SEROPREVALENCE OF Q FEVER IN DAIRY COWS IN FARMS FROM SELECTED STATES IN MALAYSIA

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SEROPREVALENCE OF Q FEVER IN DAIRY COWS IN FARMS FROM SELECTED STATES IN MALAYSIA

By

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_____

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SEROPREVALENCE OF Q FEVER IN DAIRY COWS IN FARMS FROM SELECTED STATES IN MALAYSIA

ABSTRACT

Query (Q) fever is a bacterial zoonotic disease that occurs at a global scale which is caused by *Coxiella burnetii*. The disease mostly affects small ruminants and occasionally in large ruminants such as cattle. Dairy animals, especially dairy cattle shed the organism in raw milk that could be consumed by humans thus causing direct oral transmission of the bacteria to humans. This study was carried out to investigate the status of Q fever in Malaysia among dairy cattle herds in selected states. About 120 serum samples were collected from dairy farms across several states in Malaysia. All samples were tested using an indirect ELISA test kit for the detection of antibodies against *C. burnetii*. The results showed Pahang has the most seropositive of Q fever (11.76 %) followed by Johor (8.82%), Perak (3.45%) and lastly Sabah (0%). The results conclude that dairy cattle is likely to have minimum risk of contracting *C. burnetti* infection, however care must be properly taken whilst handling the animals due to its potential zoonotic exposure to farmers.

Keywords: zoonotic, bacteria, ruminants, dairy, antibodies

SEROPREVALENCE OF Q FEVER IN DAIRY COWS IN FARMS FROM SELECTED STATES IN MALAYSIA

ABSTRAK

Demam Q ialah penyakit zoonosis bakteria yang berlaku pada skala global yang disebabkan oleh *Coxiella burnetii*. Penyakit ini kebanyakannya menyerang ruminan kecil dan kadang-kadang pada ruminan besar seperti lembu. Haiwan tenusu, terutamanya lembu tenusu menghasilkan organisma dalam susu mentah yang boleh diminum oleh manusia sekali gus menyebabkan jangkitan bakteria secara oral kepada manusia. Kajian ini dijalankan untuk menyiasat status demam Q di Malaysia kepada kumpulan lembu tenusu di negeri terpilih. Kira-kira 120 sampel serum telah dikumpulkan dari ladang tenusu di beberapa negeri di Malaysia. Semua sampel telah diuji menggunakan kit ujian ELISA tidak langsung untuk pengesanan antibodi terhadap *C. burnetii*. Keputusan menunjukkan Pahang merekodkan paling seropositif demam Q (11.76%) diikuti oleh Johor (8.82%), Perak (3.45%) dan terakhir Sabah (0%). Hasilnya menyimpulkan bahawa lembu tenusu berkemungkinan mempunyai risiko minimum dijangkiti jangkitan *C. burnetti*, namun penjagaan mesti diambil dengan betul semasa mengendalikan haiwan tersebut kerana potensi pendedahan zoonosisnya kepada penternak.

Kata kunci: zoonosis, bakteria, ruminan, tenusu, antibodi

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Thank you.

DEDICATION

I would like to dedicate this study to my family for their unwavering support for my journey towards pursuing this degree.

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LIST OF ABBREVIATIONS

℃	Degree Celsius
BSL	Biosafety level
CDC	Centers for Disease Control and Prevention
CFT	Complement Fixation Test
DNA	Deoxyribonucleic acid
DVS	Department of Veterinary Services
EIF	Enhanced immunofluorescence
ELISA	Enzyme-linked immunosorbent assay
FPV	Faculty of Veterinary Medicine
G	Gauge
ID	Innovative Diagnostics
IFA	Immunofluorescence assay
IgG	Immunoglobulin G
IHC	Immunohistochemistry
LCV	Large cell variant

LPS	Lipopolysaccharide
MSST	Multispacer sequence typing
O.D.	Optical density
OA	Orang Asli
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PPE	Personal protective equipment
Q	Query
QFS	Q fever fatigue syndrome
S/P	Sample-to-positive ratios
SCV	Small cell variant
SST	Serum Separator Tube
ST	Sequence type
USA	United States of America
WOAH	World Organisation for Animal Health

LIST OF SYMBOLS

%	Percent
≤	Less than or equal to
≥	Greater than or equal to

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CHAPTER 1

INTRODUCTION

Query (Q) fever is a zoonotic disease caused by the bacterium *Coxiella burnetii*. This disease typically causes a mild infection in animals and usually affects ruminants such as cattle, sheep and goats. The disease is currently listed as one of the notifiable diseases by the Department of Veterinary Services (DVS) and the World Organisation for Animal Health (WOAH) due to its status as a possible bioterrorism agent as it has low infectious dose, stability in the environment and ability of aerosol dispersion according to Radostits *et al*., (2007). In most animals and humans, the prognosis is good as many infected animals or individuals would recover without treatment or show any signs or symptoms of the disease. In severe cases, it may cause abortion, which in this case can cause a decrease in milk production performance in dairy cattle which will lead to economic losses. Seropositive animals may continue to shed the organism for at least 2 years, prompting early diagnosis to allow proper management and control measures.

On the global scale, the first ever exceptionally large outbreak of Q fever occurred in The Netherlands in 2007 which occurred for 4 years and more than 4000 human cases were reported according to Schneeberger *et al*., (2014). Several recent studies have shown that there is a variable seroprevalence of Q fever in animals in Europe such as high seroprevalence in Italy and low seroprevalence in Poland. Ferrara *et al*., (2022) stated that the findings indicate that *C. burnetii* is prevalent in bovine populations; therefore, there could be a number of explanations for the variation in seroprevalence, including the features of the ELISA kit that was employed. In Asia, South Korea reported a rise in Q fever cases in humans, with occupational contact with animals or

animal products serving as the main risk factor. The seroprevalence of Q fever in dairy cattle was reported to be moderate to high according to Yun *et al*., (2023). Rai *et al*., (2011) stated that the first ever documented zoonotic case of Q fever in Malaysia was in Penang in 2007 in which the man had pyrexia of unknown origin and managed a farm with abortion cases in goats. A more recent study conducted in Malaysia by Khor *et al*., (2018) which involved the indigenous people showed evidence of Q fever seropositive in the population. The factors influencing the seroprevalence include physical contact with farm animals and exposure to ticks.

