SEROPREVALENCE OF BLUETONGUE VIRUS INFECTION AND ASSOCIATED RISK FACTORS IN GOATS IN KELANTAN, MALAYSIA

By

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SEROPREVALENCE OF BLUETONGUE VIRUS INFECTION AND ASSOCIATED RISK FACTORS IN GOATS IN KELANTAN, MALAYSIA

ABSTRACT

Abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan in requirement on the course DVT 55204 - Research Project.

Bluetongue disease is an infectious viral vector-borne disease of ruminants and camelid species, which is mainly transmitted via biting midges of *Culicoides* genus. Sheep are the major hosts of Bluetongue virus, while other affected ruminant hosts such as goats manifest as subclinical, regarded as amplifying reservoir hosts in endemic areas. The latest retrospective study in Peninsular Malaysia was conducted in the year 2019 without risk factors analysis. This study aims to investigate the current seroprevalence of the Bluetongue disease among the domesticated goats in selected farms in Kelantan, Malaysia and its associated risk factors. A total of 40 serum samples from goats were collected from two ruminant farms in Kota Bharu and Bachok area, and the samples were tested using competitive Enzyme Linked Immunosorbent Assay (c-ELISA) to detect Bluetongue antibodies. The results showed that 33 goat serum samples (82.50%) were positive for Bluetongue virus, and revealed the significant risk factors are associated with vector control, animal source and farm management. In conclusion, this study presented a high seroprevalence of Bluetongue disease among the goat farms in Kelantan, suggesting this disease is still circulating within ruminant farms in Kelantan and could possibly cause a huge economic impact due to trade restrictions.

Keywords: Bluetongue, *Culicoides*, seroprevalence, goat, c-ELISA, Kelantan

SEROPREVALENSI DAN ANALISIS FAKTOR RISIKO PENYAKIT *BLUETONGUE* DI ANTARA KAMBING DI KELANTAN

ABSTRAK

Abstrak daripada penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan bagi memenuhi syarat daripada keperluan kursus DVT 55204 - Projek Penyelidikan.

Penyakit Bluetongue merupakan penyakit viral bawaan vektor yang dijangkit oleh serangga agas daripada genus *Culicoides* kepada haiwan ruminan. Biri-biri merupakan hos utama penyakit Bluetongue, manakala spesies ruminant yang lain seperti kambing merupakan hos takungan yang menunjukkan simptom sub-klinikal sahaja. Kajian retrospektif yang terkini di tahun 2019 hanya menunjukkan seroprevalensi penyakit Bluetongue di Semenanjung Malaysia tanpa analisis faktor risiko tersebut. Projek penyelidikan ini bertujuan untuk mengenalpasti seroprevalensi penyakit Bluetongue yang terkini dan faktor risiko yang berkaitan dalam kalangan kambing di Kelantan, Malaysia. Sejumlah 40 sampel serum telah dikumpul daripada dua ladang ruminan di kawasan Kota Bharu dan Bachok. Sampel serum tersebut telah digunakan untuk mengesan antibodi Bluetongue melalui ujian c-ELISA. Keputusan c-ELISA telah menunjukkan keputusan positif daripada 33 sampel serum kambing (82.50%), dan membuktikan bahawa pengawalan vektor, sumber haiwan dan pengurusan ladang merupakan faktor risiko yang penting dalam penyebaran penyakit viral tersebut. Kesimpulannya, kajian penyelidikan ini telah menunjukkan seroprevalensi yang tinggi di antara kambing di Kelantan, Malaysia, dan kemungkinan penularan penyakit ini yang masih berterusan di ladang ruminan dalam Semenanjung Malaysia akan menyebabkan kerugian ekonomi yang tinggi akibat sekatan perdagangan.

Kata kunci: Bluetongue, *Culicoides*, seroprevalensi, kambing, c-ELISA, Kelantan

CERTIFICATION

It is hereby to certify that we have read this research paper entitled of "**Seroprevalence of Bluetongue Virus Infection and Associated Risk Factors In Goats In Kelantan, Malaysia**", by Leong Zhi Zong and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirements for the course of DVT 55204 – Research Project.

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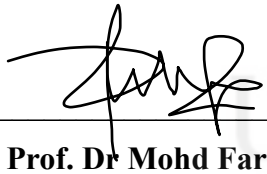

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Thank you so much.

DEDICATIONS

I would be honored to dedicate my work to my lecturers, family, and friends.

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LIST OF ABBREVIATIONS

AGID	Agar Gel Immunodiffusion Test
BT	Bluetongue disease
BTV	Bluetongue virus
c-ELISA	Competitive enzyme-linked immunosorbent assay
CFL	Compact fluorescent lamp
CI	Confidence Intervals
DVS	Department Veterinary Services
NSAID	Non steroidal anti inflammatory drugs
NS	Non-structural
O.D.	Optical density
OR	Odds Ratios
P	p-values
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
S/N	Sample-to-negative ratio
VP	Viral protein
WOAH	World Organization for Animal Health

LIST OF SYMBOLS

%	Percentage
℃	Degree Celsius
µL	Microlitre
X^2	Chi Square
nm	Nanometre

CHAPTER 1

INTRODUCTION

1.1 Research Background

Bluetongue disease (BT) is an infectious, vector-borne disease of ruminants and camelid species with worldwide distribution that has caused a significant economic impact in most tropical and subtropical countries (Saminathan, 2020). BT is a member of the Orbivirus genus in the Reoviridae family with a complex non-enveloped virus with a capsid and double-stranded RNA genome consisting of 10 segments of different sizes (Patel A. 2014). There are currently 27 serotypes of BTV recognised by the World Organization for Animal Health (WOAH) in 2021, but only 6 serotypes were isolated in Malaysia including serotypes 1, 2, 3, 9, 16, and 23 (Daniel et. al., 2004). It is mainly transmitted via biting midges (*Culicoides* genus) by feeding on susceptible ruminants, although direct vertical transmission, and venereal transmission were documented too.

In the recent outbreak in November 2023, the first BT was detected in Kent, England (NDCC, 2024). Temporary control zones in Kent, Norfolk and parts of Suffolk have been established as part of the disease mitigation measures. As a result of cases of BTV-3 in England, all moves of ruminants and their germinal products including semen, and embryos of ruminants from Great Britain to the island of Ireland were suspended.

The last BTV outbreak experienced in Malaysia was in October 1987, originating from a shipment of sheep imported from South Australia (Chiang, 1989). Since then, vigilant monitoring and control measures, including extensive serological surveys and restrictions on animal movement, were implemented to manage the spread of the disease. In 2009, antibodies for BTV were detected in a batch of imported sheep in Peninsular Malaysia (DVS, 2014). Subsequently, authorities in Peninsular Malaysia have implemented annual active surveillance programs.

Sheep are the major hosts of the BTV where the incubation period is approximately a week with a range of 2-10 days , while other affected ruminant hosts such as cattle and goats

commonly manifest as subclinical, with these species regarded as amplifying reservoir hosts in endemic areas (Jesse, 2020). However, the BTV-8 outbreaks in Europe during 2006 resulted in clinical disease affecting both goats and cattle (Wilson & Mellor, 2009).

