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DETECTION OF HAEMOPARASITE IN SEMI-COMMERCIAL AND SCAVENGING VILLAGE CHICKEN

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DETECTION OF HAEMOPARASITE IN SEMI-COMMERCIAL AND SCAVENGING VILLAGE CHICKEN

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In partial fulfilment of the requirement for the

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CERTIFICATION

It is hereby certified that we have read this project entitled "Detection of haemoparasite in Semi-commercial and scavenging village chicken, by Nur Izzati binti Aman and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD4999-Project.

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DEDICATION

This project paper is dedicated to Allah SWT, who had created me and made all things possible,

To my family,

Father,

Mother,

Brothers, Sisters

Lecturers,

And to all my friends who have committed themselves towards this project.

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LIST OF ABBREVIATION

%	Percentage
°C	Degree celcius
µl	Micrometer
ml	Milimeter
IACUC	Institutional Animal Care and Use Committee
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
rpm	Rotation per minute
RT-PCR	Reverse-transcriptase Polymerase Chain Reaction

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ABSTRAK

Abstrak daripada kertas projek yang di kemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek

PENGESANAN PARASIT DARAH DALAM AYAM KAMPUNG SEPARA KOMERSIAL DAN PEBANGKAI

Oleh

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2017

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Ayam kampung (*Gallus gallus domesticus*) biasanya dikaitkan dengan system pemeliharaan lepas bebas yang terdedah kepada alam sekitar dan penyakit, dan juga kepada bacteria dan parasitdijumpai di dalamtanah, burungdanhaiwan lain. Jangkitan parasite darah adalah lebih lazim dan memberi impak dalam ayam kampung. Perbandingan di buat di antara ayam kampong separa komersial dan pebangkai untuk tahap jangkitan parasit. Sebanyak 24 sampel darah telah diambil di sebuah kampung yang terletak di Simpang Renggam, Kluang, Johor. 12 sampel darah telah diambil dari ayam kampong separa komersial dari lading Aqil Aqilah dan 12 sampel darah telah di ambil daripada ayam kampung di sekeliling kawasan kampung. Dalam kajian ini,

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kami menggunakan teknik mikroskopik dan molekul analisis untuk memeriksa sampel darah jika terdapat parasite darah. Smer darah nipis yang di warna dengan Giemsa di sediakan dan di periksa. Pengesanan antigen telah dilakukan dengan menggunakan konvensional transcriptase membalik berantai polymerase konvensional (RT-PCR) untuk kedua-dua kumpulan sampel darah dan spesifik primer di reka untuk sasaran mitokondria gen sitoktrom b parasit. Semasa pemeriksaan mikroskopik smer darah nipis, tiada parasite darah di dalam semua sampel.RT-PCR juga menunjukkan hasil negative dalam semua sampel darah yang diuji. Kesimpulannya, tidak ada perbezaan dalam jangkitan parasite darah dalam separa komersial ayam kampung dan ayam kampung pebangkai.

Kata kunci: jangkitan parasit darah, ayam kampung, RT-PCR, pewarna Giemsa

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ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 – Project

DETECTION HAEMOPARASITE IN SEMI-COMMERCIAL VILLAGE CHICKEN AND SCAVENGING VILLAGE CHICKEN

By

NurIzzati Binti Aman

2017

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Village chicken (*Gallus gallus domesticus*) is usually associated with free-range system that exposed to the environment and seasonal outbreaks of disease, as well as to bacteria and parasites found in the soil, wild birds, and other animals. Haemoparasites infections are more prevalent and contribute significantly in village chicken. Therefore, the presence of haemoparasite infection in semi commercial village chicken and scavenging village chicken were compared. A total of 24 blood samples were collected in a village located in Simpang Renggam, Kluang, Johor. 12 blood samples were collected from semi-commercial village chicken from Aqil Aqilah Farm and 12 blood samples were collected from village chicken that were found scavenged around within the village. In this study, we used both microscopic and