C. burnetii is maintained in wild animals through ticks. The infection occurs when the animal comes in contact with the infected tick excreta or tick bites. Transmission between ruminant hosts occurs through indirect contact via airborne or fomites or direct contact. Transmission via direct contact may include ingestion or contact with body secretions such as placenta from infected animals. Humans may get infected by inhaling bacteria-contaminated aerosols. In Malaysia, there are limited studies on Q fever detection in dairy cattle. Most research was done on small ruminants in Malaysia possibly due to rare abortion cases in cattle. Nonetheless, dairy cattle can shed *C. burnetii* in unpasteurised milk which will be consumed by humans and the main shedding route for cattle is milk. Anderson *et al*., (2013) stated that because Q fever symptoms are often nonspecific and difficult to diagnose, the illness has long been underreported and underdiagnosed. By conducting this study, it may shed some light to increase our knowledge of the prevalence of Q fever in dairy cattle in Malaysia to allow proper diagnosis, management and control measures to be taken in both animals and humans.

CHAPTER 2

2.1 Research problem

Several studies have been conducted on the seroprevalence of Q fever in ruminants in Malaysia. However, these studies were mainly focusing on small ruminants. There is insufficient documentation on the seroprevalence of Q fever among dairy cattle in Malaysia.

2.2 Research questions

2.2.1 What is the seroprevalence of Q fever in dairy cows in farms from selected states in Malaysia?

2.2.2 Which of the selected states in Malaysia has the highest seroprevalence of Q fever among dairy cattle?

2.3 Hypothesis

2.3.1 There is moderate to high seroprevalence of Q fever in dairy cows in farms from selected states in Malaysia.

2.3.2 The state of Pahang has the highest Q fever seroprevalence in dairy cows in Malaysia.

2.4 Objectives

2.4.1 To determine the seroprevalence of Q fever in dairy cows in farms from selected states in Malaysia.

2.4.2 To identify the state with the most dairy cows with seroprevalence positive for Q fever in Malaysia.

CHAPTER 3

LITERATURE REVIEW

3.1 Description of *Coxiella burnetii*

According to WOAH, *Coxiella burnetii* is the obligate intracellular Gram-negative bacterium that causes Q fever. It has evolved to live within the phagolysosome of the phagocyte. Several important characteristics of this bacteria include antigenic variation or phase variation, morphological forms, passive entry into host cells by phagocytosis and survival in phagolysosomes. WOAH (2018) described that *C. burnetii* has two antigenic forms: the pathogenic phase I, isolated from infected animals or humans, and the attenuated phase II, obtained by repeated in-ovo or in-vitro passages. These forms were determined by the change of lipopolysaccharide (LPS) molecule in its cell wall. Phase I has full-length LPS O-chains while phase II has truncated LPS. WOAH also described that *C. burnetii* produces a small, dense, highly resistant spore-like form which has been attributed to the existence of *C. burnetii* developmental cycle variants described from in vitro studies which included large-cell variants (LCV), small-cell variants (SCV), and small dense cells (SDC). The SDC and SCV represent the small morphological variants of the bacteria likely to survive extracellularly as infectious particles which allows it to persist in the environment.

According to Ullah *et al*., (2022), the avb3 integrin primarily mediates the bacteria's passive entry into the host cells. Virulent bacteria only require the avb3 integrin for attachment to phagocytic cells like monocytes and macrophages while avirulent bacteria require both the avb3 integrin and complement receptor CR3 for attachment. Phase variation determines whether

bacteria can survive inside phagocytic cells; Phase I bacteria can survive inside phagocytic cells while Phase II bacteria are eliminated. Additionally, Phase I bacteria undergo less phagocytosis than Phase II bacteria. The acidic environment of the phagolysosomes, which is ideal for the growth of this bacteria, allows it to survive. By the phagocytosing action of the monocytes and macrophages, the SCV enter the phagolysosomes where they fuse with the lysosomal material to become metabolically active, go through vegetative growth, and eventually change into LCV. Only Phase I bacteria are affected by this, as Phase II bacteria are rapidly destroyed. The availability of nutrients necessary for *C. burnetii* growth is ensured by the acidic pH, which also shields it from the effects of various antimicrobials.

3.2 Common strains of *Coxiella burnetii* worldwide

The virulence of *C. burnetii* is associated with the type of strain involved in the disease. The multispacer sequence typing (MSST) technique identified four distinct lines of *C. burnetii* which include sequence type (ST) 8 was isolated from sheep in France, ST 15 from goats in France, ST 16 from ticks in Montana (USA), and ST 20 from cattle in France according to Ullah *et al*., (2022).

3.3 Details of the disease

3.3.1 Global

The Netherlands experienced the world's first Q fever outbreak of extraordinary proportions in 2007. Over 4,000 human cases were involved in the four-year outbreak. Although no single cause for the worldwide outbreak could be found, research revealed that people who live near goat farms are more likely to contract acute Q fever. Several studies by Ferrara *et al*., (2022)

revealed the seroprevalence rate of Q fever in animals in Europe as summarized in **Table 3.3.1** below:

Table 3.3.1 Seroprevalence rate (%) in several European countries

Country	Seroprevalence rate (%)
Italy	11.7
Albania	7.9
Germany	7.8
Spain	6.7
Ireland	6.2
Poland	4.18

These results show that *C. burnetii* is prevalent in bovine populations, though slight variations may be caused by variations in epidemiologic circumstances, sample size, and the features of the ELISA kit that was used.

3.3.2 Asia

In one study from South Korea by Yun *et al*., (2023), between 2011 and 2017, Q fever infection reported 0.07 cases per 100,000 people nationwide. By 2017, that number had sharply increased to 0.19 cases per 100,000 people. A quarter of all cases that were reported had to do with working with animals or animal products. Goats (60.0%) and dairy cattle (32.0%) are the most common sources of *C. burnetii* exposure in Q fever cases with a history of animal contact. The seroprevalence of *C. burnetii* among workers in high-risk occupations with increased animal

contact was 1.2% in 2007 and 1.4% in 2012. The seroprevalence of *C. burnetii* in dairy cattle ranged from 7.1–24.2%, while in goats, it was 8.6–19.1%, according to ELISA studies. In South Korea, a molecular test or antigen test is used to diagnose the disease if an animal exhibits reproductive system symptoms like abortion or stillbirth. Tetracycline-based antibiotic treatment and quarantine are the current methods used to control Q fever.

