UNIVERSITI PUTRA MALAYSIA

SEROPREVALENCE OF BOVINE ANAPLASMOSIS AMONG DAIRY CATTLE IN FOSTER FARMS OF THE FACULTY OF VETERINARY MEDICINE, UNIVERSITI PUTRA MALAYSIA

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JOY LEE XING PEI

A project paper submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
in partial fulfilment of the requirement for the
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It is hereby that we have read this project paper entitled “Seroprevalence Of Bovine Anaplasmosis Among Dairy Cattle In Foster Farms Of The Faculty Of Veterinary Medicine, Universiti Putra Malaysia”, by Joy Lee Xing Pei and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of requirement for the course VPD 4999 – Final Year Project.

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LIST OF ABBREVIATIONS

cELISA	Competitive Enzyme-Linked Immunosorbent Assay
CF	Complement fixation
^{o}C	Degree celcius
GST	Gluthathione S-transferase
IEs	Infected erythrocytes
MBP	Maltose binding protein
µ	micron
ml	Milliliter
nm	Nanometer
OD	Optical density
PCV	Packed cell volume
% I	Percentage of inhibition
PCR	Polymerase chain reaction
p	Probability
rMSP5	Recombinant major surface protein 5
Se	Sensitivity
Sp	Specificity
SPSS	Statistical Product and Service Solutions
UPM	Universiti Putra Malaysia
OIE	World Health Organization for Animal Health

ABSTRAK

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

SEROPREVALENS ANAPLAMOSIS BOVIN DALAM KALANGAN LEMBU TENUSU LADANG ANGKAT FAKULTI PERUBATAN VETERINAR, UNIVERSITI PUTRA MALAYSIA

Oleh

JOY LEE XING PEI

2017

Penyelia: Prof. Dr. Rasedee Abdullah

Penyelia bersama: Prof. Madya Dr. Faez Firdaus Jesse Abdullah

Anaplasmosis bovin adalah penyakit disebab oleh *Anaplasma marginale* menjangkiti eritrosit, membawa kepada eritrofagositosis, dan menyebabkan anemia. Mengikut kajian lepas, anaplasmosis adalah antara penyakit ketara yang melibatkan lembu di Malaysia. Bagaimanapun, kini tiada rekod terhadap prevalens penyakit ini di kalangan lembu Ladang Angkat Fakulti Perubatan Veterinar, Universiti Putra Malaysia (UPM). Justeru, kajian ini dijalankan untuk menentukan seroprevalens anaplasmosis pada lembu tenusu Ladang Angkat. Kajian ini juga membandingkan kepekaan dan kekhususan di antara kaedah serologi mengguna cELISA dan kaedah mikroskopi dalam pengesanan dan diagnosis anaplasmosis. Sampel darah diperolehi melalui vena daripada empat-puluh (45) ekor lembu yang dipilih daripada empat (4)

ladang berasaskan pensampilan mudah laku. Darah dalam heparin diguna untuk membuat saput darah nipis terwarna Giemsa, penentuan hematokrit, dan pengesanan mikroskopi anaplasmosis,. Serum diperolehi daripada darah tergumpal untuk ujian serologi mengguna Anaplasma Antibody Test Kit (VMRD Inc.). Seroprevalens anaplasmosis bovin dalam populasi lembu kajian ini ialah 51.11%. Terdapat perkaitan tererti ($p<0.05$) di antara kaedah pengesanan anaplasmosis mikroskopi dan serologi. Kaedah serologi menunjukkan kepekaan and kekhususan yang lebih tinggi daripada kaedah pengesanan mikroskopi. Kesimpulannya ialah, kadar prevalens anaplasmosis yang rendah pada lembu Ladang Angkat ini adalah hasil daripada program kesihatan yang baik yang telah dikendalikan di bawah penyeliaan Fakulti Perubatan Veterinar, UPM.

Katakunci: anaplamosis bovin, *Anaplasma marginale*, seroprevalens, cELISA, pengesanan mikroskopi.

ABSTRACT

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

SEROPREVALENCE OF BOVINE ANAPLASMOSIS AMONG DAIRY CATTLE IN FOSTER FARMS OF FACULTY OF VETERINARY MEDICINE, UNIVERSITI PUTRA MALAYSIA

By

JOY LEE XING PEI
2017

Supervisor: Prof. Dr. Rasedee Abdullah
Co-Supervisor: Associate Prof. Dr. Faez Firdaus Jesse Abdullah

Bovine anaplasmosis is a disease primarily caused by *Anaplasma marginale* that infects erythrocytes, leading to erythrophagocytosis causing anaemia. According to past prevalence studies, anaplasmosis is a significant disease affecting cattle in Malaysia. However, currently there is no record on the prevalence of this disease among cattle of the Foster Farms of the Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). Thus, this study was carried out to determine the seroprevalence of anaplasmosis among dairy cattle in the foster farms. This study also compared the sensitivity and specificity between the serological method using cELISA and the microscopic method in the detection and diagnosis of anaplasmosis. Blood samples were collected via jugular venipuncture from forty-five (45) cattle selected based on convenience sampling from four (4) farms. Heparinized whole blood was used to prepare Giemsa-stained thin blood smears, for determination of haematocrit, and microscopic detection of anaplasmosis. Serum was extracted from coagulated blood for serological testing using the Anaplasma Antibody Test Kit (VMRD, Inc.). The seroprevalence of bovine anaplasmosis in the study cattle

population was 51.11%. There was a significant ($p<0.05$) association between microscopic detection and serological method for anaplasmosis. The serological method had a higher sensitivity and specificity than the microscopic detection method. In conclusion, the low prevalence rate of anaplasmosis in the dairy cattle of the Foster Farms is the result of the good herd health programme instituted under the supervision of the Faculty of Veterinary Medicine, UPM.