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molecular analysis techniques to examine blood samples for diagnosing avian haemoparasites. Giemsa-stained thin blood smears were prepared and screened for the presence of haemoparasites. Antigen detection using conventional reverse transcriptase PCR for both groups of blood samples was done and specific primer was designed targeting mitochondrial cytochrome b genes of the parasites. During microscopic examination of blood smears, it revealed that there was absence of haemoparasites in all samples obtained. RT-PCR analysis results gave negative finding in all blood samples tested. In conclusion, there are no difference in blood parasite infection in semi-commercial village chicken and scavenging village chicken.

Keyword: haemoparasite infection, village chicken, RT-PCR, giemsa stain

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1.0 INTRODUCTION

Poultry plays a major role in developing country where it is relatively inexpensive and widely available as it provides not only high-quality protein but also important vitamins and minerals (Farrell, 2013). The poultry industry has been self-sufficient since 1984 and contributes 75% of total output in Malaysia where the major production are broilers and egg (Loh, 2014).

Village chickens (*Gallus domesticus)* are a domestic breed of chicken with small heads, large bodies, bare and scaled legs. The red jungle fowl (*Gallus gallus*) is known as wild ancestor of domestic chicken and most likely to have hybridized with *Gallus sonneratii* approximately around 8000 years ago (Sawai *et al*., 2010). It is suggested that domestic chickens originated from jungle fowls in Southeast Asia (Sawai *et al*., 2010). Their plumage color, comb type, body conformation and weight are closely related to the red jungle fowl. Other than that, village chicken has slow growth rate, late sexual maturity, low production and reproductive performance (Eskindir, 2013).

There are two important production types of the poultry industry in southeast Asia which is the commercial sector where it uses highly intensive unit and has been developed rapidly over the past two decades and the traditional village-based system which relies on minimal output to try and produce high quality protein (Aini, 1990). According to Sonaiya (2007), in most tropical countries, 40-70% of chicken meat and eggs production are from integrated village farming systems and kept by rural smallholder, landless farmer, and industrial labours. Malaysia has a humid and warm climate condition will favour any insect that may serve as important vectors for

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parasite (Rahman *et al*., 2009). Other than that, ectoparasites infestations are very common in scavenging poultry and being the vector of several haemoparasites. Village chickens that are managed in free-range system are usually exposed to the environment and seasonal outbreaks of diseases, as well as to bacteria and parasites found in the soil and in wild birds and other animals. Inadequate hygiene also can be one of the factors that can provide optimum condition for maintaining the haemoparasite population as well as environment such as rainfall, humidity and ambient temperature. According to Benedikt *et al*., (2009), the most common parasites in the peripheral blood smear are *Haemoproteus, Leucocytozoon* and *Plasmodium.*

Therefore, this study was designed to examine blood parasites infection in both semi-commercial and scavenging village chicken. The objective of this study is to determine the presence of haemoparasites such as *Haemoproteus, Leucocytozoon* and *Plasmodium* and level of parasitaemia present in semi-commercial and scavenging village chickens. The hypothesis for this study is the scavenging chickens have a higher level of parasitaemia compared to semi-commercial village chickens.

2.0 LITERATURE REVIEW

2.1 Village chicken production in Malaysia

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According to USDA (2014), 90%of village chicken productions are located in peninsular Malaysia whereas the remainder are in East Malaysia and comprises of 67% of the commercial broiler, 25% of layers and 8% of breeders out of the total population. At over 40 kilograms per year, Malaysia's per capita consumption of poultry meat and/or products is among the highest in the world (Farrell, 2013).In Malaysia, village chicken contributes a small portion of chicken meat and egg production where it estimated approximately 10 million birds in total population which is comprised of 3% of total population while others 97% is from commercial broiler (Ramlah,1999). It also stated that most of southeast Asian countries including Malaysia especially in rural areas practices rearing chickens under scavenging systems of backyard farming for centuries. According to Ramlah & Shukor (1987), the indigenous village chickens are reared for both meat and eggs and due to its small body size with variable body conformations and physical characteristics, they mainly reared on the semi-intensive or free range systems.

