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TOXOPLASMA GONDII INFECTION IN LOCAL AYAM KAMPUNG IN AREAS OF SELANGOR AND MELAKA

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TOXOPLASMA GONDII INFECTION IN LOCAL *AYAM KAMPUNG* IN AREAS OF SELANGOR AND MELAKA

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A project paper submitted to the Faculty of Veterinary Medicine, Universiti Putra Malaysia in partial fulfillment of the requirement for the DEGREE OF DOCTOR OF VETERINARY MEDICINE

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CERTIFICATION

It is hereby that we have read this project paper entitled "*Toxoplasma gondii* infection in local *Ayam Kampung* in areas of Selangor and Melaka", by Mohammad Sabri Bin Abdul Rahman and in our opinion it is satisfactory in terms of scope, quality, and presentation as partially fulfillment of requirement for the course VPD4999 – Final Year Project.

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DEDICATION

I would like to dedicate this thesis to my mother, Sa'adiah Samad and my father, Abdul Rahman Autan. Without their kindness, generosity, and encouragement I would have been ploughing land in a remote village in Malaysia.

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CONTENTS

LIST OF ABBREVIATIONS

ELISA	Enzyme-linked immunosorbent assay
IFAT	Indirect fluorescence antibody test
IHAT	Indirect haemagglutination test
MAT	Modified agglutination test

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ABSTRAK

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

Jangkitan *Toxoplasma gondii* dalam Ayam Kampung di kawasan Selangor dan Melaka

Oleh

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2015

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Toksoplasmosis adalah zoonosis disebabkan oleh protozoa *Toxoplasma gondii* yang menjejaskan manusia dan haiwan. Ayam Kampung (*Gallus domesticus*) adalah salah satu haiwan yang dikatakan penting bagi epidemiologi toksoplasmosis. Kajian ini dibuat untuk menentukan kehadiran dan organ lazim dalam jangkitan *T. gondii* serta perubahan tisu yang berkaitan dengan menggunakan kaedah serologi dan histopatologi. Sebanyak 50 sampel serum ayam kampung dari Selangor (20 ekor ayam dari empat ladang) dan Melaka (30 ekor ayam dari enam ladang) dikumpul dan dianalisis dengan

menggunakan kit serologi. Secara keseluruhan sampel positif antigen *T. gondii* adalah 20% (Selangor-30%; Melaka-13%) berdasarkan ujian ELISA dan antibodi seropositif terhadap anti-*T.gondii* juga dikesan pada semua sampel positif untuk ELISA berdasarkan ujian penghemaglutinatan tidak langsung (IHAT). Dalam pemeriksaan histopatologi, sista tisu dan perubahan tisu seperti inflamasi dan degenerasi (nekrosis) yang dapat diperhatikan pada kadar 18% (9 ekor ayam) pada otak, 22% (11 ekor ayam) pada hati dan 10% (5 ekor ayam) pada limpa. Keputusan menunjukkan bahawa *T. gondii* berada di dalam hati lebih kerap daripada tisu lain. Kajian ini merupakan laporan pertama jangkitan *T. gondii* dalam ayam kampung di Malaysia.

Kata kunci: *Toxoplasma gondii*, Zoonosis, Ayam Kampung, Serologi, Histopatologi.

ABSTRACT

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

Toxoplasma gondii infection in local *Ayam Kampung* in areas of Selangor and Melaka

By

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2015

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Toxoplasmosis is a worldwide zoonosis caused by the protozoa *Toxoplasma gondii* which affects human and animals. Local *Ayam Kampung* or free-range chickens have recently been reported to be important in the epidemiology of toxoplasmosis. This study determines the presence and common sites of *T. gondii* infection and related tissue changes using serological and histopathological methods, respectively. A total of 50 serum samples from local *Ayam Kampung* (*Gallus domesticus*) from Selangor (20 chickens from four farms) and Melaka (30 chickens from six farms) were collected and analyzed by using serological kits. *T. gondii* antigen was detected in 20% (Selangor-

30%; Melaka-13%) samples using ELISA test and anti-*T. gondii* antibody was detected in all positive samples from ELISA using the indirect haemagglutination test (IHAT). From the histopathological examination, cysts and tissue changes such as inflammation and degeneration (necrosis) were observed in 18% (9 chickens) of brain samples, 22% (11 chickens) of liver samples and 10% (5 chickens) of spleen samples. The results indicate that *T. gondii* tend to localize in the liver more often than in other tissues. This is the first report of *T. gondii* infection in local *Ayam Kampung* in Malaysia.

Keywords: *Toxoplasma gondii*, Zoonosis, *Ayam Kampung*, Serological, Histopathological.