Keywords: bovine anaplasmosis, *Anaplasma marginale*, seroprevalence, cELISA, microscopic detection.

1.0 INTRODUCTION

Agriculture is one of the foundations of Malaysia's economy, contributing 8.9% to the gross domestic product (GDP) in 2015, amounting to RM94.1 billion (Jabatan Perangkaan Malaysia, 2016). One of the contributing industries to GDP is the dairy cattle farming for the production of milk. The local fresh milk production had increased from 45.5 million litres annually in 2006 to 76.0 million litres in 2015, an increase of 67.03%. However, this is not sufficient to meet local demand and there is still need for importation of milk (Agrofood Statistic, 2012).

To ensure improvement to the dairy industry, control of diseases in these cattle is of paramount importance. Among the common diseases affecting local cattle is anaplasmosis. Bovine anaplasmosis is a disease characterised by haemolytic anaemia that had a significant contributor to economic losses in the industry (Ashuma *et al.,* 2013). Cattle infected with the parasite show reduced milk production and growth and death. Treating this disease incurs veterinary and labour costs that add to the cost of production. This disease, although normally causes sporadic deaths, can occasionally affect large number of animals if herd immunity towards the parasite is compromised (Abba *et al.,* 2016).

Anaplasmosis in cattle is caused mainly by *Anaplasma marginale*. The *Anaplasma* sp. are intraerythrocytic mircoorganisms from the order of Rickettsiales. Two main species of concern are *Anaplasma marginale* and *Anaplasma centrale*, with the former being more pathogenic (Urquhart, 1987). There are variable clinical signs of the disease that can vary from subclinical to acute severe form. The contributing factors of the disease are the virulence of *Anaplasma sp.* strain, age-related host susceptibility, and breed resistance. Affected cattle can show hyperthermia, anorexia, and lethargy, which can lead to a dramatic decrease in milk production. Cattle that survived 2 to 3 days of infection, that is at the acute stage of the disease, must be observed for paleness or icterus, which are characteristic signs of the clinical form of the disease. Animals that recover from the disease may not exhibit prominent clinical signs, but may remain as a carrier for life and become reservoirs for transmission. Thus, in the management of the disease, accurate diagnosis is important for institution

of appropriate treatment regimens while continuous screening is required to minimise the spread of disease in carrier animals (Smith, 2015).

In the diagnosis of anaplasmosis, the complement fixation (CF) test is one of the most frequently used serological test. This test is usually conducted on animals prior to interstate or international shipment. However, the accuracy of the CF test is prone to error because the of high variability of results in the detection of persistent infection. A better alternative for diagnosis of anaplasmosis is the competitive enzyme-linked immunosorbent assay (cELISA) using the bovine serum or demonstration and identification of the infective agent in Giemsa-stained thin blood smears (Bradway *et. al,* 2001).

According to Pong *et al.* (2012), the infection rate of anaplasmosis in Peninsular Malaysia was 87% while in East Malaysia 60%. This study shows that the disease is prevalent in Malaysia. However, there are no records of the prevalence of this disease among foster farms (*Ladang Angkat*) of the Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). Thus, this study was undertaken to determine the seroprevalence of bovine anaplasmosis among dairy cattle in these foster farms. This study also compares the sensitivity between serological and thin blood smear methods in the detection of bovine anaplasmosis.

2.0 LITERATURE REVIEW

2.1 Natural History of Bovine Anaplasmosis

Bovine anaplasmosis, also termed as redwater or gall sickness of cattle in South Africa was first described by Sir Arnold Theiler in 1910. The disease was initially grouped with babesiosis as Texas fever that is caused by *Babesia bigemina* in cattle. Sir Theiler suspected that this is a different disease from babesiosis when cattle that recovered from *B. bigemina* infection and expected to develop immunity to pathogen, underwent a similarly clinical disease when deliberately inoculated with *B. bigemina*. Through clinical observations, microscopic observations, and post-mortem lesions of cattle experimentally inoculated with *B. bigemina* infected cattle blood (Palmer, 2009), Sir Theiler concluded that the 'marginal points' observed in erythrocytes were not stages of the *B. bigemina* life cycle as earlier suspected and that babesiosis and anaplasmosis were two separate diseases that often co-exist in the animal. These observations led to the discovery of *Anaplasma marginale* (Kocan *et al.*, 2010).

2.2 Aetiological Agent

Anaplasma marginale is an intraerythrocytic haemoparasite originally from the order of Rickettsiales (Urquhart *et al.,* 1987). Based on 16S ribosomal DNA gene sequence, the parasite was classified in the genogroup II of *Ehrlichaea* (Walker & Dumler, 1996). Currently, it is classified under the genus *Anaplasma* of the family *Anaplasmataceae* (Aubry *et al.,* 2011). Under Giemsa stain, these organisms appear as dense and rounded bodies with a diameter of approximately 0.3 – 1.0 μm located within the erythrocyte, on or near the margin of the erythrocyte.

Anaplasma-infected erythrocytes can be taken up by ticks and the parasite develops into different stages in the tick gut and salivary gland. *A. marginale* develops within membrane-bound vacuoles or colonies in the tick. Within the colony, the first

form of the organism is reticulated or vegetative where it divides by binary fission to form large colonies containing hundreds of the organism. The reticulated form transforms into a dense form that is infective and can survive outside the cell for a limited period of time. It is this dense form of *A. marginale* that infects cattle via the salivary glands secretions through bites of infected ticks (Kocan *et al.*, 2004). The organism then undergoes cycles of invasion, replication and release from erythrocytes following its transmission to cattle.