2.2 Parasitism in village chicken

Parasitism is a common factor threatening the production of village chickens. There were many studies showing that chickens that are reared under free range conditions can be exposed heavily to parasite infection (Opara *et al.,* 2014; Amin-Babjee and Lee, 1994; Rahman, 2009; Poulsen *et al., 2000*). According to Poulsen *et al.,* (2000), parasitism is common in rural chickens due to free range systems and scavenging habits (Soulsby, 1982; Pandey *et al*., 1992; Permin *et al*., 1997). However, chicken that are reared in confinement has significantly reduced level of parasitism (Rod *et.*

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al, 2008). According to Nnadi & George, (2010), parasitism caused reduced growth and egg production, emaciation, and anemia as well as high mortality compared to viral infection of poultry such as Newcastle disease virus. Common ectoparasites and endoparasites in poultry are lice, mites, fleas, ticks, helminths, and coccidia. However, Opara *et al.,* (2014) stated that haemoparasites infections are the most common type of parasitism in chickens.

2.3 Avian blood parasite

There have been reported that various species of haemoparasites infect wild and domestic birds. A research that was done by Gimba et al., (2014) showed that the common haemoparasites reported in domestic poultry and village chickens in Malaysia include *Plasmodium* sp., *Leucocytozoon* sp., *Haemoproteus* sp., *Trypanosoma* sp., and microfilaria. Haemoparasite infection is often observed in free-range poultry at the age groups of chicks and growers (Permin *et al.,* 2013). The haemoparasite infection differs from one species to another for this haemoparasites, the level of parasitemia, the host species and the physiological state of the host and it can affect the health of the host and fitness of a particular host (Clark *et al.,* 2009). Some of avian blood parasite observed have economic importance such as plasmodium that caused avian malaria and leucocytozoonosis is pathogenic in younger bird where it can cause mortality (Opara *et al.*, 2014).

2.4 Leucocytozoonosis in chicken

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Leucocytozoon is an avian genera haemoparasites of phylum Apicomplexa. *Leucocytozoon spp.* such as *Leucocytozoon caulleryi* and *Leucocytozoon sabrazesi* can infect a large number of avian hosts, including domestic chicken. Research done in Selangor revealed that there was a low average infection rates of *L. sabrazesi* and *L. caulleryi* with relatively of 0.7% and 0.5% in chicken, respectively (Gimba *et al.,* 2014). Black flies (*Simulium* sp.) is the vectors of this parasite and responsible for the transmission of *Leucocytozoon* in the birds. The organism can be found in immature erythrocytes and leucocytes. The mature gametocytes are either round or oval in shape where it pushes the nucleus of infected cells out and cause distortion of the host cell in elongated with long tapering extremities (Permin*et al.,* 1998). *Leucocytozoon* infection can range from subclinical to clinical and might eventually cause death depending on strain of parasite, host, and level of parasitaemia (Bennett *et al.,* 1994). The clinical signs that can be seen in affected birds are anorexia, anemia, ataxia, and tachypnea. It is also stated that the infection of *Leucocytozoon* in chickens can affects the reproductive tract where it is can cause inflammation of the oviduct and causeedemaleading to drop in egg production. This will cause significant loss to the farm (Lee *et al.,* 2016).