BT disease presents in reproductive forms and acute vasculitis form which affects multiple organ systems. Typically manifests sudden fever, rapid weight loss, swelling of face, and abortions (WOAH 2021). The global impact of BT was estimated to be US$ 3 billion, with an annual indirect cost of approximately US$ 125 million in 1996 (Saminathan, 2020). It was suggested that the BTV-8 outbreaks in Northern Europe likely resulted in more significant economic damage than to any previous outbreak involving a single serotype of BT. The expenses primarily stem from the trade restrictions imposed throughout the outbreak period.

While the recent study indicated that BTV is still present at a low level among domestic and wild ruminant livestock in Malaysia, the significant economic impact resulting from the outbreak emphasizes the necessity for vigilant monitoring to prevent potential future outbreaks. As small ruminant breeds have varying disease susceptibility, BTV infections of livestock can occur unobserved and be detected only by active surveillance (WOAH, 2021). BT has been listed as one of the notifiable diseases by the WOAH due to its impact on the trade socioeconomic concerns for international trade in animals and animal products. Hence, this study will be conducted to investigate the seroprevalence of BT among the domesticated goats in Kelantan and its associated risk factors.

1.2 Research Problem

There were no further updates on the local seroprevalence of BT after the recent retrospective study in 2019. Additionally, the most recent retrospective study on BT in Peninsular Malaysia did not focus on Kelantan, apart from other states in West Malaysia. Furthermore, the risk factors associated with BT in goats in Malaysia have not been well studied.

1.3 Research Questions

1. Are the goats in Kota Bharu and Bachok provinces in Kelantan exposed to BT disease ?
2. What are the risk factors associated with the prevalence of BT among the goats in Kelantan ?

1.4 Research Hypothesis

1. There is moderate to high seroprevalence of BT among the goats in selected farms in Kelantan, Malaysia.
2. The associated risk factors for BT in goats are sex, age, source of animal, vector control and farm management.

1.5 Research Objectives

1. To determine the seroprevalence of BT among the domesticated goats in selected farms in Kelantan, Malaysia.
2. To determine the risk factors associated with the prevalence of BT among the goats in Kelantan, Malaysia.

CHAPTER 2

LITERATURE REVIEW

2.1 Classification and Morphology of Virus

BT is an infectious, non contagious disease in ruminants which is caused by Bluetongue virus (BTV). BTV is a type of species from the genus *Orbivirus* within the family *Reoviridae* (Belbis, 2017). It composed of 10 linear segments of double-stranded RNA (dsRNA), and its genome comprises ten segments (Seg-1 to Seg-10) encoding seven structural proteins (VP1-VP7) and five non-structural proteins (NS1, NS2, NS3/NS3a, NS4, and S10-ORF2) (Bumbarov et al. 2020).

The outermost layer consists of diffuse proteins (VP2 and VP5), while the transcriptionally active internal core is composed of two layers: the intermediate or middle layer (VP7) and an icosahedral innermost sub-core (VP3 and three minor enzymatic proteins, VP1, VP4, and VP6) (Belbis, 2017). The VP2 protein acts as ligand for cell receptors of mammals, which facilitates clathrin mediated endocytosis and acts as a major determinant of BTV serotype as it is responsible for the stimulation of serotype-specific neutralizing antibodies and hemagglutination. VP7 is highly conserved and represents an immunodominant BTV-specific antigen that is a major determinant of serogroup that determines the several distinct phylogenetic groups. The VP7 protein enhances BTV attachment and demonstrates high infectivity to *Culicoides* midges, but it shows limited infectiousness to mammalian cells or hosts. VP7 antigen is commonly used in complement-enzyme linked immunosorbent assay (c-ELISA) to detect anti-BTV antibodies (Mertens, 2005).

Individual BTV strains are distinguished within a species based on their genotypes and neutralization tests, with 27 recognized serotypes currently identified (WOAH, 2021). Variations in the sequence of the Seg-2 genome and its translated protein VP2 are responsible for determining the serotypes, along with partial influence from Seg-6 and its translated protein VP5. High error rates characterize BTV replication, as it lacks proofreading mechanisms found in other RNA viruses. This leads to random mutations and

reassortment of genome segments among BTV strains, contributing to variability between them.

2.2 Vector and geographical distribution

BTV serotypes are distributed globally, and it depends on the availability of susceptible host and vector *Culicoides* populations within the geographical regions. *Culicoides*, measuring between 1 and 3 millimeters, are small midges that rely on blood meals from mammals and birds. They are commonly found in warm, humid, and swampy areas, and around animal sheds where organic matter is abundant, providing ample feeding opportunities for *Culicoides*. *Culicoides* possess the capability to fly short distances, typically up to 5 kilometers, or they can be passively transported over long distances, reaching up to 100 kilometers, depending on wind speed (Elbers, 2015).

BT was initially documented in the late eighteenth century on the African continent. Since then, the disease has been reported across various continents and countries, spanning South and North America, Australia, Europe, and Asia, including the Indian subcontinent, where it emerged as a spreading disease, extending beyond its country of origin, primarily due to the movement and trade of ruminant livestock. At present, the disease is present in almost all continents except Antarctica. The complex epidemiology of BT can be affected by factors such as the density and distribution of *Culicoides* midges vector populations, the composition of host species, climatic conditions, and the diversity of virus strains.

The most recent retrospective seroprevalence study of BT infection was published to study the status in Malaysia ruminant livestock in Peninsular Malaysia from 2013 to 2019. The overall status of BTV was 20.18% (1,975 / 9,787), with 30.68% in goat, whereas the sample distributions by states in Peninsular Malaysia shows that Kelantan is among the states with the highest seropositive level (38.34%) (Pauzi, 2022). However, the seroprevalence rate of BTV identified in this study remains low compared to previous findings in India (43.68%) and Nepal (45.20%).

Intensive diagnostic test was only introduced in 1987 following outbreaks in imported sheep from South Australia, in which 159 out of 2,249 imported Australian sheep were affected with clinical BT and 82 (51.6 %) of them died after 10 weeks post-arrival in Malaysia. (DVS, 2020) Since then, BT has been included in the National Animal Disease Surveillance Program, aiding in identifying and controlling significant and notifiable animal diseases. This initiative aims to safeguard the livestock industry and promote animal welfare within the country.

2.3 Host and clinical manifestation

Sheep are the major hosts of BT, in which the incubation period is approximately 2-10 days (Jesse, 2020), while other affected ruminant hosts such as cattle and goats commonly manifests as subclinical, with these species regarded as amplifying reservoir hosts in endemic areas.

BT presents in reproductive forms and acute vasculitis forms, affecting multiple organ systems. Vasculitis typically manifests with a sudden fever followed by depression, reduced appetite, and rapid weight loss (Schwartz, 2008). Animals affected by the disease may display swelling in various areas such as the lips, tongue, throat, ears, and brisket. Additional symptoms include excessive drooling and reddening or blueness of the oral mucosa, including the tongue. The reproductive aspect of the disease exhibits abortions, stillbirths, and the birth of weak, non-viable "dummy calf syndrome" (WOAH, 2021). Reluctance to move is a common symptom, with severe cases possibly experiencing torticollis.