3.3.3 Malaysia

According to Rai *et al*., (2011), Penang saw the first known zoonotic case of Q fever in Malaysia in 2007. In this instance, a number of goats had spontaneous abortions within two weeks prior to the farm owner being admitted to a private hospital for pyrexia of unknown origin. A few days before the pyrexia started, he had delivered a goat abortus without the use of any personal protective equipment (PPE). Since he bought all of his goats from nearby farms, it was found that Q fever had been circulating among his animals and the surrounding farms.

A more recent study was conducted in Malaysia by Khor *et al*., (2018) to investigate the seroprevalence of Q fever among the indigenous people of Peninsular Malaysia. According to serological testing, 9.6% of the participants had positive *C. burnetii* IgG antibodies, with seroprevalences ranging from 0% to 47.4% in the 14 villages. It was found that exposure to ticks and physical contact with farm animals were the factors influencing the seroprevalence. The OA frequently reside and work near the edges of forests where ticks, wildlife, and domestic animals can be found. Recent molecular detection has confirmed the presence of *C. burnetii* in ticks taken from domestic animals visiting the veterinary clinic, as well as wild animals captured in Malaysian

forests. This study did not specify any control or prevention measures because its goal was to describe the presence of antibodies against Q fever among indigenous people.

3.4 Economic impact of Q fever

The economic impact of Q fever involves a decrease in milk production performance in dairy cattle and animals lost through abortion. Ullah *et al*., (2022) stated that Q fever typically causes infertility in cattle as well as reproductive issues in animals, particularly small ruminants. These issues can include spontaneous abortion in late pregnancy, early delivery, stillbirth, and the birth of weak offspring. Acute Q fever in cattle typically manifests as a subclinical infection, whereas chronic infection can lead to reproductive problems. The typical range of abortions caused by *C. burnetii* infection is 3–8%. Abortion leads to a decrease in milk production performance in dairy cattle. Keshavarzi *et al*., (2020) stated that this is because the earlier start of lactation compared to what would happen in cows having normal parturitions results in an absence of development or redevelopment of the mammary glands, which would lead to lower milk yields. Additionally, subclinical mastitis may also develop according to Ullah *et al*., (2022). The organism is found in the placenta and mammary glands of pregnant dairy animals. The fetus and related structures contain the greatest concentration of *C. burnetii*. Post-parturient shedding of organisms in birth fluids is higher in small ruminants, while lower in the case of cattle.

3.5 Zoonotic potential of Q fever

Zeeshan *et al*., (2023) stated that Q fever can present clinically in a variety of ways in humans. It can be acute, most commonly as pneumonia or hepatitis, or chronic, most commonly as endocarditis. Subclinical and inapparent infections are also common. Zeeshan *et al*., (2023) also

stated that because *C. burnetii* reactivates during pregnancy, it is the cause of increased rates of low birth weight, preterm, and abortion. Another important symptom of this disease according to Ullah *et al*., (2022) is Q fever fatigue syndrome (QFS), which affects 20% of patients and is a crippling ailment that follows an acute Q fever that affects the major body systems. Although chronic fatigue is not life-threatening, it can have detrimental effects on a person's quality of life and ability to work, which can have significant social and economic ramifications.

3.6 Transmission of Q fever

There are several routes involved in the transmission of *C. burnetii* from animals to humans and among animals as illustrated in Figure 3.6 which was adapted from a study done by Celina and Cerny (2022). The major route involves inhaling contaminated aerosol whereas the ingestion of contaminated raw food materials is the minor route. On rare occasions, blood transfusions, mating, or skin or mucosal contact with contaminated products can all result in transmission. Fetal membranes, faeces, and milk are among the bodily tissues and fluids in which *C. burnetii* sheds. In cases of reproductive failure, there is a significant number of bacteria in the birth fluids and vaginal secretions. Depending on the host species and shedding routes, the length of the shedding varies. The main shedding route for cattle is milk. While asymptomatic, infected cattle can continue to excrete pathogens in their milk for several months. When bodily secretions and animal excretions containing a high concentration of *C. burnetii* dry and mix with dust, they produce contaminated aerosol, which exposes people to dust particles through the air. Ticks may actively contribute to the spread of Q fever in animals according to Ullah *et al*., (2022). Ticks are potential reservoirs for this bacterium because they can pass it both transovarially and transstadially to their progeny. Large quantities of *C. burnetii* are excreted by infected ticks in their faeces, which

contaminate host animals' skin. Ticks are therefore crucial to the environmental transmission of *C. burnetii* infection.