1.0 Introduction

Toxoplasmosis is a common infection in animals and humans caused by *Toxoplasma gondii* (Susana, 2012). *T. gondii* is an obligate intracellular protozoan that infects humans and a wide range of mammals and birds (Smith and Reduck, 2000). From Dubey (2010), *T. gondii* is a coccidian parasite with cats as the definitive host, and warm-blooded animals as intermediate hosts. There is only one species under the genus of *Toxoplasma* which is *T. gondii*. The life cycle of *T. gondii* includes asexual multiplication in the intermediate host and sexual reproduction in the definitive host. Many species of warm-blooded animals can act as intermediate hosts and, seemingly, many animal species may be carrying tissue cysts of this parasite. Cats and wild felids are the only definitive hosts that may pass oocysts through their feces which will sporulate in the environment before becoming infective (Dubey, 2010). All hosts, including humans, can be infected by three different life cycle stages of the parasite: tachyzoites, bradyzoites contained in tissue cysts and sporozoites contained in sporulated oocysts (Dubey, 2007).

Ayam Kampung or free-range chickens are considered as one of the best indicators for soil contamination with *T. gondii* oocysts because of their feeding habits and their resistance from developing clinical symptoms. Chicken meat is consumed widely all over the world, and consumption of uncooked or not properly cooked chicken meat is a risk for *T. gondii* infection in humans and other animals (Dubey, 2010). The recent trend of consumers demanding meat from organically grown free-range chickens will increase

the prevalence of *T. gondii* in humans if meat was not properly cooked (Dubey and Jones, 2008). MARDI (2012) reported that *Ayam Kampung* constitute 5% of local chicken market but there is increasing demand for *Ayam Kampung* because their meats are said to be more delicious, lean, less fat and organic.

1.1 Rationale of the study

Toxoplasmosis is a serious zoonosis caused by *T. gondii* had recently been associated with mental disorder (Lovetta *et al*., 2013). Free-range chicken meats are popular and increasing in popularity worldwide, furthermore, free-range chickens have been found to harbour *T. gondii* infection in other countries as reported by Dubey (2010). In Malaysia, no information is available on *T. gondii* infection in local *Ayam Kampung*, therefore the risks of infection is unknown.

1.2 Hypothesis and objectives of the study

T. gondii can be detected using serological or histopathological methods. The prevalence of toxoplasmosis among local *Ayam Kampung* is unknown, therefore this study was undertaken to:

1. Determine the presence of *T. gondii* infection among local *Ayam Kampung* in areas of Selangor and Melaka.
2. Determine the most common sites of *T. gondii* infection and related tissue changes.

2.0 Literature Review

2.1 Stages of *T. gondii*

T. gondii is the causative agent of toxoplasmosis, one of the most important zoonotic parasites. There are three stages of *T. gondii* which consist of oocysts, tachyzoites and bradyzoites. Oocysts are hardy and resistant therefore suitable for environmental transmission of the parasite. Oocysts are shed in cat feces; only produced by felid definitive hosts. Tachyzoites are rapidly dividing tissue stages found in all vertebrate hosts. Bradyzoites are slowly dividing, encysted tissue stages in all warm-blooded vertebrate hosts (Capcvet.org, 2015).

2.2 Host Associations, Pathogenesis and Sites of Infection

T. gondii is a two-host coccidial organism, although cats can also be infected by ingestion of oocysts. Sexual stages develop only in the intestine of cats (wild and domestic) while asexual stages or extraintestinal (tachyzoites and bradyzoites) develop in all mammalian and avian species. Tachyzoites divide rapidly within various cells, leading to cell death, tissue necrosis and granulomatous inflammation. The organism survives for extended periods in host tissues as slowly dividing bradyzoites within tissue cysts. Bradyzoites may be reactivated and transform to rapidly dividing tachzoites upon host immunosuppression. Organs and tissues commonly affected include lymph nodes, liver, lung, brain or spinal cord and eye (Capcvet.org, 2015).

2.3 Prepatent period of *T. gondii*

Cats and other felids are the only species that develop patent infections with *T. gondii*, shedding oocysts 3-10 days following ingestion of bradyzoites in raw meat and 19-48 days following ingestion of oocysts. Oocysts shed by cat sporulate and become infective in 1 to 5 days and survive for months to years in the environment (Capcvet.org, 2015).

2.4 Toxoplasmosis of humans

Toxoplasmosis can be contracted via three principal routes which are foodborne, animal-to-human (zoonotic) or mother-to-child (congenital) (CDC, 2015). Weiss and Dubey (2009) stated that the most common form of infection in humans is latent infection which is asymptomatic. Symptomatic infections are usually associated with lymphadenopathy and reticular hyperplasia. In congenital infections, first trimester of pregnant women may get severe infection leading to abortion. Newborn babies developed congenital diseases such as hepatitis, pneumonia, hydrocephalus, retinochoroiditis and cerebral calcifications if the infection persist and if the fetus survive after first trimester (Hill and Dubey, 2002; Bhopale 2003). In Malaysia, ocular toxoplasmosis has been recorded where 31 clinically suspected cases were diagnosed as congenital ocular toxoplasmosis (Lim and Tan, 1983). From 1990 to present the percentage of toxoplasma infection in pregnant women has significantly increased (Khairul Anuar *et al.*, 1991; Ravichandran *et al.*, 1998; Nissapatorn *et al.*, 2003). A study by Nissapatorn *et al.*, (2002) stated that 4.8% of toxoplasma seropositive

immunocompromised patients developed toxoplasma encephalitis. Moreover, a recent study by Lovetta *et al*., (2013) at Hospital Kajang stated that *T. gondii* infection is significantly associated with mental disorder such as schizophrenia.