2.4 Pathogenesis

Culicoides midges become infected with BT when they feed on the blood of an infected mammalian host. The virus then replicates in the midgut cells and escapes into the hemocoel. Over the course of 6 to 8 days, BTV infects and replicates in the midge's salivary glands. Once this process is complete, the midge can transmit the virus to other susceptible vertebrate hosts through its bite (Wilson & Mellor, 2009).

BTV is carried from the skin to local lymph nodes by dendritic cells, where it initially replicates. The virus then enters the bloodstream, leading to primary viremia, which distributes BTV to secondary organs including nearby lymph nodes, the spleen, and the lungs. BTV primarily replicates in vascular endothelial cells and mononuclear phagocytic cells, including macrophages and lymphocytes. This replication results in endothelial damage and the release of excessive levels of cytokines and vasoactive mediators, leading to a cytokine storm. It then causes a cytokine storm that increases vascular permeability, which leads to severe hemorrhages, edema and effusions, thrombosis, infarction, and disseminated intravascular coagulation. Furthermore, BTV infection in pregnant ruminants can lead to cerebral malformations in their offspring (Drew, 2010).

2.5 Diagnosis of Bluetongue Virus

The c-ELISA is commonly used to measure the concentration of BTV antibodies in ruminant sera. It is designed to measure BTV-specific antibodies without detecting antibodies that cross-react with other *Orbiviruses* (WOAH, 2021). The c-ELISA is considered less expensive and an ideal technique for studying BTV distribution, monitoring the vaccination status as well as planning of control and eradicating policies.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR are highly sensitive and rapid diagnostic techniques for the detection of the BTV genome in various samples when compared to virus isolation or neutralization assays (Maan, 2016). Conventional RT-PCR has an advantage over serological methods by identifying different BTV serotypes within a single isolate containing mixed serotypes. Additionally, RT-PCR is used for BTV serotyping and can detect BTV-RNA in samples up to 6 months post-infection. (De Leeuw, 2015)

2.6 Treatment available and prevention management

Currently there are no effective and specific therapeutic drugs available for animals clinically infected with BT. Symptomatic treatment, including administering antipyretics, antihistamines, or nonsteroidal anti-inflammatory drugs (NSAID), is commonly used to reduce inflammation and pain. This approach aims to decrease morbidity and mortality in

susceptible ruminants and mitigate economic losses, as animals diagnosed with BT undergoing supportive treatment have a good prognosis for survival (Gamsjäger, 2024).

Insecticides are routinely used to control adult *Culicoides* midge populations, thereby reducing the risk of BT transmission. Topical application of "pour-on" products and washing the animals with synthetic pyrethroids or organophosphate compounds are employed to control both ectoparasites and endoparasites in ruminants for up to 3-5 weeks . Saturation spraying strategies of organophosphate are regularly used to control the populations of Culicoides too (Narladkar, 2006).

CHAPTER 3

MATERIAL AND METHODOLOGY

3.1 Ethical Considerations

Ethical approvals for using animals in the current study was obtained from the Animal Ethics Committee of the Universiti Malaysia Kelantan. (UMK/FPV/ACUE/FYP/009/2024).

3.2 Methodology

3.2.1 Study area

The study area involved two goat farms in Kelantan, Malaysia. Out of the eleven provinces in Kelantan, two farms were selected from two different provinces of Kelantan, which are Kota Bharu and Bachok

3.2.2 Study design

This was a cross-sectional study design in which the selected serum samples were processed and tested for the presence of antibodies against BTV from the sample population at the point of sampling.

3.2.3 Study population

A total of forty goats (n=40) from Kota Bharu and Bachok province in Kelantan, Malaysia were sampled randomly, twenty-three (23) goats from Farm A, and seventeen (17) goats from Farm B.

3.3 Study Criteria

3.3.1 Inclusion Criteria

Two goat farms in Kota Bharu and Bachok, Kelantan were chosen by convenience sampling. Meanwhile, the subjects, the goats on the selected farms were chosen by simple random sampling, regardless of age, sex, breed, health status, and body weight.

3.3.2 Exclusion Criteria

There was no exclusion criteria listed in this study.

3.4 Sampling Method and Procedures

3.4.1 Animal Profile

The physical examination of the selected goats was performed, and the patient signalment and vital parameters were recorded in animal profile form prior to blood sampling.

Table 3.1: Animal profile form

Goat ID	Sex	Age	BCS	Vector Control Program	Farm Management	Source of Animal

3.4.2 Sample Collection

Forty blood samples were collected via the jugular venipuncture method using 1-inch 21-gauge vacutainer needles. The jugular groove was palpated with the free hand to locate the jugular vein. The thumb was pressed firmly into the jugular groove to raise the vein, positioned far enough caudally to leave adequate space for needle insertion and sample collection. The needle was then placed over the jugular groove cranial to the thumb at a 45° angle to the skin and inserted into the vein, following the direction of the jugular. The vacutainer holder was stabilized between the thumb and index finger of the hand raising the vein, while the other fingers maintained the vein's position. The vacutainer was inserted into the vacutainer holder, with the index and middle fingers placed over the holder's base, and the palm was used to press the blood tube into the holder, securing it onto the needle. While doing so, the other hand kept the needle stable in the vein. Once the vacutainer was 2/3 full, it was removed, followed by the vacutainer holder and needle. The blood samples were transferred to plain vacutainer tubes to allow serum separation without centrifugation. Each tube was labeled with an ID number using a permanent marker. After labeling, the samples were kept chilled in a cold box packed with ice packs during transportation. Upon arrival at the laboratory, the samples were stored in a chiller at 2 - 8 °C temperature.

3.4.3 Sample Processing

Blood clots were removed by centrifuging the samples at speeds between 2500 - 3000 rpm. After centrifugation, the serum was extracted using a Pasteur pipette and transferred into microcentrifuge tubes. Each tube was labeled with the corresponding identification number for accurate record-keeping. The processed samples were then stored in a chiller at 2 - 8°C temperatures.

3.4.4 Serology Detection - c-ELISA

The c-ELISA kit (IDVet, France) was used to perform serological detection of BTV from the samples. The kits included various components, reagents, a microplate coated with VP7 recombinant protein, a concentrated conjugate, positive and negative controls, dilution buffer 2, 20X wash concentrate, substrate solution, and 0.5M stop solution.

First, all apparatus and equipment, including multichannel pipettes, disposable tips, a 96-well microplate, distilled water, a wash system, and a 96-well microplate reader, were prepared. Distilled water was used to prepare a wash solution by diluting the wash concentrate (1:20). All reagents were homogenized by inversion or vortexing and brought to room temperature. A final serum dilution of 1:50 was used.