During acute infection, cell-associated rickettsaemia may reach a concentration of 10^9 infected erythrocytes per milliliter (IE/mL) blood resulting in clinical anaplasmosis. Animals that survive the acute stage will continue to have a life-long persistent infection where there will be repeated cycles of rickettsaemia ranging from $10^{2.5}$ to 10^7 IE/mL. This level of infection is low for microscopic detection.

2.3 Epidemiology

Anaplasma marginale is distributed in the tropical and subtropical regions of Europe, Africa, Asia and Australia. It has also been reported to occur in almost every state in the United States of America. Transportation and subsequent transmission from asymptomatic persistently infected animals to susceptible ones is suspected to be the main cause of the wide distribution of the organism (Aubry *et al.,* 2011). Bovine anaplasmosis is a vector-borne disease and climate has an effect of the epidemiology of the disease. In a study by Tabachnick (2010), it was shown that increase in environmental temperature causes increased population growth rate and length of transmission period of vectors. Thus, in Malaysia, the change in tick population and distribution of bovine anaplasmosis is seasonal. A seroprevalence study, conducted by Mirwan (2003) on selected dairy farms in Selangor and Pahang states of Malaysia, showed a seropositivity of 87.7%. In a more recent study, it was shown that the seroprevalence in Malaysia was of 79.4% (Pong & Nik Him, 2012).

Cattle are hosts for both *A. marginale* and *A. central* and both *Bos indicus* and *Bos Taurus* have equal susceptibility to the infection under field conditions. However, *Bos indicus* are less commonly affected because of their relative resistance to heavy

tick infestation. It has also been found that breeds with black or red fur coat colour have a higher risk of infection in geographical locations where biting flies are important sources of transmission. Dairy breeds are also more susceptible, as they are at a greater risk of iatrogenic transmission. In terms of age, it has been observed that young cattle are less susceptible to infection and clinical disease even if infected. Animals older than three years old are more commonly affected by the peracute fatal form of the disease (Radostits *et al.*, 2007).

2.4 Transmission

Anaplasma marginale can be transmitted through several modes, which is biologically, mechanically or transplacental transmission. Transmission through ticks are the major mode for biological transmission of anaplasmosis involving at least 20 different species of ticks. Some of the major tick vectors of *A. marginale* include *Boophilus spp., Dermacentor spp.,* and *Rhipicephalus spp* (Kocan *et al*., 2004). When ingested, *A. marginale* will replicate in the tick gut epithelium and salivary gland acini, which it can replicate and reach 10^4 to 10^5 organisms per salivary gland during the subsequent transmission feeding (Löhr, 2002). The high *A. marginale* organism load in the ticks had resulted in similarly high levels in blood during subsequent feeding. It is believed that biological transmission is the necessary mode for transmission from persistently infected carriers to susceptible animals. This is because the transmission is not dependent on the level of rickettsaemia of the host because ticks act as efficient bio-magnifiers of *A. marginale* when there is a low level of rickettsaemia during the chronic or carrier phase of the infection (Scoles *et al.,* 2005). Tick transmission can occur transstadially or intrastadially, but not transovarially. Intrastadial transmission of male ticks is an important mechanism of transmission because the ticks serve as a reservoir and infection can occur after just a short period of feeding (Kocan *et al.,* 2006)

Mechanical transmission can occur either via biting flies or iatrogenic means such as contaminated equipment. There are at least 12 species of biting flies that have been experimentally shown to be potential mechanical transmitters of *A. marginale*.

This includes *Stomoxys calicitrans* (stable fly), 8 species of tabanids (horse flies) and 3 species of midges of the *Culicidae* family (Aubry *et al.,* 2011). In contrast to the ticks, biting flies are presumed to be purely mechanical vectors with no involvement in the development or replication of the organism. Thus, the transmission of the disease is directly dependent on the level of rickettsaemia of the host during feeding and it is believed that transmission of anaplasmosis by flies can only occur during the acute phase of infection. The factors determining efficacy of the fly-borne transmission include feeding behaviour of the fly, size of fly population carrying *A. marginale*-infected blood, and number of *A. marginale* viable infective units maintained in fly before feeding on the second host. In the study by Scoles *et al.* (2005), it is shown that increased numbers of interrupted feedings by the biting fly and subsequent movement of the flies between hosts also provides an ideal opportunity for the transmission of the organism to uninfected hosts. This is because when blood feeding is interrupted, the flies can immediately contact a nearby host, since cattle under fly-feeding pressure tend to group closely together. The study also showed that mechanical transmission by biting flies is at least twice less efficient than tick-borne biological transmission. It was speculated that this observation is due to the loss of viability of *A. marginale* in the fly or the inefficient transfer of parasite to the host.

Iatrogenic spread of the disease is important only in within herd transmission and not between herds. Some fomites including needles, dehorning equipment, tattooing instruments, ear tagging devices, and neutering equipment could be sources of mechanical transmission (Kocan *et al.*, 2006).

There are several reports of vertical transmission of anaplasmosis through transplancental route (Aubry *et al.*, 2011). It was observed that *in utero* transmission can occur during the two trimesters of gestation. However, in the field, this mode of transmission occurs infrequently.

2.5 Clinical Signs

In the acute stage of the infection, the proportion of erythrocytes being parasitised by *A. marginale* ranges from 10 to 90% depending on the strain of *Anaplasma* and susceptibility of the host. However, at least 15% of the erythrocytes need to be parasitised to cause a clinical disease. The incubation period is generally three to four weeks with tick borne infection, and may vary with the challenge dose (Radostits *et al.,* 2007).