2.5 Avian Plasmodium

Avian plasmodium is highly pathogenic and can cause 90% mortality in poultry (Soulsby,1982). The *plasmodium* genus is specific in different host in all avian orders with the exception of the Struthioniformes (ostriches), the Coliiformes (mousebirds), and the Trogoniformes (trogons and quetzals) (Atkinson, 2008). In domestic chickens,

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the common *Plasmodium* sp. Are *Pl. gallinaceum, Pl. juxtanucleare* and *Pl. durae*. The prevalence of *plasmodium* in domestic poultry was moderate which is 2.7% (Gimba *et al.,* 2014). Another survey done in Malaysia by Amin-Babjee and Lee, (1994) reported an infection rate of 8.3% for *P. juxtanucleare* among the fighting breed of domestic chickens. The avian plasmodium is transmitted by mosquitoes, which is *Culex* and *Aedes*. The *P. gallinaceum* gametocyte and schizonts can be found in erythrocytes where it can be round, oval or irregular in shape (Permin *et al.,* 1998). Each of this schizont can produce around 8-36 merozoites. Compare to *P. juxtanucleare*, the schizonts are round, ovoid or irregular and much smaller. In tropozoites stage, the schizont is small, round to oval in large vacuole and form signet ring appearance. The presence of this parasite in the erythrocyte causes displacement of the nucleus of erythrocyte. The affected animals will show signs of paresis, paralysis, anaemia and greenish diarrhoea (Omar *et al*., 1962; Omar, 1968). Some of the wild birds are resistance towards this infection. The domestic chicken can be infected when the area are endemic (Permin *et. al*., 1998).

2.5 *Haemoproteus* in poultry.

Haemoproteus spp is the common haemoparasite in birds distributed all over the world such as pigeons, doves and raptors. It is also can infect a numerous number of species including domestic poultry.It is first detected in Malaysia according to a survey done by Gimba *et al.,* (2014) where there is 0.8% of average infection of *Haemoproteus*. This parasite is transmitted by biting flies and biting midges.

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The gametocytes of *Haemoproteus* is pigmented and found in mature erythrocytes. It partially encircles the erythrocyte nucleus to form "halter-shape" (Gupta *et al*., 2011). It is also occupy half of cytoplasm of erythrocyte and cause minimal displacement of host cell nucleus. *Haemoproteus* infection in poultry is non-pathogenic but the affected birds will have anaemia, anorexia, and depression (Islam *et al.,* 2013). Usually, the clinical sign of anaemia in chicken is due to erythrocytic parasitism when the host has immunosuppression (Cardona *et al.,* 2002). *Haemoproteus* spp is the common haemoparasite in birds and has wide distribution all over the world and can be found in species such as pigeons, doves, raptors and domestic poultry. It is first detected in Malaysia according to a survey done by Gimba *et al.,* (2014) where there is 0.8% of average infection of *Haemoproteus*. This parasite is transmitted by biting flies and biting midges.

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3.0 MATERIAL AND METHOD

3.1 Sampling area

The location of blood sampling was at Aqil Aqilah farm located in Kampung Melayu Bukit Nyamuk, Simpang Renggam, Kluang, Johor. The farm has started its operation since 2013. The farm can housed approximately 5000 village chickens in 5 different houses at one particular cycle. The farm is practising semi-intensive system where the chickens were released and allowed to roamed and scavenged around the farm during daylight. The chickens will be sold when there is demand for it and when it reaches approximately 2 months of age and weighing approximately around 1 - 1.5kg. The farm did not practice the usage of antibiotics apart from vaccination program for Newcastle disease virus (NDV) and infectious bursa disease virus (IBDV) and multivitamins supplementation.

3.2 Sampling method

12 semi-commercial and 12 scavenging village chickens were randomly picked from Aqil Aqilah Farm and nearby area within the village. The chickens were restrained adequately to allow visualization of the vein for withdrawal of blood. The feathers overlying the site was dampened with an alcohol solution or plucked to aid in visualizing the wing vein. 2ml of blood from wing vein of the chicken was withdrawn and kept in the ethylene-diamine-tetra acetic acid (EDTA) tubes. The tubes were kept in ice box to be transported and stored under 4°C in the refrigerator. This study was

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approved by the Institutional Animal Care and Use Committee (IACUC), with reference number: UPM/IACUC/FYP.2017/FPV.046.