2.5 Toxoplasmosis of animals

The factors that may contribute to animal infection by *T. gondii* oocysts are management and hygienic conditions as well as environmental condition such as humidity and temperature that favour of oocysts survival (Butty, 2009). In birds, the modes of transmission are carnivorism and fecal contamination. Tachyzoites and bradyzoites may be spread by carnivorous ingestion and sporulated oocysts are spread by cat feces (Butty, 2009). Dubey (2010) stated that chickens rarely develop clinical toxoplasmosis. In Malaysia, few studies have been conducted on toxoplasmosis in animals. A study by Dorny *et al*., (1993) on toxoplasmosis in goats revealed that 35.2% seropositive *T. gondii* by using modified agglutination test (MAT). In 2008, Chandrawathani *et al*., studied the seroprevalence of *T. gondii* antibodies in cats, local cattle, yellow cattle, dogs and goats in Peninsular Malaysia and found that 14.5%, 7.9%, 4.0%, 9.6% and 35.5% seropositive *T. gondii* respectively by using indirect fluorescence antibody test (IFAT). A recent study on free-range ducks by Puvanesuaran *et al*., (2013) managed to isolate and genotype *T. gondii*.

2.6 Diagnosis and treatment

Diagnosis is made using biological, serological, and histological methods, or by combinations of these. Clinical signs of toxoplasmosis are nonspecific and cannot be

depended upon for a definite diagnosis (Dubey, 2010). Sulfonamides and pyrimethamine are two drugs widely used for therapy of toxoplasmosis (Khan and Elsheikha, 2011). These two drugs act synergistically by blocking the metabolic pathway involving p-aminobenzoic acid and the folic-folinic acid cycle respectively (Sabin and Warren, 1942; Eyles and Coleman, 1953). These two drugs are usually well tolerated but sometimes thrombocytopenia or leucopenia may develop. The effects can be overcome by giving patients folinic acid and yeast without interfering with treatment, because the vertebrate host can utilize pre-synthesized folinic acid while *T. gondii* cannot (Dubey, 2010). Supportive care should be provided as needed.

2.7 Prevention and public health considerations.

The control measures for human to prevent *T. gondii* infection are: i) destroy tissue cysts by heat treatment of infected meat to 66°C or higher (Dubey *et al*., 1990), ii) hands as well as all the equipments used should be washed thoroughly with soap and water after handling meat, iii) exclude cats that have important role as a definitive host for this parasite, in farms to protect the farm animals (Butty, 2009), iv) pregnant women, should avoid contact with cats, soil and raw meat, v) pet cats should be fed only dry, canned, or cooked food and cat litter should be emptied everyday because oocysts require at least 24 hours to become infective (Dubey, 2010). Illness is likely to be more severe in fetuses and immunosuppressed individuals; hence, additional precautions for these higher risk individuals need to be applied.

3.0 Materials and Methods

3.1 Sampling

Cross-sectional study was conducted from 12th January until 16th January 2015. Fifty local *Ayam Kampung* (*Gallus domesticus*) were purchased from 10 different farms in areas of Melaka and Selangor. Thirty chickens were purchased from six different farms in areas of Melaka while another 20 chickens were purchased from four different farms in Selangor. Blood, brain, liver and spleen samples were taken and processed by using serological and histopathological method.

3.2 Serological method

Blood samples were collected during slaughter by using plain blood tube. Blood was allowed to clot overnight at 4°C then centrifuged for 15 minutes at 1000rpm to separate the serum from whole blood. Serum was collected and stored at -20°C for further analyses.

3.2.1 Enzyme-linked immunosorbent assay (ELISA)

Fifty serum samples were analyzed by ELISA kit. Chicken toxoplasma circulating antigen (TCA) ELISA kit CUSABIO® was used for detection of *T. gondii* antigen in vitro according to manufacturer instructions. This test kit gave qualitative results to achieve a yes or no answer indicating whether the toxoplasma antigen is present in a sample. ELISA is a sensitive and recommended method by researchers

(Casartelli-Alves *et al.*, 2014) and has been used in previous studies to determine the serological prevalence of toxoplasma in chickens (Dubey, 2010; Fitzgerald, 2011).

3.2.2 Indirect haemagglutination test (IHAT)

Twenty serum samples were analyzed by IHAT kit. ELI.H.A Toxo kit ELITech® was used for quantitative determination of anti-*T. gondii* serum antibodies by indirect haemagglutination. The kit only allows 20 serum samples to be tested. IHAT is a specific method (Casartelli-Alves *et al.*, 2014) and most commonly used for research as well as for serological diagnosis and prevalence of toxoplasma in chickens (Dubey, 2010).