The primary effect of the infection is the induction of extravascular haemolytic anemia, which is presumed to be the cause of death (Abba *et. al.,* 2016) The anaemia occurs when *A. marginale* infects mature erythrocytes through endocytosis, followed by binary fission, producing two to eight infective initial bodies, which leave the erythrocytes by exocytosis to infect other erythrocytes. Subsequently, the number of IEs will double every 24 to 48 hours. The cattle's reticular endothelial system removes parasitized erythrocytes via phagocytosis, leading to erythrocyte destruction, which results in mild to severe anaemia (Radostits *et al.¸* 2007). However, in the infected animals, there is no hemoglobinuria or hemoglobinemia, which allows clinicians to differentiate between bovine anaplasmosis from babesiosis (OIE, 2015). The degree of anaemia varies according to the proportion of parasitised erythrocytes. As the anaemia becomes more severe, the acutely infected animal can lose condition rapidly, suffer from dehydration and constipation, and the respiratory signs can be progressively more evident (Mirwan, 2003). The acutely affected animals may die shortly after reaching this phase of infection (Radostits *et al.¸* 2007).

During erythrophagocytosis there is release of acute phase inflammatory reactants, which is followed by pyrexia. Rectal temperatures tend to rise slowly but rarely exceeding 40.5°C. The temperature may then remain elevated or fluctuate with irregular periods of hyperthermia and normothermia for several days up to two weeks (Radostits *et al.¸* 2007). This discomfort can lead to the development of incomplete anorexia.

Hyperexcitability and aggression has also been reported in acutely affected cattle. The exact cause is not known but it is presumed to be due to the effect of hypoxia on the brain (Whittier *et al.*, 2007). Abortion has also been reported in

pregnant cows, while bulls may suffer from depressed testicular function for several months (Radostits *et al.,* 2007). Animals that survive the acute stage of infection can become persistently infected and are carriers of the organism for life.

2.6 Diagnostic Methods

There are a variety of methods to diagnose bovine anaplasmosis and they can be broadly categorized according to agent identification and serological method (OIE, 2015). Identification of the agent can be done by microscopic examination where blood from live animals are collected via jugular venipuncture for preparation of Giemsa-stained thin blood smears.

Polymerase chain reaction (PCR)-based methods can also be used for agent identification with this analytical sensitivity is estimated at 0.0001% IEs. The nested PCR has also been used to identify carrier cattle with IEs as few as 30/mL blood (OIE, 2015).

In serology, CFT is one of the most frequently used test in the diagnosis of anaplasmosis. However, according to Bradway *et. al.* (2001), CFT is prone to a high error rate. False-positives may be due to the normal bovine erythrocytes stroma in the CF antigen while false-negatives may be due to failure to detect bovine immunoglobulin isotypes. The cELISA method detects *A. marginale* recombinant major surface protein 5 (rMSP5) antibodies. Thus, the test recommended by the OIE is the cELISA, which is more reliable. The cELISA is of superior sensitivity in the detection of *Anaplasma marginale* with 94.8% compared to the CFT method at 26.5% (Coetzee *et. Al.,* 2007). Chung *et. al.* (2014) developed an improved cELISA by replacing rMSP5-maltose binding protein (rMSP5-MBP) with rMSP5-gluthathione S-transferase (GST), which eliminated the MBP from the antigen. The rMSP5-GST cELISA is faster and simpler to perform with higher specificity and comparable sensitivity to that of the commercially rMSP5-MBP cELISA. Indirect ELISA can also be used for detection of anaplasmosis. Its sensitivity and specificity is as good as cELISA but takes four to five hours to perform to the 2.5 hours via cELISA. The card agglutination test is a sensitive test that can yield results in a few minutes. However,

this test may produce nonspecific reactions, and prone to subjectivity when interpreting the assay reactions, which may result in great variability (OIE, 2015).

3.0 MATERIALS AND METHODS

3.1 Blood Sampling

Four (4) dairy cattle farm of the Faculty of Veterinary Medicine, UPM Foster Farm programme (*Ladang Angkat*) were used in the study. Forty-five (45) cattle were selected by convenience sampling, based on ease of restraint. The age of the cattle ranged from 2 to 9 years. The age was determined based on dentition and counter-checked with farm records.

Blood was obtained from each cattle via jugular venipuncture into labelled plain tube to obtain the serum for serological testing and with sodium heparin tubes to obtain plasma tube haematocrit (PCV) determination and making of thin blood smear for microscopic examination. The blood tubes were immediately placed in an ice box for transportation to laboratories of the Faculty of Veterinary Medicine, UPM, to be processed.

The study was conducted with the approval of the Institutional Animal Care and Use Committee [AUP No.: FYP.2016/FPV (32.50)].

3.2 Serum Extraction

Blood collected in plain tubes are centrifuged at 300 × g for 5 minutes to harvest serum. At least 0.5 mL serum each in 2 tubes was then extracted out using a pipette and transferred labelled to 1.5 mL size micro-centrifuge tubes. One tube is stored at -20ºC for serological evaluation, while the other stored at -80ºC for future use.

3.3 Serological Testing

The serum samples to be subjected analysis using the Anaplasma Antibody Test Kit cELISA v2 (Veterinary Medical Research & Development) was kept

refrigerated at a temperature between 2ºC to 7ºC prior to use. Before running the cELISA test kit, the conjugate solution was prepared by diluting 1:99 of 100X Antibody-peroxidase Conjugate concentrate and Conjugate Diluting Buffer. The wash solution was prepared by diluting 1:9 of 10X Wash Solution Concentrate and distilled water. The positive control was loaded in duplicates, while negative control triplicates prior to loading of serum samples. Fifty (50) microliter of thawed serum sample was loaded in duplicates onto the antigen-coated plate. The side of the plate was tapped several times to ensure uniform and complete sample coating of the bottom of the wells. The plate was then incubated at room temperature for one hour, followed by washing twice with the diluted wash solution that was prepared earlier on. Fifty (50) microliter of diluted Antibody-Peroxidase Conjugate was then loaded into each well. The side of the plate was tapped for several times to ensure uniform and complete sample coating of the bottom of the wells and the incubated at room temperature for 20 minutes and then washed four times with diluted wash solution. Fifty (50) microliters substrate solution was added into each well. The side of the plate again tapped several times to ensure uniform substrate coating of the bottom of the wells. The plate was reincubated at room temperature for 20 minutes before immediately stopping the reaction with 50 µL of Stop Solution. The plate was immediately read using a microplate absorbance spectrophotometer (Infinite® M200 from Tecan Trading AG) at a wavelength of 630nm.