3.3 Blood parasite examination

Thin blood smear was performed for all 24 blood samples. A drop of blood was placed onto the slide and spread evenly with cover slip that was placed and hold at an angle about 45 degrees and pushed forward firmly. The slides were allowed to dry for 10 minutes. The slides were then fixed with absolute methanol for 2 minutes in the coupling jar. The slides were then stained with Giemsa stain for 35 minutes. The slides were then washed with tap water and placed in an upright position to air dry. After that, the slides were examined under x100 oil immersion objective with a light microscope. This experiment was repeated in triplicates.

3.4 Demonstration of Antigen

3.4.1 RNA extraction

The RNA was extracted from blood samples by using TRIzol® method. 2 ml of blood samples were placed into a 15ml centrifuge tube according to groups and was labelled accordingly. 5 ml of RBC lysis buffer was added into the centrifuge tube and was allowed to stand at room temperature for 10 minutes. Then, the tube was centrifuged at room temperature at 1400 rpm for 10 minutes. The supernatant was carefully decanted and gently the pellet was resuspended in 1 ml of RBC lysis buffer. The tubes were then centrifuged at room temperature at 3000 rpm for 2 minutes. The supernatant was aspirated and the pellet was resuspended with 1 ml of sterile PBS and centrifuged

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again at room temperature at 3000 rpm for 2 minutes and was then transferred into a 1.5 ml of centrifuge tube. 2 ml of TRIzol® reagent were pipetted into the centrifuge tube and the cells were resuspended. 2ml of chloroform was added and gently vortexed and the suspension were then incubated at room temperature for 15 minutes. The tube was centrifuged at 12000 rpm for 15 minutes at 4°C. Three layers were formed and the upper layer was pipetted out and transferred into a new 1.5 ml centrifuge tube. 0.8 ml of isoprophyl alcohol was pipetted in and incubated at room temperature for 15 minutes. The tube was centrifuged at 12000 rpm for 15 minutes at 4°C. The supernatant were pipetted out, leaving the RNA pellet. The RNA pellet was then washed with 98% alcohol by pipetting it in 1 ml into the tube, gently vortex and centrifuged at 7500 rpm for 4 minutes at 4°C for two times. Finally, the RNA pellet was dissolved with 15 µl of nuclease-free water.

3.4.2 RNA concentration and purification

The extracted DNA was measured by using spectrophotometry (TECAN M200 PRC) for the concentration and purification and interpreted using i-control 1.10 software. The RNA samples were then stored at -80ºC before proceed with conventional reverse-transcriptase (RT-PCR).

3.4.3 Primer selection

In this study, the primers were designed according to several studies for detection of haemoparasites in chicken (Razmyar *et. al.*, 2016; Hellgren *et. al*., 2004; Bert *et al*., 2005). The primers were designed to target the cytochrome b gene of the haemoparasite mitochondrial genome thus enabling the detection of haemoparasite in

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avian blood sample. There are two primers that being used in this study which coded LEUCO and HAEM. The housekeeping gene used to test the blood sample is 12Sr DNA which target on mitochondrial DNA (See Appendix A).

3.4.4 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

An access RT-PCR System Mix (Promega, USA) test kit was used and the manufacturer protocol was followed to run the RNA samples. The assemble reactions was done in the biosafety cabinet where the reagents were combined in a thin-walled reaction tube on ice. The reagents of master mix were prepared as shown in Appendix B. The master mix was kept on ice during handling. The reaction was performed by adding 2µl of each RNA samples in tube 1 to tube 9 to the final volume of 50µl for each tube. 48µl of nuclease-free water was added into the tube as non-template control. The tubes were then placed in PCR thermocycler (Senso Quest lab cycler, Germany) as set in Appendix C.

3.4.5 Agarose gel preparation

2% of agarose gel was prepared by diluting 1g of agarose powder into 50ml of TAE buffer and medium and was heated in microwave (Electrolux, USA) for 3 minutes to dissolve the powder. The gel was then cooled down and 5µl gel stain was added into the gel solution. The stained agarose solution was carefully mixed to ensure no bubble is formed. The gel then poured into the gel casting apparatus and the comb was put into the gel casting apparatus. The gel then left to solidify for 20-30 minutes.