3.3 Histopathological method

Brain, liver and spleen samples were removed from the slaughtered chickens and fixed in 10% neutral buffered formalin. Then samples were sectioned, processed and stained with Haematoxylin and Eosin (H&E) and Giemsa. The presence of tissue cysts and tachyzoites were examined under oil immersion (100x). H&E stain was used to examine overall condition on tissue sections such as inflammation and degeneration of tissues and cells. Giemsa stain was used to examine and focus more on parasite itself such as tissue cysts and tachyzoites (Fitzgerald, 2011). Tissue cysts are cyst or round shaped containing bradyzoites while tachyzoites are crescent or leaf shaped and as long as the red blood cell when examined histologically (Khan and Elsheikha, 2011). The finding of tachyzoites indicates active infection while finding of tissue cysts may

indicate latent infection (Dubey, 2010). Histological examination is the gold standard for toxoplasmosis and has high specificity (Casartelli-Alves *et al.,* 2014).

3.4 Data analysis

The seroprevalence of toxoplasmosis was calculated as the number of samples positive for the antibodies of *T. gondii* from all those tested. The results from ELISA and histopathology were compared using the 2x2 table to determine the sensitivity and specificity of ELISA as compared to histopathology (gold standard).

4.0 Results

4.1 ELISA

Ten out of 50 serum samples were positive for toxoplasma by ELISA. The seroprevalence of *T. gondii* in local *Ayam Kampung* was 20.00% (95% CI: 10.03% - 33.72%) (Fig 1). Table 1 shows the number of samples examined and positive samples by location. The seroprevalence in Selangor was 30.00% rate of infection (6/20) and Melaka was 13.33% rate of infection (4/30).

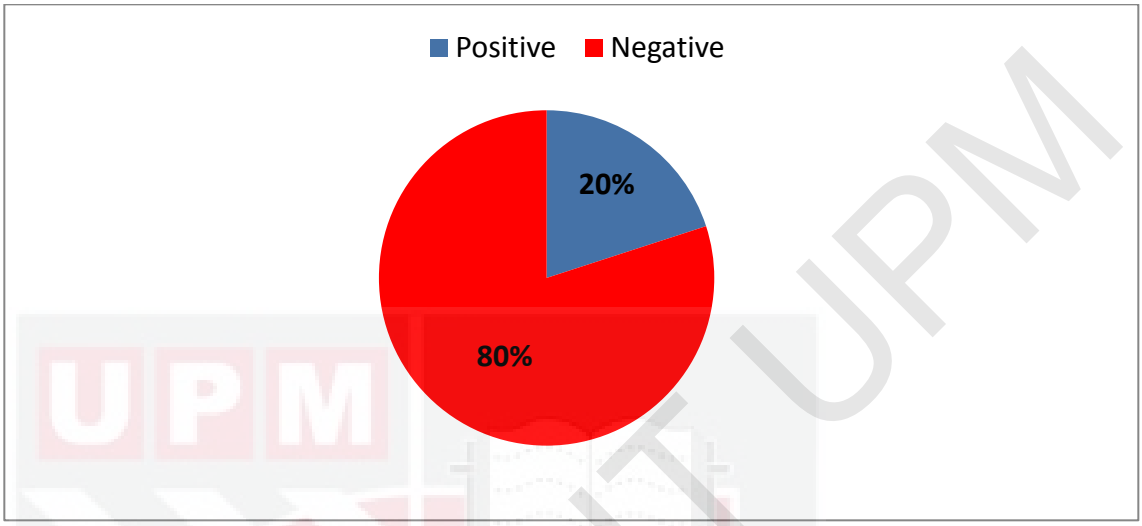

Figure 1: Seroprevalence of *T. gondii* in local *Ayam Kampung*.

Table 1: Number of positive samples and their percentages in different locations in areas of Selangor and Melaka

Location	No. of samples	No. of positive samples	% of infection
Selangor	20	6	30.00
Melaka	30	4	13.33

4.2 IHAT

Out of 20 examined samples (10 positive ELISA serum samples and another 10 serum samples randomly chosen), 10 were seropositive for toxoplasma antibody (Table 2). These samples were also seropositive using ELISA. The positive agglutination titers were 1:320 to 1:1280, which indicate presence of anti-toxoplasma antibodies in the serum samples.

Table 2: Indirect haemagglutination test (IHAT) antibodies to the *T. gondii* in local *Ayam Kampung*

Total samples	Positive	Antibody titration		
		1:320	1:640	1:1280
20	10	4	5	1

4.3 Histopathology

Histological examination from 50 chickens revealed the presence of tissue cysts of *T. gondii* in 11 (22%) liver samples (Figure 2), while tissue cysts observed in brain (Figure 3) and spleen (Figure 4) were 9 (18.00%) and 5 (10.00%) respectively as shown in Table 3. Tachyzoite (Figure 5) was observed in the one brain sample which indicates active infection. The most common tissue changes observed in infected chickens was inflammation (Figure 6 & 7) and degeneration (Figure 8).