100µL x 96 wells x 1 = 9.6 ml

≈ 10 ml

$$M1V1 = M2V2 \quad (3.1)$$

$$(10)\ V = (1)\ (10)$$

$$V1 = 1\ \text{mL}$$

*1 mL of Conjugate Concentrate 10x was diluted in 9 mL of Dilution Buffer to get Conjugate 1x

The conjugate 1x was prepared by diluting the Concentrated Conjugate 10x to 1:10 in Dilution Buffer 3. Calculation was done as stated below:

300µL x 2 x 96 = 57600 µL

≈ 60 mL of Wash Solution was used for 2 wash steps

$$M1V1 = M2V2 \quad (3.1)$$

$$(20)\ V = (1)\ (60)$$

$$V1 = 3 \text{ mL}$$

* 3 mL of Wash Concentrate (20x) was diluted in 57 mL distilled water to get Wash Solution (1x)

Next, a pre-dilution microplate was placed in the 96-well plate. Then, 5 µL of the negative control was added to wells A1 and B1, and 5 µL of the positive control was added to wells C1 and D1. Following this, 5 µL of each test sample was added to the remaining wells, and 245 µL of dilution buffer was added to each well. The plate was covered and incubated for 45 minutes ± 4 minutes at 21 °C ± 5 °C.

After incubation, the wells were emptied and washed three times with at least 300 µL of wash solution per well. Then, 100 µL of substrate solution was added to each well, and the plate was covered. The plate was incubated for 15 minutes ± 2 minutes at 21 °C ± 5 °C in a dark place. After incubation, 100 µL of Stop Solution was added to each well in the same order as before to stop the reaction. The optical density (O.D.) at 450 nm was read and recorded. Before interpreting the result, validation was determined. The test was validated if the mean value of the Negative Control optical density (O.D.) was > 0.7, and the ratio of the mean values of the Positive and Negative Control O.D. was < 0.3. After result validation, the competition percentage was calculated by S/N (%) to determine the status of the sampled goat against BTV antibodies, whether positive or negative.

Formula to determine S/N % = OD sample / OD nc x 100 (3.2)

Table 3.2 : Interpretation of BTV status by S/N %

Result	Status
S/N % < 40%	Positive
S/N % > 40%	Negative

3.5 Data Collection

The data resulting from the serological detection of the BTV was manually recorded and tabulated using Microsoft Excel.

3.6 Data Analysis

The collected data was analyzed using IBM SPSS Statistics 27 software. Categorical data was summarized by calculating frequencies and percentages. These summaries were displayed both in frequency tables and in bar graphs to facilitate comparison and visualization of the data.

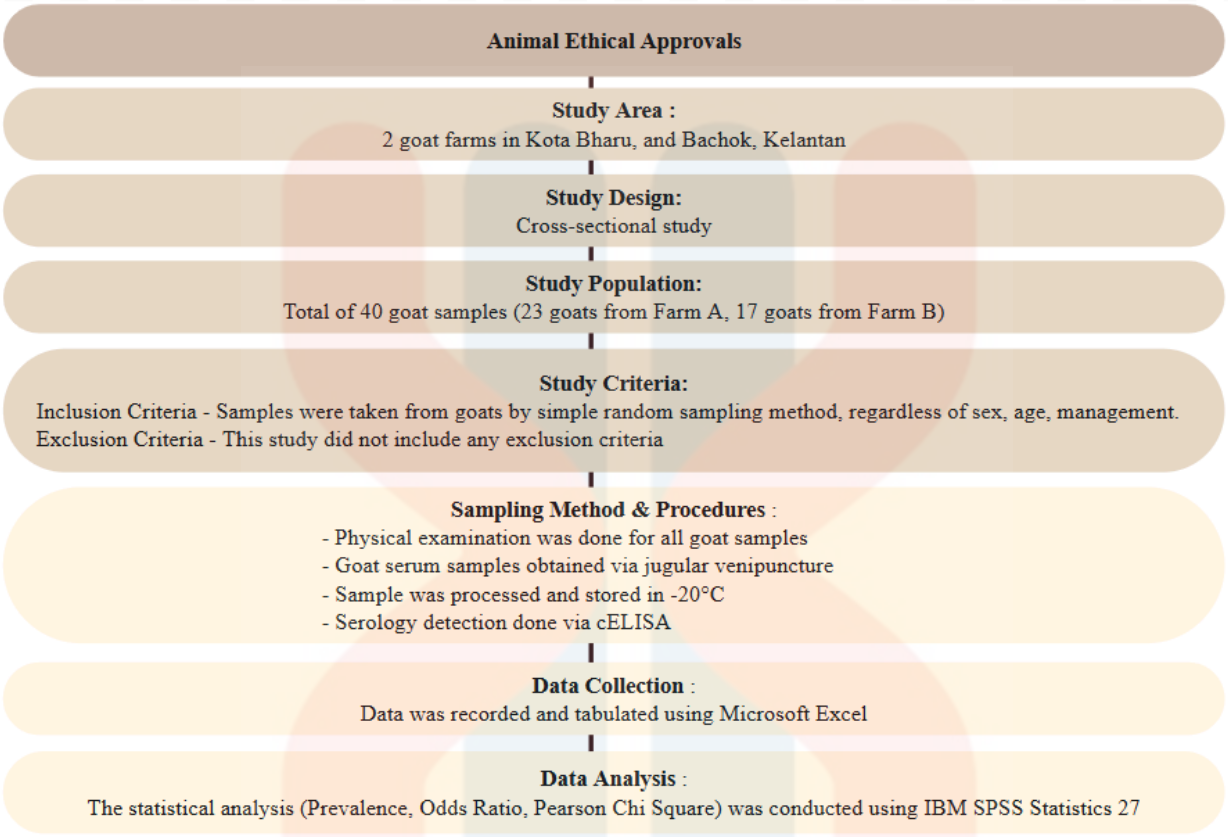

Figure 3.1 : The summary of methodology conducted in this study.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Results

In this study, a total of 40 goat serum samples have been obtained from 2 farms in Kelantan, 23 goats from Farm A and 17 goats from Farm B. The c-ELISA result revealed up to 82.50% (33/40) seropositive among this study's goat samples, suggesting high BT seroprevalence in goats in Kelantan.

Results of the univariate analysis using the Chi-square test revealed no significant association between the sex ($\chi 2 = 0.016$, $p>0.05$) and age ($\chi 2 = 1.212$, $p>0.05$) of the animals with the seropositivity of BTV, suggesting that the observed differences are likely due to random variation rather than a true effect of sex. However, the usage of vector control ($\chi 2 = 6.484$, $p<0.05$), the source of farm animals ($\chi 2 = 6.484$, $p<0.05$) and farm management ($\chi 2 = 6.484$, $p<0.05$) are statistically significant in this risk factor analysis study.

Table 4.1 : Seroprevalence of BT in goats of Kelantan in this study.