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3.4.6 Agarose gel electrophoresis

The agarose gel was transferred into an electrophoresis tank (Bio-Rad, USA). The TAE buffer was filled into the tank to immerse the gel. 1µl of 100bp DNA ladder was diluted from stock (Invitrogen, USA) into 9µl of deionized water. 2µl ladder solution was mixed with 2µl of Blue Orange 6× Loading Dye (Promega. USA) and pipetted into well number 8. 3µl of PCR products were pipetted out from each tube and mixed with 1µl Blue Orange 6× Loading Dye (Promega. USA), then pipetted into well number 1-7 and 9-14. Agarose gel electrophoresis was ran at voltage 100v for 45 minutes using an electrophoresis tank (Bio-Rad, USA). The migration of the sample was monitored. Then, the gel was placed in a Gel Doc Imaging System (Bio-Rad, USA) under UV exposure. The image was captured and analysed with Image Lab™ software.

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4.0 RESULTS

4.1 Blood smears examination

The microscopic examination of the blood smears of 24 blood samples was done and revealed there is absence of *Leucocytozoon, Haemoproteus* and *Plasmodium* in all samples tested (Table 1).

Table 1: Number of blood parasites in blood samples.

Group of chicken	Sample number	Blood parasites that present
Semi-commercial	1	0
	2	0
	3	0
	4	0
	5	0
	6	0
	7	0
	8	0
	9	0
	10	0
	11	0
	12	0
Scavenging	1	0
	2	0
	3	0
	4	0
	5	0

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	6	0
	7	0
	8	0
	9	0
	10	0
	11	0
	12	0

4.2 Demonstration of antigen using RT-PCR

All blood samples tested shown negative result upon tested with primer designed.

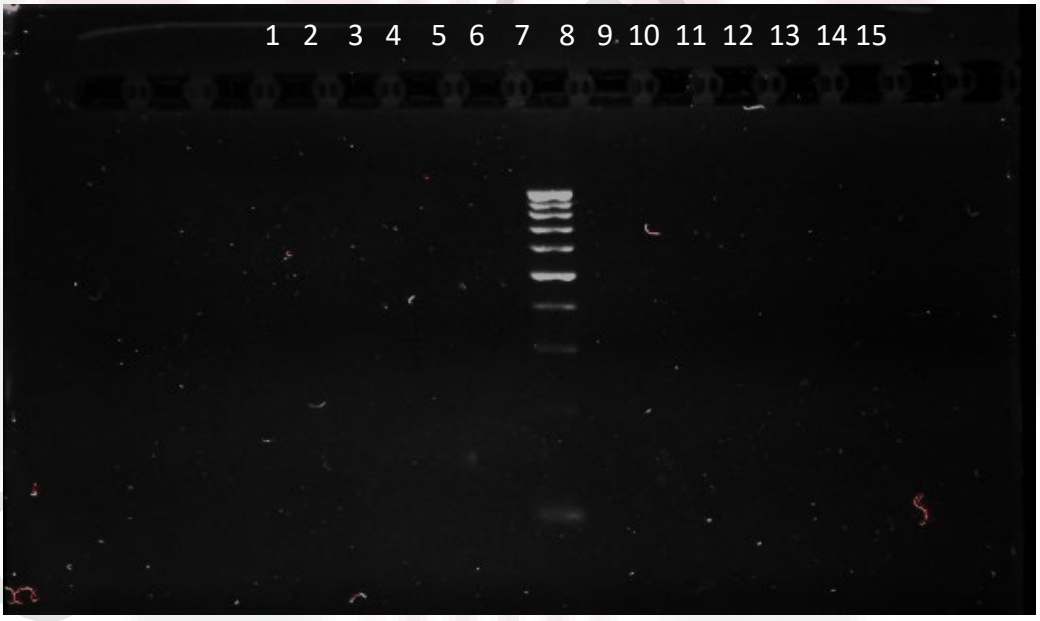

Figure 1: RT-PCR of blood samples, 1-6) Blood samples for HAEM, 7&15). Non template control 8)100bp ladder, 9-14) Blood samples for LEUCO