Table 3: Histopatology results for tissue cysts of *T. gondii* in various organs

Organs	No. of samples	Tissue cysts (containing bradyzoites)	% infection
Liver	50	11	22.00
Brain	50	9	18.00
Spleen	50	5	10.00

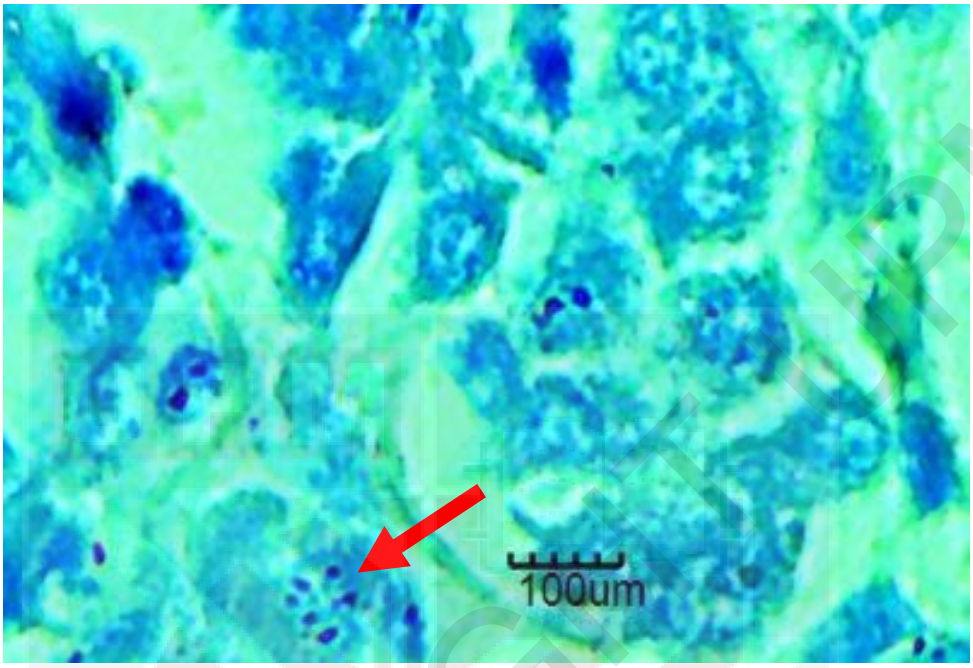

Figure 2: Tissue cyst of *T. gondii* in liver, stained by Giemsa. 100x(oil lens).

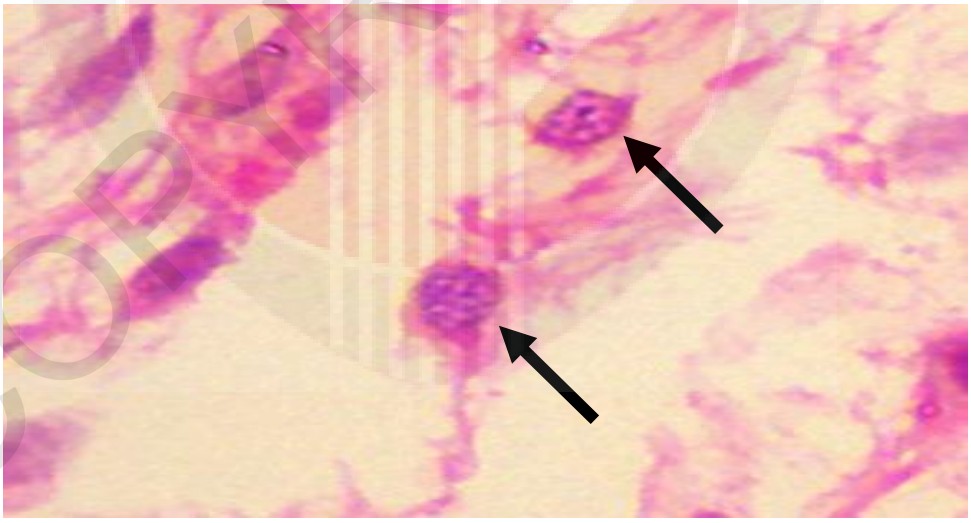

Figure 3: Tissue cysts of *T. gondii* in brain, stained by H&E. 100x(oil lens).