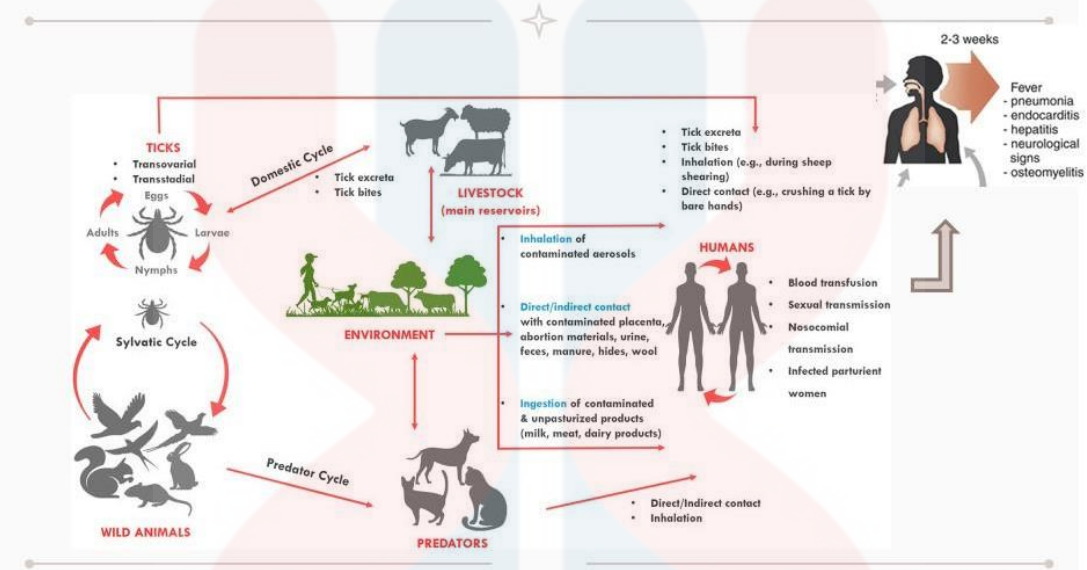

Figure 3.6 Transmission of Q fever

Source: Seyma and Jiri, 2022

3.7 Laboratory diagnosis of Q fever

3.7.1 Available methods for diagnosis

According to Ullah *et al*., (2022), there are four different categories of diagnostic techniques that can be used which include isolating and propagating the organism using tissue culture, embryonated chicken eggs, or laboratory animals; serodiagnostic tests like Immunofluorescence Assay (IFA), Complement Fixation Test (CFT) and Enzyme-linked Immunosorbent Assay (ELISA); antigen detection assays like Immunohistochemical Staining (IHC); and genomic detection assays like Polymerase Chain Reaction (PCR). Combining different laboratory tests, such as PCR for nucleic acid detection and ELISA for serology, is highly suggestive of a confirmatory diagnosis of Q fever. According to DVS (2011), the samples required for the isolation and detection of the organism may include the liver, lungs, and placenta.

3.7.2 Comparison between each method of diagnosis

According to Ullah *et al*., (2022), *C. burnetii* may be isolated and multiplied using a variety of techniques, such as tissue culture, chicken embryos, and lab animals. One potential benefit of tissue culture is that human and animal samples can be used with equal effectiveness. The drawback is that because of its intracellular growth, standard biological media are inappropriate for its growth and instead call for a cell culture system called shell vial cell culture. One potential drawback with embryonated chicken eggs is that PCR confirmation may be necessary. One benefit of using lab animals could be that the samples collected could be used to inoculate chicken embryos or cell culture systems in order to isolate *C. burnetii*. However, it also requires confirmation by other diagnostic methods such as PCR.

Next, Caraguel *et al*., (2020) stated that because *C. burnetii* infection and shedding are temporary, serology is frequently used in animal studies to identify infections and past exposure to the parasite. Several serological methods available may include IFA, CFT and ELISA. For IFA, it is frequently used to diagnose Q fever in people and makes it simple to distinguish between suspected acute and chronic forms of the illness by measuring Phase I and Phase II antibody titers. The lack of a commercial Q fever IFA kit for the identification of *C. burnetii* in animals is one of the drawbacks, though. Herd-level or extensive screening cannot be conducted using species-specific IFA. Because of the delay in the development of antibody titers, it is also unsuitable for the detection of early infection of acute Q fever. To properly interpret the results of IFA, a high level of expertise and very specific, expensive instruments are needed. For the CFT, the possible drawbacks are its low sensitivity in comparison to other tests and the existence of

anticomplementary activity in multiple samples, which impedes antibody titer estimation even after several tries.

For ELISA, Wegdam-Blans *et al*., (2014) stated that it demonstrates greater specificity and sensitivity as it is even more sensitive than the immunofluorescence assay which may be among its many benefits and Gürtler *et al*., (2014) stated that ELISA is superior to complement fixation in terms of specificity and sensitivity when diagnosing Q fever. It is thought to be a useful technique for seroepidemiological surveys and convenient for herd-level screening according to Porter *et al*., (2011). Antibodies against both antigenic phases of *C. burnetii* can be detected in serum, and this process can also be performed on a variety of animal species. It can be used to determine the serodiagnosis of Q fever because it offers a cumulative interpretation of results as seropositive, suspicious, or seronegative status according to Zeeshan *et al*., (2023). However, Ullah *et al*., (2022) stated that due to a lag in the development of antibody titers, it cannot be used in the first two weeks following the onset of clinical signs.

Other than that, according to Ullah *et al*., (2022), one antigen detection assay that is available is IHC. Nevertheless, because it only provides a tentative indication of *C. burnetii* infection and necessitates additional confirmation by other diagnostic techniques shows that it lacks specificity. Last but not least, PCR is available for genomic detection assay. PCR has a number of benefits, including the ability to work with a variety of biological specimens and being quick, sensitive, and highly specific. *C. burnetii* shedders can be successfully identified using quantitative real-time PCR. However, Ullah *et al*., (2022) stated that the inability of PCR to

identify *C. burnetii* DNA in blood after two weeks of the onset of clinical symptoms may be one of its drawbacks.

3.8 Treatment of Q fever

3.8.1 Treatment of Q fever in dairy cattle

According to DVS, all pregnant animals from the infected herds are quarantined and treated with oxytetracycline for 8 mg/kg/day in drinking water for 5 days before parturition and all animals that are tested positive for Q fever are also quarantined and treated with oxytetracycline through drinking water or feed for two to four weeks. However, Yun *et al*., (2023) stated that veterinarians administering antibiotics to animals infected with *C. burnetii* must perform labor-intensive work and run the risk of coming into contact with the infection even though they are wearing personal protective equipment.

3.8.2 Treatment of Q fever in humans

According to Ferrara *et al*., (2022), during the Dutch outbreak, doxycycline was the antibiotic that was prescribed the most. The study's conclusions demonstrated that patients treated with antibiotics thought to be effective against *C. burnetii*, such as doxycycline and fluoroquinolones, had a lower risk of hospitalization than patients treated with beta-lactams or azithromycin. Patients receiving 200 mg/day of doxycycline had the lowest risk of hospitalization. This validates the generally advised use of antibiotics in cases of acute Q fever. Yun *et al*., (2023) stated that when treating chronic Q fever that presents as endocarditis or vascular infection, long-term therapy lasting about two years may be necessary, along with surgery.