3.4 Test Validation and Results Interpretation

The tests were validated by using negative and positive controls. Tests are valid when the mean optical density (OD) of the negative controls is > 0.40 and ≤ 2.10 while the mean of positive controls ≥ 30% inhibition.

Percentage inhibition (I%) was calculated using the following formula:

$$I\% = 100\ [1- (OD_{Sample} / OD_{Negative\ control}]$$

Where OD_{Sample} is the OD of the sample and $OD_{Negative\ control}$ the OD of the negative control.

Test samples with < 30% of inhibition are negative for bovine anaplasmosis, while those with ≥ 30% inhibition are positive.

3.5 Thin Blood Smear Preparation And Microscopic Examination

Blood from heparinised tubes was used to prepare thin blood smears on the same day of blood sampling. Thin blood films were air-dried, fixed with absolute methanol for 30 seconds and Giemsa-stained using the standard procedure (OIE, 2015). The slides are examined under oil-immersion light microscope. The *Anaplasma marginale* is identified as dense, rounded and deeply stained intraerythrocytic bodies with a diameter of approximately 0.3 – 1.0 μm, while *Anaplasma centrale* have a similar appearance at a more central location in the erythrocyte. A total of at least 50 fields at the feather edge of the blood smear of each slide were examined. The blood smear is considered to be positive when the organism is visualized and identified.

3.6 Haematocrit

The heparinized blood samples were thoroughly mixed on a tube rotator. One hematocrit capillary tube is filled to at least three-quarters full, before sealing one end of the capillary tube. The capillary tubes were spun at the microhaematocrit centrifuge (Haematokrit 20, Hettich Instruments) 5 minutes, and the hematocrit (PCV) determined by the standard method.

In this study, the PCV was “Low” and “Normal” groups if the value is < 0.24 and ≥ 0.24 L/L, respectively.

3.7 Statistical Analysis

Chi-square test was used to analyse the data using IBM SPSS Statistics V22.0 to determine association between age and PCV level with seropositivity, as well as to compare between the serological and microscopic detection methods.

4.0 RESULTS

4.1 Seroprevalence Of Bovine Anaplasmosis

Results in this study showed that the seroprevalence of bovine anaplasmosis among dairy cattle in Foster Farms (*Ladang Angkat*) under Faculty of Veterinary Medicine, UPM is 51.11% (Table 1).

Table 1: Bovine anaplasmosis seroprevalence among dairy cattle in Foster Farms of Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Farm	Samples (Number)	Seropositive animals (Number)	Seropositive animals (%)
Farm A	12	10	83.33
Farm B	12	5	41.67
Farm C	13	3	23.98
Farm D	8	5	62.50
Total	**45**	**23**	**51.11**

True prevalence is estimated from apparent prevalence using the following formula (Thrusfield, 2007):

$$\text{True prevalence} = \frac{Apparent\ prevalence + Specificity - 1}{Sensitivity + Specificity - 1}$$

In the Foster Farms, the true prevalence of bovine anaplasmosis was 52.81%

4.2 Seropositivity According To Age Group Of Cattle

Cattle in the age group of 3 years or more had a higher percentage of seropositive cattle compared to cattle of the younger age group (Table 2). There was no statistically ($p<0.05$) significant association between the age of cattle and its seropositivity.

Table 2: Bovine anaplasmosis seroprevalence according to age among dairy cattle in Foster Farms of Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Age (years)	Sample (Number)	Seropositive animal (Number)	Seropositive animal (%)
< 1	0	0	0.00
1 – 3	13	6	46.15
> 3	32	17	53.13
Total	**45**	**23**	**51.11**

4.3 Haematocrit

There were only four out of 45 cattle with low PCV levels, where only 25% of the cattle with low PCV level was seropositive (Table 3). There was a higher percentage of 53.66% of cattle that were seropositive despite having normal PCV levels. There was no statistically significant association between the PCV level of cattle and its seropositivity at $p < 0.05$, according to the Chi-Square test.

The mean and standard deviation PCV levels among seropositive and seronegative cattle are 0.35 ± 0.08 and 0.30 ± 0.07 L/L, respectively.