Farm	Total number of samples (n)	Positive (n)	Negative (n)
A	23	22	1
B	17	11	6
Total (n)	**40**	**33**	**7**
Total Percentage	**100 %**	**82.50 %**	**17.50 %**

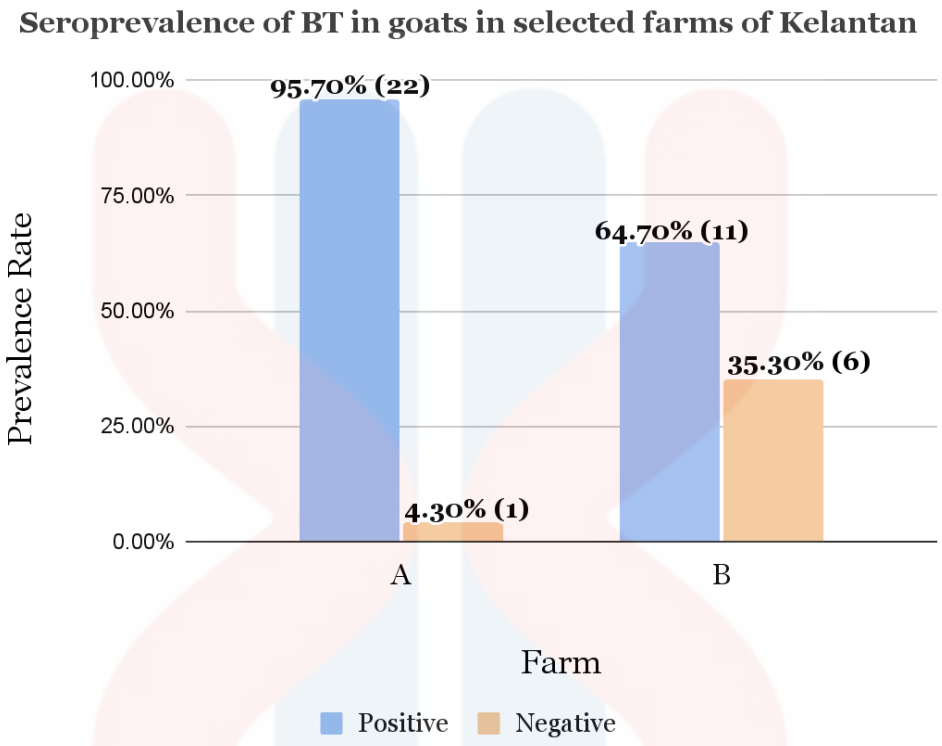

Figure 4.1: Bar chart of seroprevalence of BT in selected goats of Kelantan in this study.

Table 4.2 : Relationship between BTV seropositive levels with the potential risk factors.

Variables	Total Number of Samples (n)	Positive Samples (n)	Total Seropositive Level (%)	χ2	p	OR	95% CI
Age (Years)				1.212	0.271		
<1	5	5	100%			-	-
1-6	35	28	80.0%			-	-
Sex				0.016	0.900		
Male	22	18	83.3%			1	
Female	18	15	81.8%			0.90	0.173-4.669
Vector Control				6.484	0.011*		
No	17	11	64.7%			1	
Yes	23	22	95.7%			0.083	0.009-0.781
Source of Animal				6.484	0.011*		
Local	17	11	64.7%			1	
Acquired	23	22	95.7%			0.083	0.009-0.781
Farm management				6.484	0.011*		
Semi Intensive	17	11	64.7%			1	
Intensive	23	22	95.7%			0.083	0.009-0.781

**p value < 0.05 is considered significant.*

Chi Square (χ^2), p-values (p), Odds Ratios (OR), 95% Confidence Intervals (95% CI)

4.2 Discussion

The findings of this study revealed a high seropositivity rate of BTV among goats sampled in Kelantan, with 82.50% (33/40) testing positive for BT antibodies. This result contrasts sharply with the recent study by Pauzi (2022), which reported a much lower BTV seroprevalence rate of 38.34% (125/326) across Peninsular Malaysia. The low detection of BTV in Pauzi (2022) retrospective study is likely attributed using the Agar Gel Immunodiffusion Test (AGID). A study has shown that AGID has lower sensitivity and specificity (38.23%, 84.83%) compared to c-ELISA in detecting BTV antibodies, in which c-ELISA revealed 100% of the AGID positive samples, with additional positive samples that AGID did not detect (Shringi, 2005). This limitation may have resulted in false-negative results, increasing the likelihood that animals with prior exposure to BTV were incorrectly classified as negative. In addition, c-ELISA also has the advantage of detecting BTV-specific antibodies (sensitivity and specificity >95%) without cross-reacting with other Orbiviruses by using only a single BT serogroup monoclonal antibody, which appear to bind to the amino-terminal region of the core protein VP7 (Shringi, 2005; Niedbalski, 2011).

Foreign livestock animals that were imported from Thailand and Australia were reported to have higher BTV seroprevalence than the farm which uses native livestock in this study. Several reports from these countries have shown the possibility of active circulation of BT among ruminant livestock. Recent research conducted on molecular detection of BTV in *Culicoides* midges from western Thailand has shown that 9 out of 72 samples were positive by BTV RT-PCR analysis, and the latest seroprevalence study in Thailand reported up to 73% BTV seropositivity in goats of Thailand (Apiwatnakorn, 1995; Fujisawa, 2021). Furthermore, a BTV seroprevalence study in New South Wales, Australia in 2023 also revealed up to 42.7% of BTV seropositive sheep (Kirkland, 2024). In addition, according to DVS (2020), only live small ruminants including sheep and goat from Australia are required to screen for BT 6 months prior to exportation. However, the same preventive measures were not taken for the live animals imported from Thailand. These suggest that the goat samples collected have possibly been exposed to BT from their origin countries prior to the animal importation process into Malaysia.

Vaccination of BT is practiced in several countries to limit direct losses, minimize the circulation of BTV and allow safe movement of animals. Prior BT vaccination will induce production of specific antibodies that serology tests can detect. However, serological tests like c-ELISA cannot differentiate antibodies between the BT vaccine strain and the wild strains among the seropositive goats. Serotyping of the virus strain using RT-PCR by employing serotype-specific primers is required if the investigation interests are towards obtaining specific information regarding isolated serotypes (Maan, 2012). But Malaysia is still hesitant to adopt the use of any BT vaccine and does not implement it locally, likely due to the inability of the vaccine to provide cross-protection against other strain of BTV, and the concerns over live attenuated vaccines including the potential for virus spread from the vaccinated herd via *Culicoides* vectors, lead to virulence reversion or reassortment of vaccine virus genes with wild-type virus strains (Ferrari, 2005; DVS, 2014; WOAH, 2021).

The statistical analysis indicates an unusual trend where animals in the vector-controlled group have a higher prevalence and contradicts most of the research papers that suggest vector control practice significantly reduces the rate of BTV infection in the farm (Caporale, 2014 ; Benelli, 2017 ; Pauzi, 2022). The goat samples from farm A in this study which practices usage of red compact fluorescent lamp (CFL) as vector control were shown to have a higher prevalence rate (95.7% , 22/23) compared to the group without vector control (64.7%, 11/17). The result could be influenced by the control measures' effectiveness that impact vectors' presence. Bargini (2010) has done an entomology study on the attraction of insects with different light wavelengths, and revealed that flying insects are less attracted by dim and longer wavelength colors of light, such as red color with a wavelength of 620–750 nm. However, the emission of UV radiation from CFL is likely to increase insect attraction as compared to UV‑absent LED light, which contradicts with the initial goal of installing artificial light for insect-repellent purposes (Wakefield, 2016). Nocturnal insects, including *Culicoides* midges are more drawn to chemical and olfactory cues rather than visual cues, which suggests usage of the conventional vector control measures such as the application of insecticide chemical and electrical flying traps are more effective with sufficient supportive scientific data (Borkakati, 2019).