3.9 Control and prevention of Q fever

3.9.1 Control and prevention of Q fever in dairy cattle

DVS (2011) has provided a guideline to prevent, control and eradicate this disease in animals to increase farm productivity and prevent zoonotic spread. The proposed strategies in control and prevention of Q fever in dairy cattle included detection and report, quarantine of affected animals, biosecurity measures, antibiotics treatment, import control, and movement control. For detection and report, all abortion cases must be reported to the state DVS or district DVS and every case must be managed by taking blood samples for serological testing, and vaginal swabs from the cow and organ samples such as the liver, lungs and placenta from the fetus. If there is a positive result from the tests done, blood samples from all animals in the herd must be taken for herd-level detection by using methods such as ELISA.

For the quarantine of affected animals, animals that tested positive will be separated and quarantined and every movement of quarantined animals is monitored by DVS. For biosecurity measures, animals that tested positive will be treated with Oxytetracycline and all pregnant animals are separated and confined to properly manage any cases of parturition or abortion. All tools and surfaces are disinfected according to the routine disinfection procedure and any contaminated waste including amniotic fluid, bedding, feces and feed are disinfected with routine disinfection and buried immediately. For the antibiotic treatment, the treatment used is oxytetracycline with the stated regime.

For import control, all animals to be imported must have the veterinary health certificate that shows that the animal is from a herd or farm free from Q fever, not vaccinated against Q fever or vaccinated for more than 2 years before the importation, tested negative for Q fever and had no clinical signs of Q fever upon importation. For movement control, animals that were determined to be from a herd that is free from Q fever, and from the free zone, do not require any testing if the farm has a good biosecurity and is under DVS surveillance. For animals with unknown health status, antibiotics are given for 2 weeks and determined to have no clinical signs within 14 days before movement is commenced. Animals to be moved must be separated from other animals.

3.9.2 Control and prevention of Q fever in humans

The proposed strategies by the DVS (2011) in the control and prevention of Q fever in humans may include occupational safety, record, and awareness campaigns. For occupational safety, BSL-3 must be followed when handling tissue samples from infected herds. All staff and workers must undergo health examinations, and comply with personal hygiene and good animal husbandry practices. Personal protective equipment (PPE) such as masks and gloves must be worn. High-risk individuals may include abattoir workers and farm workers. For the record, the DVS must keep records on the disease, disease-free herds, infected herds, culled animals and tests done. For the awareness campaigns, DVS must advise the farmers, abattoir workers and laboratory staff on Q fever, especially regarding occupational safety. Other safety measures may include practicing routine disinfection procedures for clothing and tools, changing from work attire when leaving the farm, reporting to the employers when showing symptoms of Q fever such as fever and coughing, and avoiding selling raw milk to customers, especially from the infected herds.

CHAPTER 4

MATERIALS AND METHODS

4.1 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 FPV Animal Ethics Committee: UMK/FPV/ACUE/RES/002/2021.

4.2 Methodology

4.2.1 Study area

The blood samples for serum collection will be obtained from multiple dairy cattle farms from selected states in Malaysia including Sabah, Perak, Pahang and Johor.

4.2.2 Study design

The study design for this project is a cross-sectional study to investigate the seroprevalence of Q fever in dairy cattle in farms from selected states in Malaysia.

4.2.3 Study population

This study targets the population of dairy cattle in farms in several states in Malaysia. The states were chosen because they had among the largest dairy cattle population in Malaysia which include Sabah, Perak, Pahang and Johor. About 34 samples were collected from several farms in Pahang, 23 samples were collected from several farms in Sabah, 34 samples were collected from several farms in Johor and 29 samples were collected from several farms in Perak. The total

samples collected were 120. All these samples were collected based on the farm availability and cooperation of the animals.

4.2.4 Selection criteria

4.2.4.1 Inclusion criteria

The inclusion criteria for this study are dairy cows that were selected at random, with no specific age in states with many dairy farms within 5 months.

4.2.4.2 Exclusion criteria

There are no exclusion criteria in this study.

4.3 Sampling method and procedure

4.3.1 Coccygeal vein blood sampling

The equipment required for this procedure included gloves, vacutainer holder, vacutainer needle size 18G, and Serum Separator Tubes (SST). SST was used to allow serum separation without centrifugation.

The procedure done was according to a guideline provided by the University of Bristol (2020). Firstly, the gloves were worn before starting the blood collection procedure. A vacutainer needle size 18G, a plastic vacutainer holder and SST were selected. The position was by standing behind the cow and slightly to one side of the tail. Then, the tail was held approximately a third of the way from the base with the non-dominant hand and lifted until the ventral aspect of the tail was clearly visible and accessible. The groove which was situated in the midline on the ventral

aspect of the tail was located and palpated as the coccygeal vein and artery run in this groove. The back of the fingers was rested against the base of the tail with the vacutainer holder held in the dominant hand to steady the hand. The needle was inserted into the midline groove at 90° to the tail surface and continued to advance to approximately halfway along its length. The vacutainer holder was stabilized with the thumb, index and middle fingers and held with the 4th and 5th fingers and against the palm. The SST was pushed onto the needle to allow the needle to pierce the bung. The SST was removed when it was about ⅔ full and the vacutainer holder and needle were removed afterwards. The needle was recapped and discarded. The blood samples were stored in a polystyrene box with ice packs. All of the blood samples collected were stored in a polystyrene box with ice packs during transportation and upon arrival at the laboratory, the samples were stored at -20℃.

4.3.2 Jugular vein blood sampling

Some blood samples were also collected using the jugular vein blood sampling method. The equipment required for this procedure also included gloves, vacutainer holder, vacutainer needle size 18G, and SST. SST was used to allow serum separation without centrifugation.