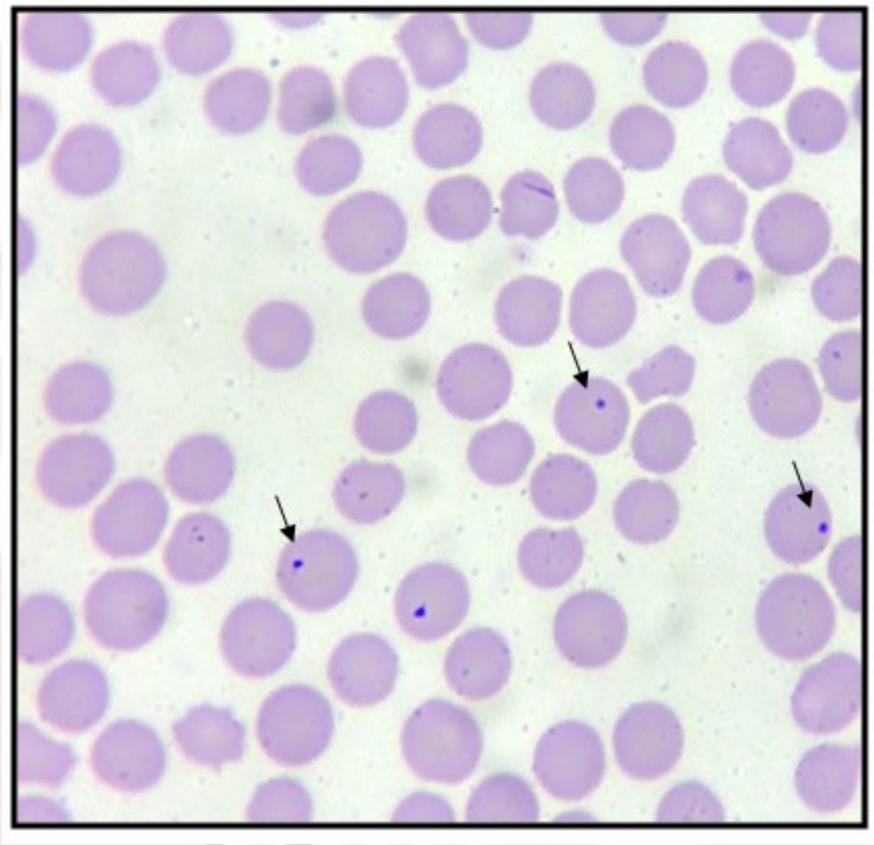
Table 3: Bovine anaplasmosis seroprevalence according to haematocrit among dairy cattle in Foster Farms of Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Haematocrit	Sample (Number)	Seropositive animal (Number)	Seropositive animal (%)
Low (<0.24 L/L)	4	1	25.00
Normal (>0.24 L/L)	41	22	53.66
Total	**45**	**23**	**51.11**

4.4 Microscopic Detection

Using microscopic detection, 16 samples were detected as positive samples. However, among the 16 samples, only 12 samples were found to be true positive samples when counter-checked with the serological result, while the remaining 4 samples are deemed as false positive samples which may be as a result of debris on the glass slides. Among the seropositive animals, only 52% was detected as positive by the microscopic detection method (Figure 1).

Figure 1: Erythrocytes infected with *Anaplasma marginale* (arrows)



4.5 Comparison Between Serological And Microscopic Detection Method

There was a statistically ($p>0.05$) significant association between thin blood smear detection method and the seropositivity (Table 4).

Table 4: Comparison between serological method (cELISA) and microscopic detection methods

		cELISA	
Microscopic detection	Positive (n)	Negative (n)	Total (n)
Positive (n)	12	4	16
Negative (n)	11	18	29
Total (n)	**23**	**22**	**45**

n = number

The *Anaplasma* antibody cELISA test kit has a sensitivity of 95% and specificity of 98%. The sensitivity and specificity of thin blood smear detection method in this study was calculated based on the formula of Thrusfield (2007):

$$Se = (\text{True Positive}/\text{Total diseased}) \times 100$$

$$Sp = (\text{True negative}/\text{Total non-diseased}) \times 100$$

Based on this formula, the sensitivity and specificity of the microscopic detection method was 52.17% and 81.82%, respectively, showing that the serological is more sensitive and specific than the microscopic detection method.

5.0 DISCUSSION

5.1 Seroprevalence Of Bovine Anaplasmosis

At 51.11%, the prevalence of bovine anaplasmosis in dairy cattle of Foster Farms, UPM was lower than that reported in previous seroprevalence studies on the disease conducted in Malaysia. Mirwan (2003) showed that cattle from farms in Selangor and Pahang had a seroprevalence of 87.7% while a more recent study (Pong & Nik Him, 2012) conducted in 10 states of Malaysia not including Malacca, Perak and Negeri Sembilan, reported the prevalence of 74.9%. All three studies used the same cELISA kit method where samples with inhibition of ≥ 30% are taken as positive (Table 5).

Table 5: Comparison of three bovine anaplasmosis seroprevalence studies in Malaysia.

	Current study	Mirwan (2003)	Pong & Nik Him (2012)
Seroprevalence	51.1%	87.7%	74.9%
Sample size (n)	45	180	212
Farms (n)	4	19	Not reported

n = number

Although geographical locations of these studies differ, the comparison of prevalence of anaplasmosis between these studies is still relevant. Our results showed the farms had low anaplasmosis prevalence than that reported earlier. The Farms in this study are adopted under the Faculty of Veterinary Medicine, UPM in the foster farm programme. Under this programme, the Faculty through visits by clinicians advises the farm on herd health program (HHP) that included waste management, parasite control, drug management, and disease monitoring.

Ticks such as *Boophilus spp.* and *Rhipicephalus spp.* and biting flies such as *Stomoxys calicitrans* and tabanids are important vectors of bovine anaplasmosis

(Kocan *et al.*, 2006; Aubry *et al.*, 2011). With improvement in the general HHP and management of the farms under the Foster Farm programme, the prevalence anaplasmosis in these farms had decreased, contributing to the lower seroprevalence of the disease in these farms. A study by Jesse *et al.* (2015) observed that parasite control showed the third highest score in terms of farmer compliance towards HHP. This could be associated with the realization that parasite infestation can adversely affect farm productivity; thus, farmers are more cognizant of the need for parasite control (Jesse *et al.*, 2015). As a result, it must have been one of the factors for the low seroprevalence of anaplasmosis in the Foster Farms.