The risk factor analysis in this study also shows that there is a significant association between farm management and the seropositivity of BT, which revealed intensive farming practice has higher seroprevalence than semi-intensive practice in this study. It is in contrast with other studies that revealed higher seropositivity in animals reared under semi-intensive farm practice, likely due to controlled environments that can be achieved in intensive farming, including feeding management, exposure to outdoor pasture and easier monitoring of the physical condition of the animal. However, improper waste management of the farm such as piling of feces and increase moisture on the feces is also a risk of providing a breeding ground for the *Culicoides* vector as more than half of its species bred specifically in the animal dung which is rich in organic matter as their food source (Zimmer, 2014). It is suggestive that these *Culicoides* midges are observed close to the livestock animals because they provide an easier opportunity for blood meal and oviposition sites. In addition, both farms are also practicing an open farming system where there is no physical barrier to inhibit entry of the vectors. Therefore, the farm management factor in this study is likely influenced by other risk factors such as vector control practice and animal source.

There is no association between the age and the seroprevalence of the BTV in these goat samples. Previous studies have indicated that adult groups of animals exhibit a higher rate of seropositivity, which researchers attribute to the increased likelihood of these adult animals being released to pastures for grazing, enhancing exposure to vectors and subsequent BT infection (Adam, 2014). In that context, farm management is more likely to contribute to the seropositivity instead of the age factor, which suggests that goats raised under similar farm practices throughout their life cycle are uniformly exposed to the risk of infected vectors. Furthermore, the statistical analysis in this study also finds no significant relationship between the sex factor and the seroprevalence of BTV, which align with most of the recent studies that concluded there is no difference in seroprevalence of antibodies to BTV in male and female animals (Adam, 2014; Sohail, 2019).

Although the seroprevalence rate in this study showed high seropositivity, all the seropositive goats appeared bright, alert and responsive during the sampling period with no

observable clinical signs suggesting of BT. It has been studied that the goat can become infected with BT but often asymptomatic. In sheep, however, the disease can vary from a hyper-acute form with up to 30% mortality to a mild or subclinical form where recovery happens within a few days (Schwartz, 2008). The virus persists in the bloodstream of sheep and lasts up to 30 days, but it can remain for more than 60 days in goats (Maclachlan, 2015). The level of viremia in infected goats is comparably higher than in infected sheep, suggesting that BTV can replicate extensively in goat's tissues, act as a viral reservoir without causing cellular damage, likely due to a lack of harmful viral or immune responses in goats (Caporale, 2014).

The risk of BT transmission from asymptomatic reservoirs, such as goats, to other susceptible ruminant livestock can lead to economic losses due to production declines resulting from a potential outbreak. DVS should be informed about the high seropositivity of BTV in this study, serving as an early warning to facilitate timely preventive measures in the future. If any ruminant livestock presented with related clinical signs including spiking fever, edema of the lips, tongue, throat, excessive salivation, mucosa hyperemia or cyanosis, active surveillance of BT via serological test should be conducted (Newcomer, 2021). According to DVS (2020) Bluetongue disease protocol, the seropositive samples are required to perform a serum neutralization test (SNT) as a confirmatory diagnosis test. Vector surveillance within 150km of the surveillance zone to confirm the presence of vector species (*C.peregrinus, C.orientalis, C.shortti, C.insignipennis, C.flavescens, C.gewertzi dan C.brevitarsis*) by morphological identification or polymerase chain reaction (PCR). Further extensive entomology study on the distribution of *Culicoides* vector is required, including research activities based on the geographical location, density of vector and animals, and seasonal distribution in that area to predict the potential of BT outbreaks (Figure 4.2) (DVS, 2014). Current studies revealed that vector-borne diseases could spread more rapidly than before due to climate change, as prolonged high temperatures combined with rainfall lead to higher humidity levels that encourage the breeding of *Culicoides* species (Benelli, 2017).

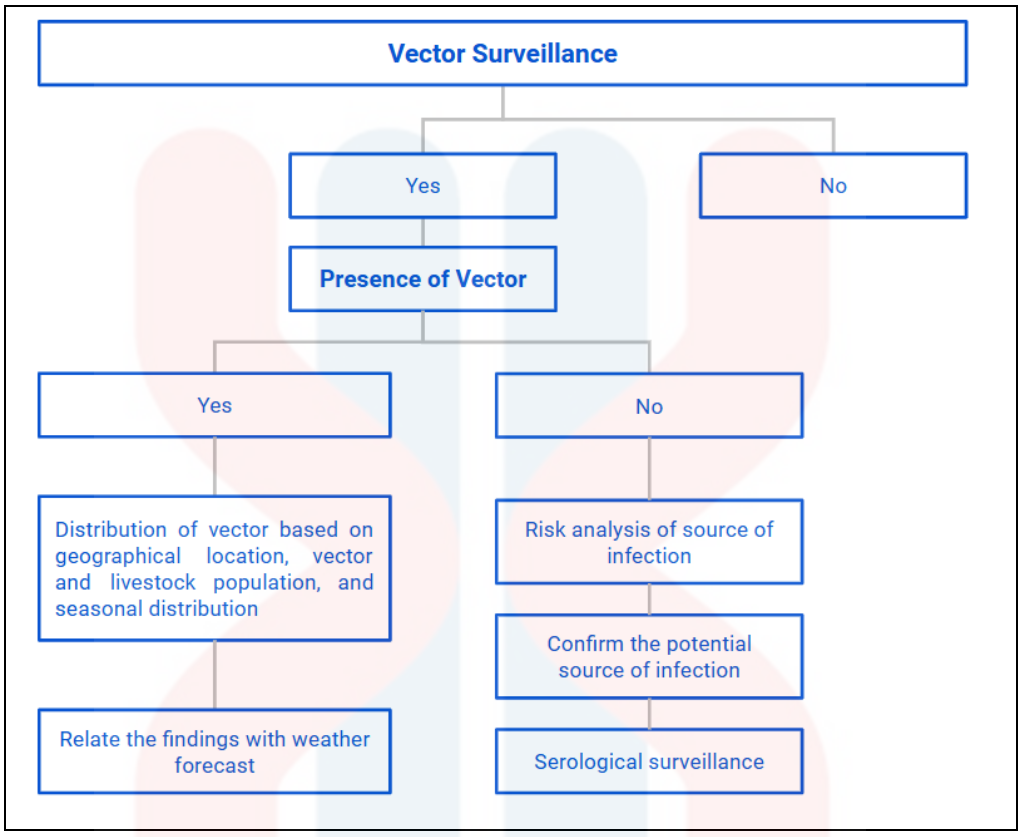

Figure 4.2: Decision tree of vector surveillance in Bluetongue disease (adapted from DVS, 2014)

Several limitations in this study restrict the insights of the findings to a wider population in Kelantan, including small sample size, and variability of the sampled farm, and species. The limited number of samples could affect the statistical power, which lead to reduced detection ability of the trends and relationships between the seroprevalence of BT and the associated risk factors. Furthermore, the limited geographic coverage, with farms sampled from only two specific provinces in Kelantan rather than across the entire state may restrict the ability to generalize the results to the broader agricultural landscape of Malaysia.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The data obtained in this study concluded that BT may have been circulating within the goat population since the first outbreak in Malaysia, and presented with high seroprevalence of BTV in these selected farms in Kelantan. The risk factors analysis shows significant association with the source of animal, vector control practice and farm management. No association was shown between the sex and age of the animal towards the seroprevalence of BT in this study. In addition, none of the seropositive goats exhibited clinical signs indicative of BT at time of sampling.