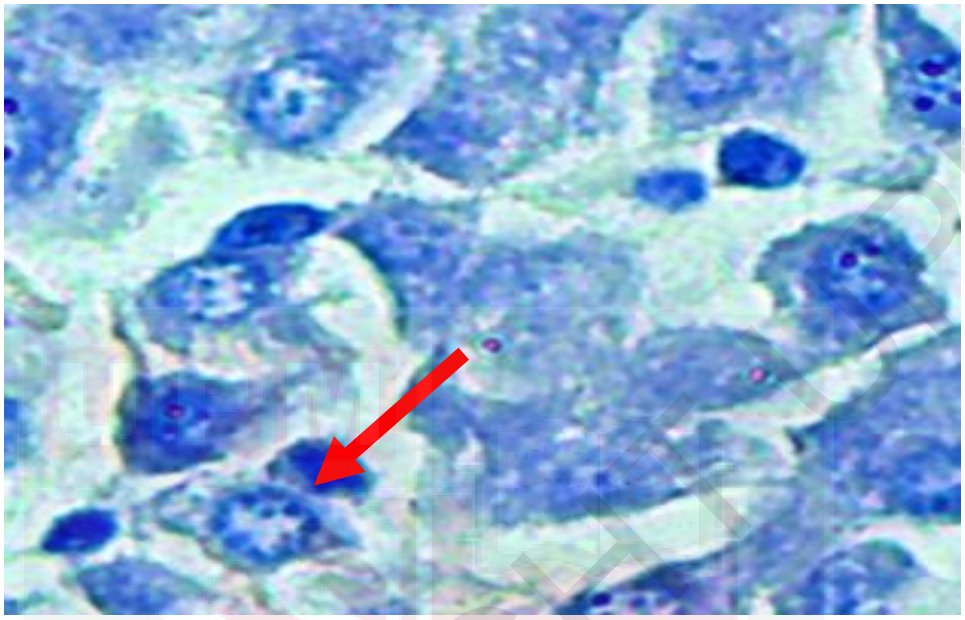

Figure 4: Tissue cyst of *T. gondii* in spleen, stained by Giemsa. 100x(oil lens).

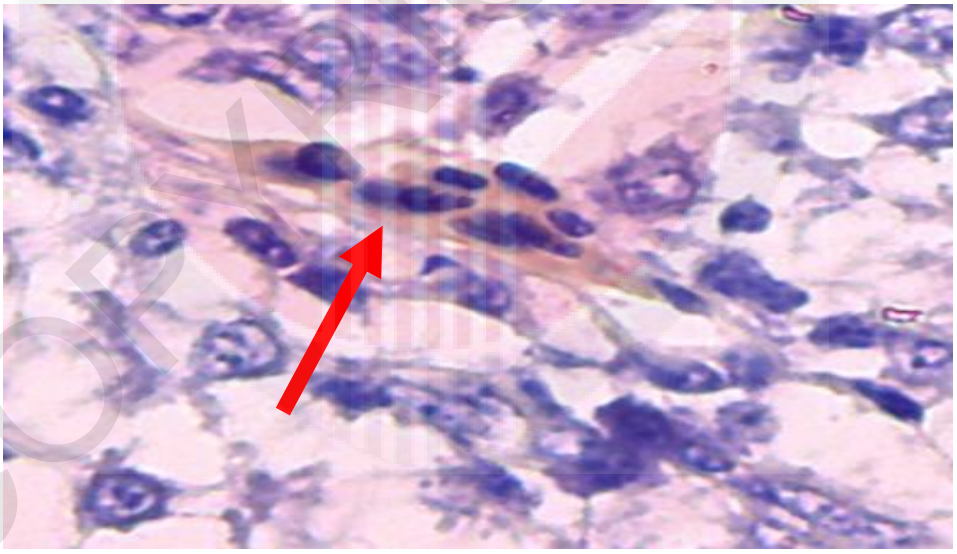

Figure 5: Tachyzoite of *T. gondii* in brain, stained by Giemsa. 100x(oil lens).

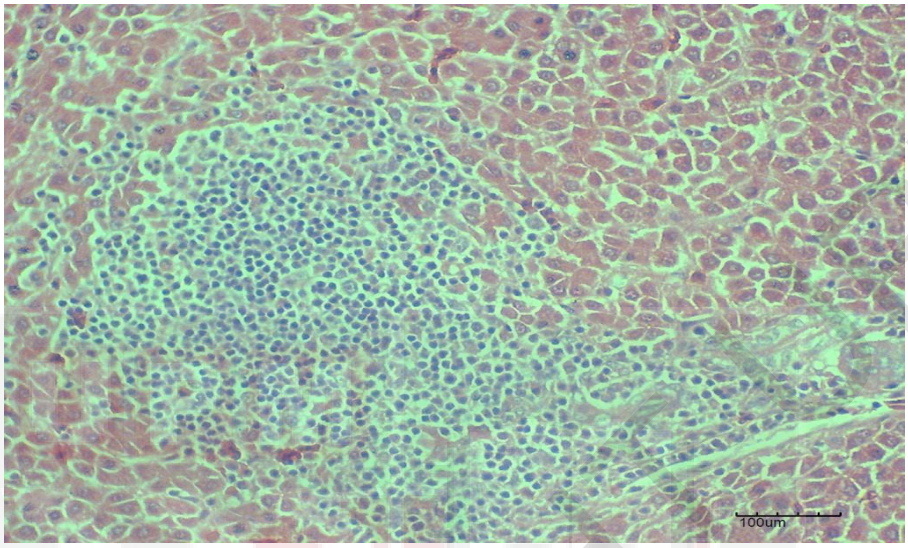

Figure 6: Inflammatory neutrophils in the liver, stained by H&E. 40x.