The procedure done was according to a guideline provided by the University of Bristol (2020). Firstly, the gloves were worn before starting the blood collection procedure. A vacutainer needle size 18G, a plastic vacutainer holder and SST were selected. Then, the jugular groove was palpated with the free hand to determine the location of the jugular vein. The jugular vein was raised with the free hand by firmly pressing the thumb into the jugular groove. The thumb position was far enough down the groove caudally to leave enough space to insert the needle and collect

the sample. The needle was positioned over the jugular groove cranial to the thumb and held at 45° to the skin surface. The needle was pushed through the skin and into the jugular vein following the direction of the jugular with the needle. The vacutainer holder was stabilized between the thumb and index finger of the hand that was raising the vein. The free fingers of that hand continued to raise the jugular vein. The SST was inserted into the vacutainer holder with the index and middle fingers over the base of the holder and the palm of the hand was used to push the blood tube up into the holder and firmly onto the needle. The other hand maintained the position of the needle in the jugular vein by stabilizing the vacutainer holder. The SST was removed once it was ⅔ filled with blood and the vacutainer holder and the needle were removed afterwards. The needle was recapped and discarded. The blood samples were stored in a polystyrene box with ice packs. All of the blood samples collected were stored in a polystyrene box with ice packs during transportation and upon arrival at the laboratory, the samples were stored at -20℃.

4.4 Detection of antibodies using Indirect ELISA kit

An indirect ELISA test kit for the detection of antibodies against *Coxiella burnetii* (ID Screen, Q Fever Indirect Multi-species, France) in serum samples was used to test the serum obtained from the blood samples collected. The components provided in the kits included reagents, microplate coated with phase I and II *Coxiella burnetii* antigens, Concentrated Conjugate (10x), Positive Control, Negative Control, Dilution Buffer 2, Dilution Buffer 3, Wash Concentrate (20x), Substrate Solution and Stop Solution (0.5M). The materials required but not provided in the kit included mono- and multi-channel pipettes capable of delivering volumes of 5µL, 100µL and 500µL, disposable tips (10µL, 200µL and 500µL), 96-well pre-dilution microplate, distilled or deionized water, manual wash system and 96-well microplate reader.

The testing procedure done was according to the guidelines provided by the ELISA test kit. Firstly, all reagents were allowed to come to room temperature (21℃ ± 5℃) before use. All reagents were homogenized by inversion. All samples were tested at a final dilution of 1:50. 5µL of the Negative Control to wells C12 and D12, 5µL of the Positive Control to wells A12 and B12, 5µL of each sample to be tested in the remaining wells except for wells E12, F12, G12 and H12 and 245µL of the Dilution Buffer 2 to each well were added in a 96-well pre-dilution microplate.

100µL of the pre-diluted Negative Control to wells C12 and D12, 100µL of pre-diluted Positive Control to wells A12 and B12 and 100µL of each pre-diluted sample to be tested in the remaining wells except for wells E12, F12, G12 and H12 were transferred into the ELISA microplate. The plate was covered and incubated for 45 minutes at 21℃ ± 5℃. The wells were emptied and each well was washed 3 times with 300µL of the Wash Solution. The wells were not dried between washes. The Conjugate 1x was prepared by diluting the Concentrated Conjugate 10x to 1:10 in Dilution Buffer 3. The calculation was done as stated below:

$$M_1V_1 = M_2V_2$$

$$(10)V_1 = (1)(20)$$

$$V_1 = 2 \text{ mL}$$

*2 mL of Conjugate Concentrate 10x is diluted in 20 mL of Dilution Buffer to get Conjugate 1x

$$(10)(60) = (1)V_2$$

$$V_2 = 600 \text{ mL}$$

100 µL x 96 well x 2 plates = 19 200 µL

= 19.2 mL of Conjugate 1x is required

100µL of the Conjugate 1x was added to each well. The plate was covered and incubated for 30 minutes at 21℃ ± 5℃. The wells were emptied and each well was washed 3 times with 300µL of the Wash Solution. The wells were not dried between washes. 100µL of the Substrate Solution was added to each well. The plate was covered and incubated for 15 minutes at 21℃ ± 5℃. 100µL of the Stop Solution was added to each well, in the same order as in step No.8, to stop the reaction. The optical density (O.D.) at 450nm was read and recorded.

The test was validated as the mean value of the Positive Control optical density O.D. (OD_{PC}) was greater than 0.350 and the ratio of the mean values of the Positive Control O.D. to the Negative Control O.D. ($OD_{PC}\ to\ OD_{NC}$) was greater than 3.

For each sample, the S/P percentage (S/P%) was calculated using the formula stated below:

$$S/P\% = \frac{OD_{sample} - OD_{NC}}{OD_{PC} - OD_{NC}} \times 100$$

The S/P% obtained was then interpreted as stated below:

Table 4.4 Interpretation of S/P%

Result	Status
S/P% ≤ 40%	Negative
40% ≤ S/P% ≤ 50%	Doubtful
50% ≤ S/P% ≤ 80%	Positive
S/P% ≥ 80%	Strong positive

CHAPTER 5

RESULTS

Based on Table 5.0 and Figure 5.0, Pahang recorded 3 positive results and 1 strong positive result for the serum samples that were tested for the presence of antibodies against *Coxiella burnetii*. Sabah recorded no positive or strong positive result. Johor recorded 1 positive result and 2 strong positive results and Perak recorded 1 positive result for the presence of antibodies against *Coxiella burnetii*.

Table 5.0 Results of indirect ELISA test kit for the detection of antibodies against Q fever in serum samples of dairy cows

Result	Pahang	Sabah	Johor	Perak	**Total**
Negative	29	23	31	28	111
Doubtful	1	0	0	0	1
Positive	3	0	1	1	5
Strong positive	1	0	2	0	3
Total samples	34	23	34	29	120