5.2 Seropositivity And Cattle Age Group

In this study, although there was no significant ($p>0.05$) association between age group and the seroprevalence, cattle from the older age group, i.e. aged > 3 years were, in number, more anaplasma seropositive than younger cattle. According to Radostits *et al.* (2007), animals infected after three years of age are commonly affected by the peracute fatal form of anaplasmosis. Persistently affected carriers that survive the acute phase of infection will remain seropositive, and may become carriers of the disease (Aubry *et al.*, 2011).

5.3 Haematocrit

One of the hallmarks of bovine anaplasmosis is anaemia, which can be quantitatively determined by PCV level. The anaemia is characterised by pale mucous membranes and increased respiratory rate (Kocan *et al.*, 2010). During the acute stage of infection, that is the parasite burden had reached 10^9 cells/mL blood, there is massive blood loss from phagocytosis of the IEs by the monocyte-phagocytic system. In a case, the acutely infected cattle the PCV decreases to as low as 10 L/L (Abba *et al.,* 2016). According to the Merck Veterinary Manual (Merck), the normal PCV range for cattle is 0.24 to 0.46 L/L. Thus, at this PCV value the cattle is severely anaemic.

5.4 Comparison Between Serological And Microscopic Detection Method

Both the sensitivity and specificity of microscopic detection method is lower than that of the serological cELISA kit method. This is in agreement with earlier studies that reported that the microscopic detection method is of low sensitivity and specificity (Noaman and Shayan, 2010; Ekici and Sevinc., 2011). The difference in efficiency in the detection of disease can be due to the nature of the causative organism as well as stage of infection. In acutely affected cattle, the number of IEs can be as high as 10^9 cells/mL blood, while in persistently infected carriers, the number of IEs fluctuates from 10^3 to 10^5 IEs/mL blood over the 10- to 14-day period. The parasite burden is in persistently infected cattle is much lower than that in acutely affected cattle (Kocan *et al.*, 2004). Conversely, the microscopic analysis of Giemsa-stained blood smears can detect parasitaemia when the parasite level is at least 10^6 IEs/mL blood. Thus, the microscopic anaplasmosis detection method is not suitable for pre-symptomatic or persistently affected carrier animals (Noaman and Shayan, 2010).

The microscopic detection method is not only dependant on level of parasitaemia and well-stained blood smears, but also requires a well-trained parasitologist to perform for accurate diagnosis. However, the advantage of this method is that it is more convenient and inexpensive for routine laboratory diagnosis (Noaman and Shayan, 2010). In their study, it was concluded that blood microscopic examination can give a good diagnosis of acute cases, but it is not useful for the detection of cattle carriers of *Anaplasma*.

The cELISA is one of the recommended serological methods for anaplasmosis (OIE, 2015). The assay has been reported to be able to detect experimental anaplasmosis in cattle as long as 6 years after infection. However, the kit is expensive and the time to perform the procedure at 2.5 hours is significantly longer than that of the microscopic detection method. However, one major advantage of the cELISA method is its ease in conducting the test, large number of sera that can be processed at one time, and accuracy of the results (Ekici & Sevinc, 2011).

6.0 CONCLUSION

In conclusion, the seroprevalence rate of bovine anaplasmosis among the foster farms is 51.11%, which is lower than that reported by other studies in Malaysia. This study was conducted on Foster Farms that had the advisory and clinical services on HHP from the Faculty of Veterinary Medicine, UPM, the rate of anaplasma infection in the cattle is well controlled. Thus, it is expected that the rate of infection in the farms is much lower than farms of the pervious study.

This study showed that there is no significant ($p>0.05$) association between age and PCV of cattle and anaplasma seropositivity. Microscopic detection was found to be significantly ($p<0.05$) associated with serological method, although of lower sensitivity and specificity.

7.0 RECOMMENDATION

It is recommended that the study to be conducted on larger number in greater number of locations in Malaysia. This will provide a truer picture of the prevalence and seropevalence of bovine anaplasmosis in the country.

It is also recommended to the farmers involved in this study to practice more stringent preventive measures in their farms. This includes vector control and prevention of iatrogenic transmission of the disease such as the reusing of needles or sharing or unsterilized surgical equipment to minimise the transmission of the disease within the herd.

REFERENCES

Abba, Y., Jesse, F.A.J.., Sadiq, M., Ibrahim, H.H., Chung, E.LT.., Bitrus, A.A., Mohd Lila M.A. & Hambali, I.U. (2016). Clinical management and gross pathological findings of a severe anaplamosis in a dairy cow. *Journal of Advanced Veterinary and Animal Research, 3*(2): 195-199.

Abdullah, F.F J., Rofie, A. M., Tijjani, A., Lim, E. L.T., Mohammed, K., Sadiq, M.A. Sahare A.A & Abba, Y. (2015). Survey of goat farmers' compliance on proper herd health program practices. *International Journal of Livestock Research, 5*(11), 8-14.

Agrofood Statistics (2012). Ministry of Agriculture and Agro-based Industry Malaysia, http://www.moa.gov.my/documents/10157/b4c836ee-7e98-49f9-a141-1c48643624b5 Accessed on 18 February 2017.

Ashuma., Sharma, A., Singla, L. D., Kaur, P., Bal, M. S., Batth, B. K., & Juyal, P. D. (2013). Prevalence and haemato biochemical profile of *Anaplasma marginale* infection in dairy animals of Punjab (India). *Asian Pacific Journal of Tropical Medicine, 6*(2), 139-144.

Aubry, P., & Geale, D. W. (2011). A review of bovine anaplasmosis. *Transboundary and Emerging Diseases, 58*(1), 1-30.