5.2 Recommendation For Future Work

It is suggestive that more extensive future studies should be done by including a larger sample size, and more variety of animal species, and ruminant farms across different provinces of Kelantan to provide a comprehensive understanding with higher statistical power of the epidemiology of BT in ruminant livestock. Furthermore, c-ELISA is recommended as the main serological diagnostic method for future BT surveillance studies as it provides higher sensitivity and specificity in BTV antibody detection. Active surveillance of *Culicoides* vector species around the radius of the farm with seropositive animals should be conducted, followed by vector virus isolation via RT-PCR to evaluate the potential of disease circulation and outbreak in Peninsular Malaysia.

APPENDICES

Figure 6.1 : Group photos of the team who went for blood sampling in the farms

Figure 6.2 : Red compact fluorescent lamp (CFL) that was used by farm A as vector control.

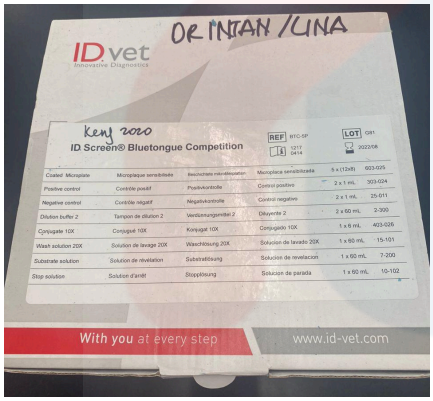

Figure 6.3: ID Screen Bluetongue c-ELISA kit was used in this study

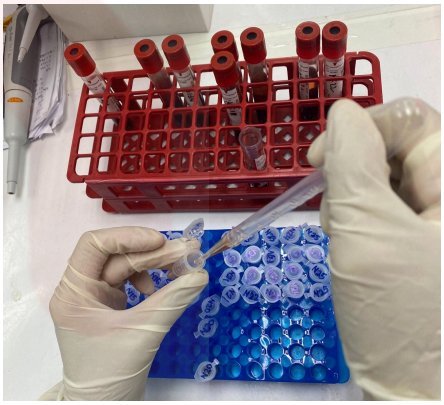

Figure 6.4 : Serum extraction from the whole blood samples collected.

Figure 6.5 : Adding Stop solution into the test kit

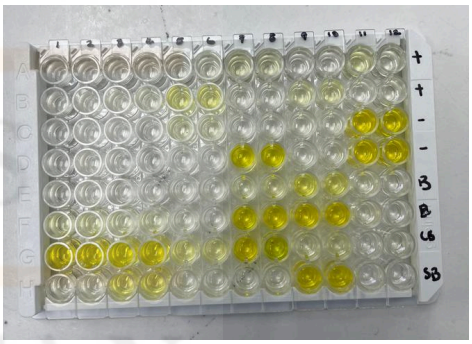

Figure 6.6 : Bluetongue c-ELISA test kit result

REFERENCES

Adam, I. A., Abdalla, M. A., Mohamed, M. E., & Aradaib, I. E. (2014). Prevalence of bluetongue virus infection and associated risk factors among cattle in North Kordufan State, Western Sudan. BMC veterinary research, 10, 94. https://doi.org/10.1186/1746-6148-10-94

Apiwatnakorn, B., Bura, P., & Pasavorakul, O. (1995). Serological study for Bluetongue in Thailand. In Bluetongue Disease in Southeast Asia and the Pacific: Proceedings of the First Southeast Asia and Pacific Regional Bluetongue Symposium, Greenlake Hotel, Kunming, P.R. China, 22-24 August 1995.

Belbis, G., Zientara, S., Bréard, E., Sailleau, C., Caignard, G., Vitour, D., & Attoui, H. (2017). Bluetongue Virus: From BTV-1 to BTV-27. Advances in virus research, 99, 161–197. https://doi.org/10.1016/bs.aivir.2017.08.003

Benelli, G., Buttazzoni, L., Canale, A., D'Andrea, A., Del Serrone, P., Delrio, G., Foxi, C., Mariani, S., Savini, G., Vadivalagan, C., Murugan, K., Toniolo, C., Nicoletti, M., & Serafini, M. (2017). Bluetongue outbreaks: Looking for effective control strategies against Culicoides vectors. Research in veterinary science, 115, 263–270. https://doi.org/10.1016/j.rvsc.2017.05.023

Borkakati, R. N., Venkatesh, M. R., Saikia, D. K., & Sasanka, S. B. (2019). A brief review on food recognition by insects: Use of sensory and behavioural mechanisms. Journal of Entomology and Zoology Studies, 7, 574-579.

Bumbarov, V., Golender, N., Jenckel, M., Wernike, K., Beer, M., Khinich, E., Zalesky, O., & Erster, O. (2020). Characterization of bluetongue virus serotype 28. Transboundary and emerging diseases, 67(1), 171–182. https://doi.org/10.1111/tbed.13338

Caporale, M., Di Gialleonorado, L., Janowicz, A., Wilkie, G., Shaw, A., Savini, G., Van Rijn, P. A., Mertens, P., Di Ventura, M., & Palmarini, M. (2014). Virus and host factors affecting the clinical outcome of bluetongue virus infection. Journal of virology, 88(18), 10399–10411. https://doi.org/10.1128/JVI.01641-14

Chiang, B. (1989). An outbreak of Bluetongue in imported sheep. Proceedings of the First Congress, MSAVA. 49–52.

Daniel, P., Pritchard, L., & Eaton, B. (2004). Global situation. Veterinaria Italiana, 40(3). Retrieved from https://www.izs.it/vet_italiana/2004/40_3/20.pdf.

De Leeuw, I., Garigliany, M., Bertels, G., Willems, T., Desmecht, D., & De Clercq, K. (2015). Bluetongue virus RNA detection by real-time rt-PCR in post-vaccination

samples from cattle. Transboundary and emerging diseases, 62(2), 157–162. https://doi.org/10.1111/tbed.12100

Department of Veterinary Services, 2014. Protokol Veterinar Malaysia-Penyakit Bluetongue. Ministry of Agriculture and Food Industry Malaysia. No Dokumentasi: PVM1(3):1/2014

Department of Veterinary Services, 2020. Requirements For The Importation Of Sheep And Goats Into Malaysia. Ministry of Agriculture and Food Industry Malaysia. Revised 30 October 2020.

Drew CP, Heller MC, Mayo C, Watson JL, Maclachlan NJ. (2010). Bluetongue virus infection activates bovine monocyte-derived macrophages and pulmonary artery endothelial cells. Vet Immunol Immunopathol. 136(3–4): 292–296.

