted at least 2 to 3 months later. Last, sentinel herds should be set up and monitored regularly for EHDV with the weather, distribution and spread of *Culicoides* spp.

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APPENDIX

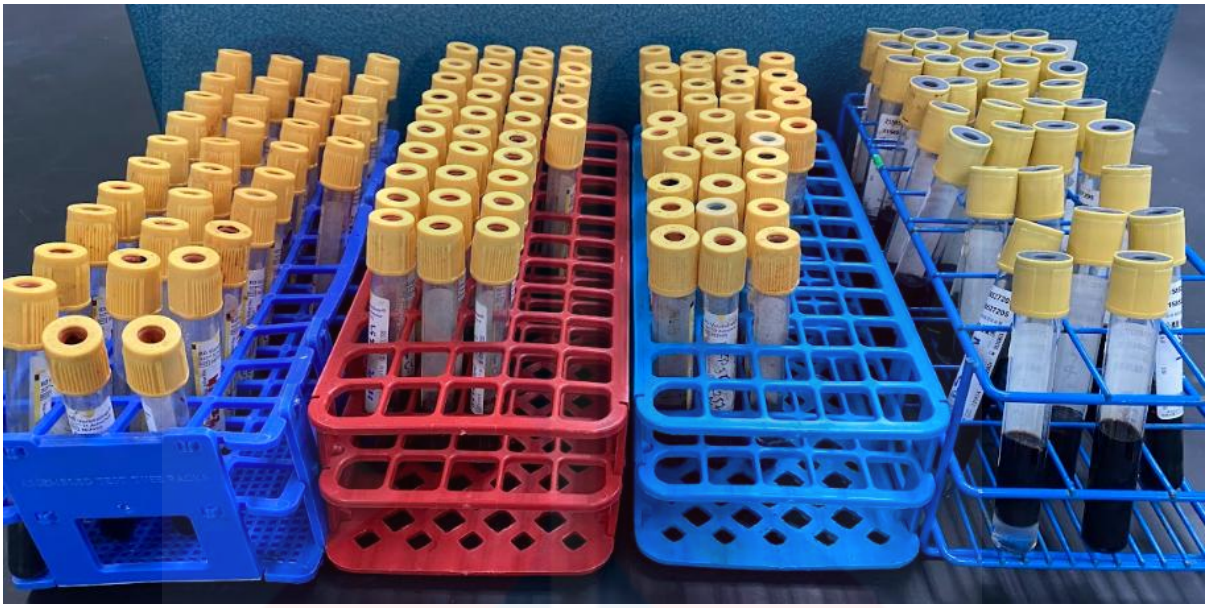

Figure 3: Bovine serum samples

Figure 4: Wash solution preparation

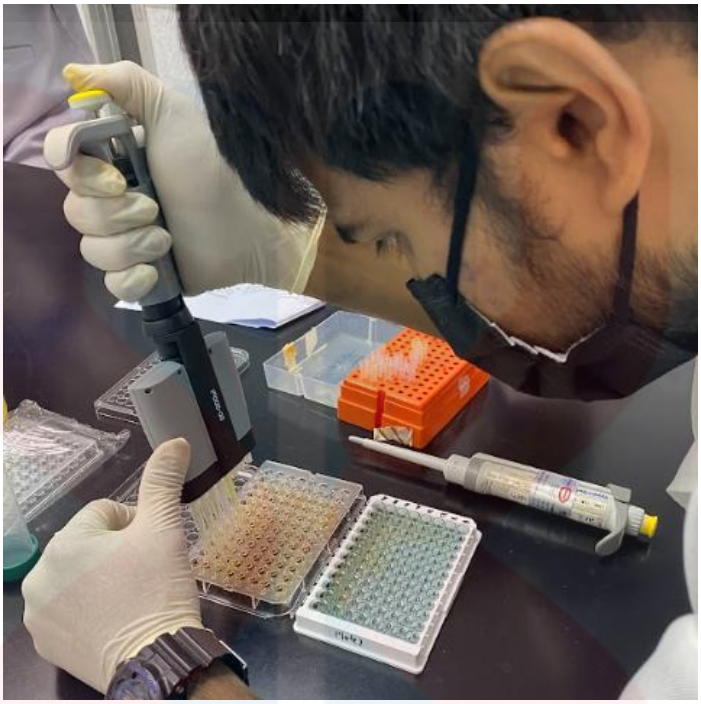

Figure 5: Transferring 96-well containing the test and control samples into an ELISA micro-plate using a multi-channel pipette

Figure 6: absence of antibodies, a blue solution appears which becomes yellow after addition of the stop solution while in the presence of antibodies, no coloration appears.