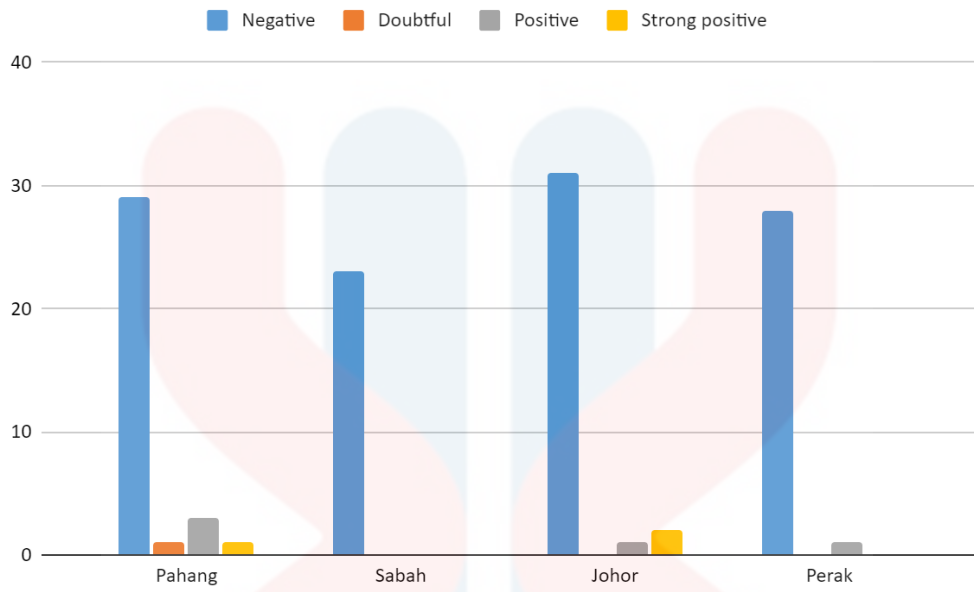

Figure 5.0 Bar chart representing the results of Indirect ELISA test kit for the detection of antibodies against *Coxiella burnetii* in serum samples of dairy cows from selected states in Malaysia

Based on Table 5.1 and Figure 5.1, Pahang was observed to have the highest herd prevalence % among all 4 states involved in this study with 11.76% followed by Johor with the second highest herd prevalence % at 8.82%, that was followed by Perak with herd prevalence % of 3.45% and lastly Sabah with 0.00%.

Table 5.1 Herd prevalence % of Q fever by states in a Q fever seroprevalence study

State	Herds Free from Infection	Herds Infected	Total Herds	Herd Prevalence %
Farm A (Pahang)	29	4	34	11.76
Farm B (Sabah)	23	0	23	0.00
Farm C (Johor)	31	3	34	8.82
Farm D (Perak)	28	1	29	3.45
National	111	8	120	24.04

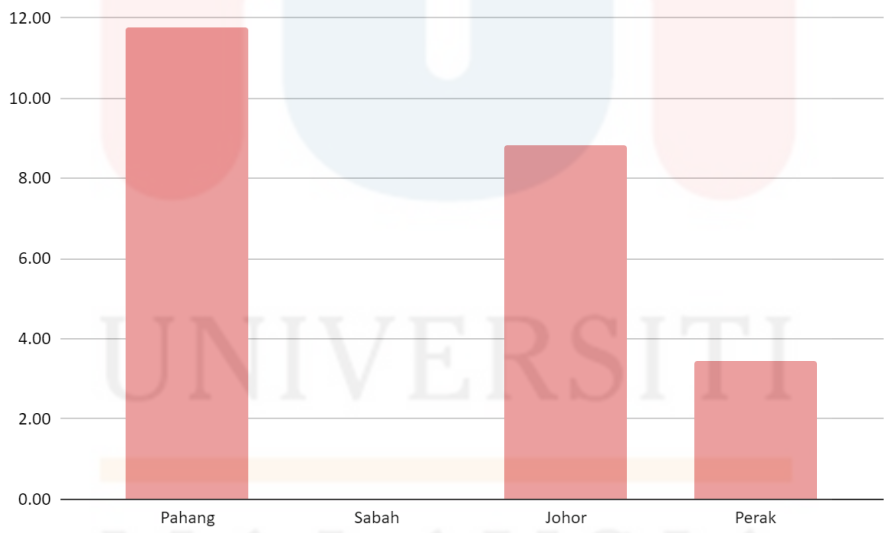

Figure 5.1 Bar chart representing the herd seroprevalence % of Q fever

CHAPTER 6

DISCUSSION

This study is crucial to determine the seroprevalence of Q fever in dairy cattle in farms from selected states in Malaysia. We can detect the magnitude of this disease in dairy cattle which is important data for the DVS to identify the associated risk factors. This can act as a reference to impose effective preventive and control measures on the farm. This is important as Q fever may cause economic losses and disease transmission to humans.

Pahang has been recorded as the state with the highest Q fever seroprevalence in dairy cows in Malaysia. The finding is very interesting, as Pahang state has the most dairy cattle population in Peninsular Malaysia. This farm, which has a total cattle herd of about 3000, allows the animals to graze freely in the pasture within the farm. Jesse *et al*., (2020) stated that it might be possible that these animals were continuously exposed to the tick vector carrying the bacteria. Previous studies in other neighboring states such as Terengganu and Negeri Sembilan, reported high findings of hard ticks within small ruminant populations. Therefore, we elucidate that these ticks may have migrated from one farm to another and had contact with humans or farm workers or other animals may spill the bacterium to the Pahang dairy cattle population.

As for Johor and Perak, the movement of workers may have contributed to the moderate to high Q fever seroprevalence in dairy cows. Some companies own farms in several different states which may have included Pahang, Johor and Perak. Thus, workers may be transported from one farm to another according to need. These workers may accidentally introduce the bacteria into

the farm due to contaminated clothing or tools. The frequency or number of workers involved in the movement may determine the level of contamination which will contribute to the seroprevalence.

Sabah has recorded all negative results for the detection of Q fever in dairy cows. This can be attributed to their main focus on their own local breeding program, and import from only several selected breeders from Australia. The lack of movement of cattle from Peninsular to Sabah may contribute to the negative results as the disease was not imported from the states in Peninsular such as Pahang. Next, as they only import from several selected breeders from Australia, they have proper documents on the health status of the animal and they are able to maintain with only importing animals with the best health condition.

Positive and strong positive results indicate the presence of antibodies against *C. burnetii* which may be acquired from active infection or passive infection. Active infection is more likely because tick vectors carrying *C. burnetii* may come into contact with the animals according to Jesse *et al.*, (2020). On the other hand, passive infection may occur through mother-fetus transmission, which are able to remain as carriers for life. In a previous study conducted by González- Barrio *et al*., (2015) to study the presence of maternal antibodies in dairy cattle calves. The presence of antibodies in dairy cows infected with *C. burnetii* is detectable throughout the gestation period and even after delivery, and these antibodies are passed on to newborn calves through colostrum. However, it is unclear if maternal antibodies offer protection against *C. burnetii* infection. We cannot rule out the possibility that the positive and strongly positive results are caused by the presence of maternal antibodies against Q fever, even though it was stated that

the maternal antibodies against *C. burnetii* would only last for less than a year. This is because there were no exclusion criteria in this study and the samples were collected from randomly selected dairy cattle on the farm without regard to age.