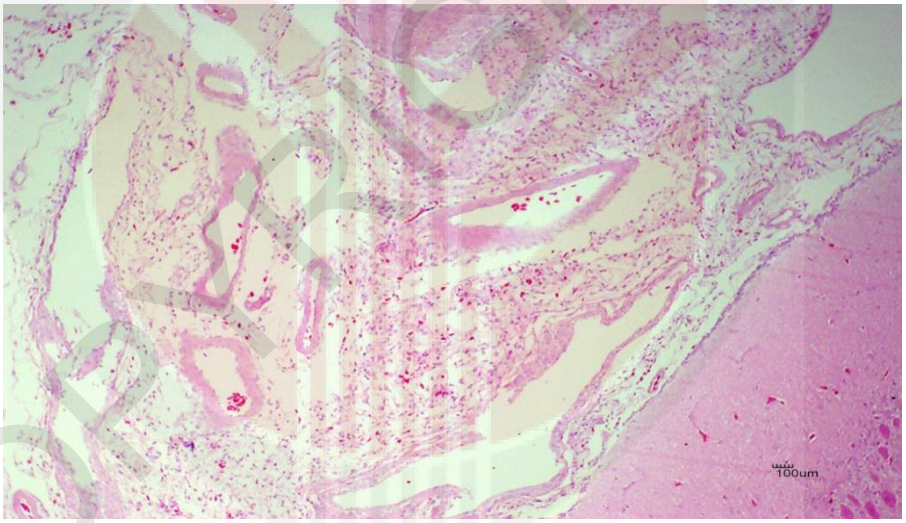

Figure 7: Severe meningitis, stained by H&E. 10x.

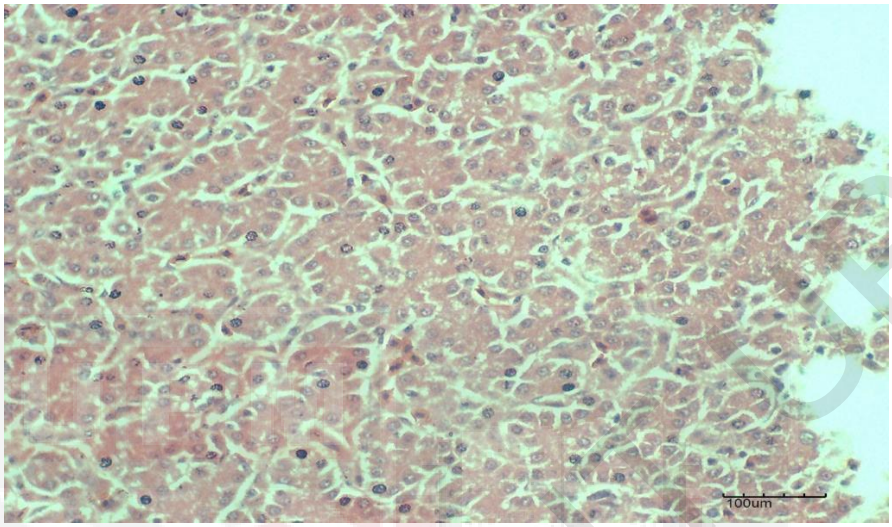

Figure 8: Multifocal necrosis (degeneration) in the liver, stained by H&E. 40x.

4.4 Comparison between ELISA and Histopathology

Table 4 shows comparison between ELISA and histopathology by using 2x2 tables. The results reveal the sensitivity of the test is 90.91% and the specificity of the test is 100%. Both sensitivity and specificity of the test were high; suggesting that ELISA is an ideal test for screening.

Table 4: Comparison between ELISA and histopathological findings for toxoplasmosis in chickens

ELISA	Histopathology		Total
	Positive	Negative	
Positive	10	0	10
Negative	1	39	40
Total	11	39	50

Sensitivity = (10/11) x 100% = 90.91%

Specificity = (39/39) x 100% = 100.00%

5.0 Discussion

Chickens have not been investigated for evidence of toxoplasma infection in Malaysia. The epidemiological role of chickens in toxoplasma infection is unknown. Previous studies in the United States have found that toxoplasma may be found in free-ranging chickens at prevalence of up 86.0% (Dubey, 2010). This study is a pilot study exploring the role of native *Ayam Kampung* in the epidemiology of *T. gondii*. The study found a high seroprevalence of toxoplasmosis (20.0%) in the local *Ayam Kampung* using ELISA kit. The finding is similar to that found in Indonesia (24.2%), Peru (26.0%) and Portugal (27.1%) and was higher than Kenya (13.3%) and Mexico (6.2%) by using MAT (Dubey, 2010). The differences in the prevalence may be associated with the geographical location and type of serological test (Karatepe *et al*., 2011).