5.0 DISCUSSION

In this study, the results from blood smear examination and molecular analysis of Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) revealed that there were no blood parasites found in all blood samples of 12 semi-commercial and 12 scavenging village chickens. The finding of this study differs from the number of previously reported studies of avian haemoparasites in village and domestic chickens in Malaysia (Hong *et al*., 2010; Gimba *et al.,* 2014). In these studies, the most common genera of avian haemoparasites were of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* species.

However, in the present study, there was an absent of any haemoparasite species from all samples examined. The difference in findings could be influenced by several factors that affecting the haemoparasite infection in the chickens. According to Opara *et al.,* (2014), modern confinement system has reduced parasite infection but haemoparasite infection is common in free-range birds and backyard flocks. This is because the biosecurity in free-range birds and backyard flocks is inadequate to minimize or prevent the haemoparasite infection in poultry (Rod *et al*., 2008).

Apart from that, the vector or arthropod that responsible for haemoparasite infection could greatly influence the presence of haemoparasite infection in the farm. The availability and breeding of the vector can be affected by poor hygiene practice and rapid urbanization and drastic land use (Gimba *et al*., 2014). The breeding of the vectors is likely cause by the climate of the farm area. Study done by Chege (2014) stated that during the wet season more chicken (79.17%) were infected with

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haemoparasites as compared during the dry season (62.50%). Immunity of the host is important as it can cause to haemoparasite infection in the chicken. As mentioned by Lobato *et al*., (2011), during breeding season, there will be demand of high energy and causing rise in haemoparasitic infection due to compromised immune system.

As in this study, the difference in findings could be as a result of management of the farm that had been practiced by the farmer. In semi-commercial village chicken farm, the owner practice a combination of good hygiene practice, flies control, sound biosecurity and giving supplement to the chickens and this could contribute towards the protection of the animals in this farm from possible diseases including haemoparasitic infection. Insecticide was sprayed around the farm every month to minimize the vector and effective microbes was used and sprayed on the floor where it can remove foul smell and minimize the incidence of flies and mosquitoes swarm. The chicken in the farm has also been supplemented with garlic as traditional remedy. It is said that the usage and application of garlic at concentrations of 1% or 2% in diet can treat parasitic on chicken (Nyugen *et al.,*2016). Besides that, no history of haemoparasite infection and mortality caused by any other diseases was recorded in this farm.

An absent of blood parasite in scavenging village chicken can be due to variation between environmental climatic conditions and presence of vectors (Permin *et al.,* 2002). A study done by Papern *et al*., (2005) found that in cold environment such as in Cameron Highlands is not favourable for the breeding of midget flies or biting midges (Culicoides spp.), which the vector of *Haemoproteus* sp. comparing to other

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areas. This can be applied in this study as the availability of vector of parasites is differs from location to location. In addition, village chickens are known to be more adapted to harsh environment with poor husbandry care (Ahlers et. al., 2009) where it can be resistant toward parasitic infection.

There are several problems with microscopic procedure to detect haemoparasite in blood smear. Microscopic procedure is less sensitive compare to polymerase chain reaction (PCR) as it can detect and determine haemoparasite infection (Richard *et al*., 2002) when the number of gametocyte that presence in the peripheral blood is low (Aysul, 2013). As for *Leucocytozoon* sp., it only can be detectable for short period of time in peripheral blood (Valkiunas, 1997) and thus, it is hard to detect any infection of this haemoparasite by using microscopic examination. Thus, demonstration of antigen using RT-PCR in this study was done.