Elbers, A. R., Koenraadt, C. J., & Meiswinkel, R. (2015). Mosquitoes and Culicoides biting midges: vector range and the influence of climate change. Revue scientifique et technique (International Office of Epizootics), 34(1), 123–137. https://doi.org/10.20506/rst.34.1.2349

Fujisawa, Y., Homat, T., Thepparat, A., Changbunjong, T., Sutummaporn, K., Kornmatitsuk, S., & Kornmatitsuk, B. (2021). DNA barcode identification and molecular detection of bluetongue virus in Culicoides biting midges (Diptera: Ceratopogonidae) from western Thailand. Acta tropica, 224, 106147. https://doi.org/10.1016/j.actatropica.2021.106147

Gamsjäger, L., & Chigerwe, M. (2024). Clinical presentation, medical management, and outcomes in 35 hospitalized sheep diagnosed with bluetongue virus disease. Journal of veterinary internal medicine, 38(1), 514–519. https://doi.org/10.1111/jvim.16944

Jesse, F.F., Hashi, H.A., Chung, E.L., & Lila, M.A. (2020). Review on Current Seroprevalence Status of Common Small Ruminants' Bacterial and Viral Diseases in Tropics Focusing in Malaysia: A Holistic of Current Status Essential to be Study and Known. Advances in Animal and Veterinary Sciences, 8.

Kirkland, P. D., Finlaison, D. S., Biddle, A., Parsons, M., Austin, H., Boland, S., Roach, G., McKinnon, R., Braddon, E., & Britton, S. (2024). Bluetongue disease in sheep in New South Wales - April 2023. Australian veterinary journal, 102(1-2), 26–29. https://doi.org/10.1111/avj.13292

Maan, S., Maan, N. S., Belaganahalli, M. N., Potgieter, A. C., Kumar, V., Batra, K., Wright, I. M., Kirkland, P. D., & Mertens, P. P. (2016). Development and Evaluation of Real Time RT-PCR Assays for Detection and Typing of Bluetongue Virus. PloS one, 11(9), e0163014. https://doi.org/10.1371/journal.pone.0163014

Maan N., Maan S., Belaganahalli M., Ostlund E., Johnson D., Nomikou K. & Mertens P. (2012). Identification and differentiation of the twenty-six bluetongue virus serotypes by RT-PCR amplification of the serotype-specific genome segment 2. PLoS One, 7 (2): e32601. DOI: 10.1371/journal.pone.0032601

Maclachlan, N. J., Mayo, C. E., Daniels, P. W., Savini, G., Zientara, S., & Gibbs, E. P. (2015). Bluetongue. Revue scientifique et technique (International Office of Epizootics), 34(2), 329–340. https://doi.org/10.20506/rst.34.2.2360

Mertens P.P.C., Maan S., Samuel A. & Attoui H. (2005). Orbiviruses. In: Virus Taxonomy. Classification and Nomenclature of Viruses; Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U. & Ball L.A., eds., Elsevier Academic Press, Amsterdam, Netherlands, 466–483.

Narladkar B.W., Deshpande P.D., Vaniprasad V., Shivpuje P.R., Deshpande A.R. (2006). Integrated management of Culicoides sp. of domesticated animals. J Vet Parasitol. 20(2):125–128.

National Disease Control Centre (NDCC), UK (2024). Bluetongue Virus Update. Department of Agriculture, Food and the Marine. Number 2 of 2024. Retrieved from https://assets.gov.ie/291701/78ef6444-9678-4225-8bcb-d2854675e6e5.pdf

Newcomer, B. W., Cebra, C., Chamorro, M. F., Reppert, E., Cebra, M., & Edmondson, M. A. (2021). Diseases of the hematologic, immunologic, and lymphatic systems (multisystem diseases). Sheep, Goat, and Cervid Medicine, 405–438. https://doi.org/10.1016/B978-0-323-62463-3.00025-6

Niedbalski W. (2011). Evaluation of commercial ELISA kits for the detection of antibodies against bluetongue virus. Polish journal of veterinary sciences, 14(4), 615–619. https://doi.org/10.2478/v10181-011-0091-y

Patel, A., & Roy, P. (2014). The molecular biology of Bluetongue virus replication. Virus research, 182, 5–20. https://doi.org/10.1016/j.virusres.2013.12.017

Pauzi, N. A. S., Roslina, H., Khoo, C. K., Roshaslinda, D., Siti Surayahani, M. S., Norlina, D., Zunaida, B., Mohd Hasrul, A. H., & Hafizah, M. Z. (2022). Seroprevalence of Bluetongue Infection Among Ruminant Livestock in Peninsular Malaysia. Malaysian Journal Of Veterinary Research. Veterinary Research Institute, Department of Veterinary Services. Volume 12 No 2 Dec 2021. pages 37-45

Saminathan, M., Singh, K. P., Khorajiya, J. H., Dinesh, M., Vineetha, S., Maity, M., Rahman, A. F., Misri, J., Malik, Y. S., Gupta, V. K., Singh, R. K., & Dhama, K. (2020). An updated review on bluetongue virus: epidemiology, pathobiology, and

advances in diagnosis and control with special reference to India. The veterinary quarterly, 40(1), 258–321. https://doi.org/10.1080/01652176.2020.1831708

Schwartz-Cornil, I., Mertens, P. P., Contreras, V., Hemati, B., Pascale, F., Bréard, E., Mellor, P. S., MacLachlan, N. J., & Zientara, S. (2008). Bluetongue virus: virology, pathogenesis and immunity. Veterinary research, 39(5), 46. https://doi.org/10.1051/vetres:2008023

Shringi, S., & Shringi, B. N. (2005). Comparative efficacy of standard AGID, CCIE and competitive ELISA for detecting bluetongue virus antibodies in indigenous breeds of sheep and goats in Rajasthan, India. Journal of veterinary science, 6(1), 77–79.

Sohail, T., Yaqub, T., Abbas, T., Rabbani, M., Nazir, J., Maqbool, S. M., Yaqub, S., Habib, M., Ul-Rahman, A., Mukhtar, N., Shahbaz, M., Zahoor, M. Y., & Shabbir, M. Z. (2019). Seroprevalence of Bluetongue virus in small and large ruminants in Punjab province, Pakistan. Acta tropica, 189, 22–29. https://doi.org/10.1016/j.actatropica.2018.09.020

Wakefield, A., Broyles, M., Stone, E. L., Jones, G., & Harris, S. (2016). Experimentally comparing the attractiveness of domestic lights to insects: Do LEDs attract fewer insects than conventional light types?. Ecology and evolution, 6(22), 8028–8036. https://doi.org/10.1002/ece3.2527

Wilson, A. J., & Mellor, P. S. (2009). Bluetongue in Europe: past, present and future. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 364(1530), 2669–2681. https://doi.org/10.1098/rstb.2009.0091

World Organization of Animal Health (WOAH), (2021). Bluetongue (Infection with Bluetongue virus). Chapter 3.1.3. Terrestrial Manual 2021.

Zimmer, J. Y., Brostaux, Y., Haubruge, E., & Francis, F. (2014). Larval development sites of the main Culicoides species (Diptera: Ceratopogonidae) in northern Europe and distribution of coprophilic species larvae in Belgian pastures. Veterinary parasitology, 205(3-4), 676–686. https://doi.org/10.1016/j.vetpar.2014.08.029