The finding of high prevalence of toxoplasmosis in the local *Ayam Kampung* is expected given the prevalence of infection among local cats. Chandrawathani *et al*., (2008) found 14.5% of cats were infected with *T. gondii*. Chickens become infected mostly during feeding on the ground contaminated with oocysts (Zia-Ali *et al*., 2005). Oocysts may survive in soil and environment for months to years (Capcvet.org, 2015), therefore chickens may become infected from multiple exposure to contaminated soil. The high prevalence of toxoplasmosis found in this study signifies that the infection is common among cats which contaminate the environment.

Histopathological examination revealed that the highest rate of infection was in the liver (22.0%). Mohammed and Abdullah (2013) also reported that the highest rate of infection in the liver (38.46%), then brain (33.33%) and lowest in spleen (12.82%). The organotropism of tissue cysts varies in different intermediate host species. In many hosts, tissue cysts have a high affinity for neural and muscular tissues. They are located predominantly in the central nervous system, the eye as well as skeletal and cardiac muscles. However, to a lesser extent they may also be found in visceral organs, such as lungs, liver, and kidneys (Dubey, 2010). The most common tissue changes seen in infected chickens are; i) inflammation such as neutrophils infiltration, and ii) degeneration such as multifocal necrosis as stated by Fitzgerald (2011).

The comparison between ELISA and histopathology as gold standard revealed the sensitivity of ELISA test as 90.91% and specificity of 100%. This suggests that ELISA is an ideal test for screening due to its convenience and rapidity.

The liver is usually consumed undercooked or half-cooked by some population; therefore, tissue cysts of the parasite may survive, hence poses serious public health risk among humans especially those who immunocompromised or pregnant. *Ayam Kampung* has a potential role in transmitting toxoplasmosis to humans in Malaysia and can be sentinel for monitoring environmental contamination with toxoplasma oocysts. With the increasing popularity of free-range chickens, this risk needs to be acknowledged and addressed appropriately.

6.0 Conclusion and Recommendation

Toxoplasma gondii infection is detected in 20% among local *Ayam Kampung* in areas Selangor and Melaka. Tissue cysts appear to localize in the liver more often than in other tissues of naturally infected chickens. This study suggest the potential risk of local *Ayam Kampung* as a source of *T. gondii* infection in humans and other animals and need to be further studied. This study did not collect any information on the management of the farms which can elucidate factors that influences the occurrence of the organism.

7.0 References

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9.0 Appendices

Appendix 1 –Serological method

Chicken toxoplasma circulating antigen (TCA) ELISA Kit CUSABIO®

PRINCIPLE OF THE ASSAY

This assay employs the qualitative enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with antibody. Samples are pipetted into the wells with Horseradish Peroxidase (HRP) conjugated antibody. Following a wash to remove any unbound reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Chicken TCA bound in the initial step. The color development is stopped and the intensity of the color is measured.

ELI.H.A Toxo Kit ELITech®

PRINCIPLE

ELI.H.A Toxo is based on the indirect haemagglutination principle (IHAT). The sensitized red blood cells consist of sheep red blood cells covered with a toxoplasma antigen. The presence of serum antibodies against *Toxoplasma gondii* results in agglutination of the sensitized red blood cells resulting in a cloudy red/brown deposit coating the well. In the absence of specific antibodies, the red blood cells form a ring-like deposit at the bottom of the well.

Appendix 2- Histopathological method

Haematoxylin and Eosin (H&E) Staining Protocol

1. Start
2. Submerge slides in Xylene (5 mins)
3. Submerge slides in 100% Alcohol (5 mins)
4. Submerge slides in 70% Alcohol (5 mins)
5. Rinse
6. Submerge slides in Haematoxylin (5 mins)
7. Rinse (3-5 times)
8. Dip slides in 1% Acid Alcohol (3 secs)
9. Running tap water (5 mins)
10. Submerge slides in Eosin (1 min)
11. Spray slides with 95% Alcohol, clean and leave to dry
12. Mount with DPX
13. Ready for viewing
14. End

Giemsa's Staining Protocol for Tissue Sections

Method

1. Bring sections to distilled water
2. Stain with diluted Giemsa's stain made up fresh (see technical point 1)
3. Rinse in distilled water
4. Differentiate with 0.5% aqueous acetic acid (see technical point 2)
5. Dehydrate rapidly
6. Clear and mount

Technical Points

1. Step 2 - usually the staining is performed at room temperature overnight, however, increasing the stain temperature shortens staining time. Sections stained at 37°C for several hours, (staining time assessed by microscopical examination), produce better results than sections stained at 60°C for a shorter period. The higher the staining temperature, the greater the intensity of blue staining, but without the equivalently increased red staining - see technical point 2 below.

2. Differentiation with acetic acid will vary according to the staining time and temperature, but it is generally achieved within 30 secs. The differentiating agent removes only the blue dye component, thus increasing the apparent intensity of the red component.