In this study, RT-PCR results revealed negative finding for all sample tested. It may be attributable to many factors, including low amount of template RNA in the test sample, inadequate removal of PCR inhibitors, ineffective release of RNA content from the host cells and poor RNA recovery after extraction and purification steps (Gonçalves-de-Albuquerque *et al*., 2014). The sample collected with low parasite burden may also contain minimal amounts of a parasite's DNA. Therefore, it may affect the amount of DNA degraded or losses during pre-PCR stages (Gonçalves-de-Albuquerque *et al*., 2014). Besides, there is no positive control that being used in this study which it is usually used to check whether the primer set or primer–probe set works and that the reaction has been set up correctly or not ("PCR Protocols

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&Applications - QIAGEN", 2017). Apart from that, the cycle condition for each primer was not optimized before RT-PCR due to time constraint and thus resulted in negative expression of the antigen.

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6.0 CONCLUSION

In this study, it can be concluded that there were no haemoparasites found in the blood smear examination for semi-commercial and scavenging village chicken indicate absence of haemoparasites infection in the chicken at the farm. For molecular detection (RT-PCR), there no haemoparasite DNA detected in all blood samples tested from semi-commercial and scavenging village chicken. Based on the results, this could be results from good management and biosecurity measures by the farmer that being practiced in the farm.

7.0 RECOMMENDATION

There are no significant results can be seen in this study which is absence of haemoparasites in both groups of chickens in the blood sample taken. Therefore, further recommendation is to test every chicken in the farm and increase the sample size to determine more significant and accurate results. To determine the prevalence of haemoparasite in the study area, more samples can be taken at the others farm surrounding the study area. Apart from that, optimization of cycle condition of primers that used in this study should be performed before RT-PCR being conducted. Other than that, the farmer should be advised to continue consistency control of the intermediate host to break the transmission of the parasite and prevent the breeding of the vectors.

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APPENDIX

Appendix A: Primer set for detection of haemoparasites in blood samples.

Primer	**Sequences**	**References**
LEUCO Forward	5'-TCTTACTGGTGTATTATTAGCAAC-3'	Razmyar *et al.*, (2016)
LEUCO Reverse	5'-AGCATAGAATGTGCAAATAAACC-3'	
Haem Forward	5'-ATGGTG CTT TCGATA TAT GCA TG-3'	Hellgren *et al.*, (2004)
Haem Reverse	5'- GCA TTA TCT GGA TGT GAT AAT GGT-3'	
12SrDNA Forward	5'- CGA TTA GAT ACC CCA CTA TGC -3'	Bert *et al.*, (2005)
12SrDNA Reverse	5'- AGG GTG ACG GGC GGT ATG TAC G -3'	

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Appendix B: Master Mix preparation

Reagent	**Volume (µl)**
Nuclease-Free water	31
AMV/ *Tfl* 5 × Reaction Buffer	10
dNTP Mix (10mM)	1
25mM $MgSO_4$	2
AMV Reverse Trancriptase (5µ/µl)	1
***Tfl* DNA Polymerase (5µ/µl)**	1
Forward primer	1
Reverse primer	1
Vortex gently before adding RNA samples	
RNA samples	2

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Appendix C: Optimized cycling condition of RT-PCR assay for detection of haemoparasites (*Haemoproteus, Plasmodium*, and *Leucocytozoon)*.

Steps	**Time**		**Temperature**		**Cycle**	
	LEUCO	HAEM	LEUCO	HAEM	LEUCO	HAEM
Reverse transcription	45 min	45 min	45ºC	45ºC	1	1
Initial heat activation	3 min	30 sec	94ºC	94ºC		
Denaturation	30 sec	30 sec	94ºC	94ºC	35	20
Annealing	30 sec	45 sec	52ºC	50ºC		
Extension	1 min	10 min	72ºC	72ºC		
Final extension	1 min		72ºC		1	1
Soak			4ºC	4ºC	∞	∞

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