Additionally, while the incidence of abortion caused by *C. burnetii* infection in cattle only varies from 3–8%, Q fever in cattle typically manifests as a subclinical infection and can result in subclinical mastitis, and chronic infection can lead to infertility, passive infection can arise from prior exposure that was overlooked according to Ullah *et al*., (2022).

According to the protocol by Department of Veterinary Services (DVS, 2011), animals to be imported must have a veterinary health certificate that shows that they had not been vaccinated against Q fever, or had been vaccinated against Q fever more than 2 years ago, thus the antibodies detected in this case would not be from active immunity from the vaccination and it was also stated that animals to be imported into Malaysia must also test negative for serology or PCR for the detection of *C. burnetii*.

As for the sensitivity of ELISA, many studies that were conducted on the diagnosis of Q fever indicated that ELISA has high sensitivity when compared to other tests which may include Porter *et al*., (2011) had findings indicated that the ELISA test has a higher level of sensitivity than the CF and IFA tests. Next, Wegdam-Blans *et al*., (2011) claimed that ELISA showed to be more dependable, sensitive, and quick when compared to the CF test.

Some problems relating to the results may arise from the kit. The kit used was ID Screen, Q Fever Indirect Multi-species which was based on a French bovine isolate, which is one of the commercial kits available for the detection of antibodies against *C. burnetii*. Since the majority of commercial ELISAs now on the market are based on whole cell lysates of various *C. burnetii* strains, cross-reactions to other pathogens may affect the tests' specificity. Because housekeeping proteins and other highly conserved proteins are present in whole cell antigens, cross-reactions with other pathogens, like *Chlamydia* spp., can affect specificity and result in "Doubtful" results according to Stellfeld *et al*., (2020). Other than that, issues may arise due to wrong calculations as some steps in the kit required calculations to get the correct amounts of components to be used based on the amounts of samples available. Pipetting errors may also have risen during the process.

Despite the mentioned drawbacks, the best method to confirm the presence of *C. burnetii* is ELISA or PCR. Based on WOAH, although there isn't a gold standard method available just yet, serological ELISA and direct detection and quantification by PCR should be taken into consideration as the preferred approaches for clinical diagnosis. It was mentioned that ELISA is reliable, automated, and suggested for routine animal serological testing for Q fever. Serological assays can be used to screen flocks or herds, but individual animals cannot be interpreted as free; a herd or flock can only be considered free if all of its members are free and have no history of Q fever, either clinical or serological. Real-time PCR has significantly improved diagnostic and research methods by enabling the detection and quantification of *C. burnetii* DNA. However, due to animal variability in shedding, including varying routes and possibly intermittent shedding, PCR cannot be used to establish the infection status.

According to WOAH (2018), Q fever is classified as an occupational zoonosis because it can pose a serious risk to people, particularly those who handle aborted materials because they contain the highest concentration of the organism, such as farm workers who handle aborted fetuses. The CDC has classified it as a group B biological agent and views it as a potential bioterrorism agent due to a number of factors, including its capacity to spread naturally as an aerosol, resistance in the environment as pseudo-spores, and the ability to inflict crippling disease in large populations.

As for the economic importance, it could affect both the workers involved and the production of the dairy industry. One of the symptoms of Q fever in humans is Q fever fatigue syndrome (QFS) as described by Ullah *et al*., (2022). Although it is not a fatal illness, it has major social and economic repercussions because it lowers a person's quality of life and makes it impossible for them to work. As Q fever is considered as occupational zoonosis, farm workers are exposed to this risk. As mentioned above, *C. burnetii* infection may lead to subclinical mastitis in dairy animals and in abortion cases, the lower milk yields would result from the mammary glands not developing or redeveloping as a result of lactation starting earlier than would happen in cows with normal parturitions according to Keshavarzi *et al*., (2020). This would present a threat that would significantly impact the dairy industry.

CHAPTER 7

CONCLUSION AND FUTURE WORK

In conclusion, most states showed seropositivity against Q fever or *C. burnetti* infection in dairy cattle except for Sabah. The detection of Q fever in this study revealed the possible transmission of the disease within the animal populations and serves as occupational risk and possible zoonotic potential to the farm workers which calls for a major public health attention.

Recommendation and future work

For future study, samples should be extended targeting multiple farms across the states to produce a meaningful result which can be used to calculate the prevalence of Q fever in the states, rather than using a single farm to represent the state. Secondly, the study can be enhanced by collecting other relevant samples such as tick, vaginal discharges, or birthing products in order to the *C. burnetti* via molecular method to correlate the findings from serology using ELISA which to further verify the status of the disease in the farm. Lastly, data collection involving farm survey may beneficial to study the related risk factors that leads to the transmission of Q fever in the farm.

CHAPTER 8

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CHAPTER 9

APPENDIX

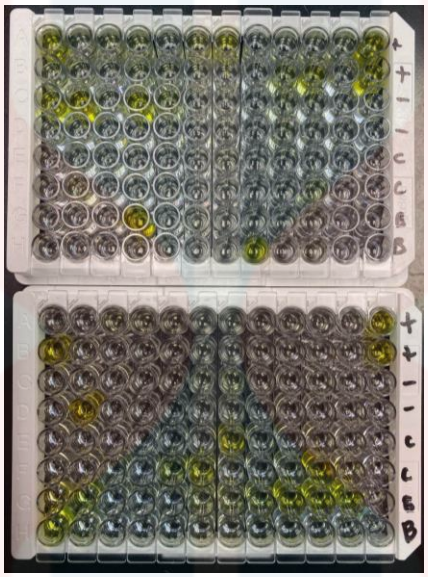

Figure 8.1 Indirect ELISA test kit result

Figure 8.2 Sample preparation for Indirect ELISA test kit