Bradway, D. S., Torioni de Echaide, S., Knowles, D. P., Hennager, S. G., & Mcelwain, T. F. (2001). Sensitivity and specificity of the complement fixation test for detection of cattle persistently infected with *Anaplasma Marginale*. *Journal of Veterinary Diagnostic Investigation, 13*(1), 79-81.

Chung, C., Wilson, C., Bandaranayaka-Mudiyanselage, C.B. Kang, E., Adams, D. S., Kappmeyer, L. S.,Knowles, D.P., McElwain, T.F., Evermann, J.F., Scoles,G.A. & Mcguire, T. C. (2014). Improved diagnostic performance of a commercial Anaplasma antibody competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5–glutathione S-transferase fusion protein as antigen. *Journal of Veterinary Diagnostic Investigation, 26*(1), 61-71.

Coetzee, J. F., Schmidt, P. L., Apley, M. D., Reinbold, J. B., & Kocan, K. M. (2007). Comparison of the complement fixation test and competitive ELISA for serodiagnosis of *Anaplasma marginale* infection in experimentally infected steers. *American Journal of Veterinary Research, 68*(8), 872-878.

Ekici O.D & Sevinc F. (2011). Comparison of cELISA and IFA tests in the serodiagnosis of anaplasmosis in cattle. *African Journal of Microbiology Research, 5*(10), 1188-1191.

Jabatan Perangkaan Malaysia (2016). Selected Agricultural Indicators. http://www.publicnow.com/view/F338E7B32E78BF2C12A6664D71B31FC22ECD8972?2016-12-30-05:00:19+00:00-xxx8281 Accessed on 18 February 2017.

Kocan, K. M., de la Fuente, J., Blouin, E. F., & Garcia-Garcia, J. C. (2004). *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): recent advances in defining host–pathogen adaptations of a tick-borne rickettsia. *Parasitology, 129,* Suppl: S285-300.

Kocan, K. M., Blouin, E. F., & Barbet, A. F. (2006). Anaplasmosis Control. Past, present, and future. *Annals of the New York Academy of Sciences, 916*, 501-509.

Kocan, K. M., de la Fuente, J., Blouin, E. F., Coetzee, J. F., & Ewing, S. A. (2010). The natural history of *Anaplasma marginale*. *Veterinary Parasitology*, *167*(2-4), 95-107.

Löhr, C. V. (2002). Specific expression of *Anaplasma marginale* major surface protein 2 salivary gland variants occurs in the midgut and is an early event during tick transmission. *Infection and Immunity, 70*(1), 114-120.

Mirwan, N. M. A. (2003). Prevalence of bovine anaplasmosis in Malaysian farms. http://psasir.upm.edu.my/11660/1/FPV_2003_14_A.pdf Accessed on 18 February 2017.

Noaman, V., & Shayan, P. (2010). Comparison of Microscopy and PCR-RFLP for detection of Anaplasma marginale in carrier cattle. *Iranian Journal of Microbiology, 2*(2), 89-94.

Palmer, G. (2009). Sir Arnold Theiler and the discovery of anaplasmosis: a centennial perspective : tick-borne diseases. *Onderstepoort Journal of Veterinary Research, 76*(1), 75-79.

Pong, S., & Nik Him, N.A.I.I. (2012). Seroprevalence of bovine anaplasmosis caused by Anaplasma marginale in Malaysia. *Proceedings of the 2nd Annual International Conference Syiah Kuala University & The 8th IMT-GT Uninet Bioscience Conference, Banda Aceh, 22-24 November, 2012*.

Radostits, O., Gay, C., Hinchcliff, K & Constable P. (2007). *Veterinary medicine: A textbook of the diseases of cattle, sheep, pigs, goats, and horses*, 10th Edition. Edinburg: Saunders Elsevier., pp1455-1458.

Reinbold, J. B., Coetzee, J. F., Hollis, L. C., Nickell, J. S., Riegel, C. M., Christopher, J. A., & Ganta, R. R. (2010). Comparison of iatrogenic transmission of *Anaplasma marginale* in Holstein steers via needle and needle-free injection techniques. *American Journal of Veterinary Research, 71*(10), 1178-1188.

Scoles, G. A., Broce, A. B., Lysyk, T. J., & Palmer, G. H. (2005). Relative efficiency of biological transmission of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) by *Dermacentor andersoni* (Acari: Ixodidae) compared with mechanical transmission by *Stomoxys calcitrans* (Diptera: Muscidae). *Journal of Medical Entomology, 42*(4), 668-675.

Smith, B. P. (2015). *Large animal internal medicine,* 5th Edition. St. Louis, MO: Mosby., pp1054-1056.

Tabachnick, W. J. (2010). Challenges in predicting climate and environmental effects on vector-borne disease episystems in a changing world. *Journal of Experimental Biology, 213*, 946-954.

Thrusfield, M. V. (2007). *Veterinary Epidemiology*. 3rd Edition. Oxford: Blackwell Science.

Urquhart, G. M., Armour, J., Duncan, J. L., Dunn, A. M., & Jennings, F. W. (1987). *Veterinary Parasitology*. Essex, England: Longman Scientific & Technical., pp243-245

Walker, D. & Dumler J.S. (1996). Emergence of the ehrlichioses as human health problems. *Emerging Infectious Diseases, 2*(1), 18-29.

World Organization for Animal Health, OIE (2015). Bovine Anaplasmosis. *OIE Terrestial Manual.* http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/2.04.01_BOVINE_ANAPLASMOSIS.pdf Accessed on 18 February 2017.

Whittier, D., Currin, N., & Currin, J.F. (2007). Anaplasmosis in beef cattle. Virginia Tech Cooperative Extension, Blacksburg, pp 400-465. https://pubs.ext.vt.edu/400/400-465/400-465_pdf.pdf Accessed on 18 February 2017.