MOLECULAR IDENTIFICATION OF *EIMERIA* SPP. OOCYSTS FROM BROILER CHICKENS IN KELANTAN

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CERTIFICATION

This is to certify that we have read this research paper entitled **'Molecular Identification of *Eimeria* Spp. Oocysts From Broiler Chickens in Kelantan'** by Lim Chien Yee, and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course DVT 5436 – Research Project.

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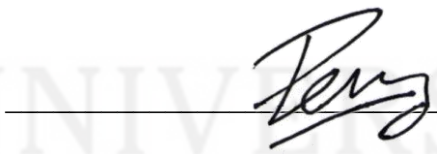

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I dedicate my work to God. A special feeling of gratitude for every blessing from God keeps me strong and optimistic throughout the entire Doctor of Veterinary Medicine program.

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement on for the course DVT 5436 – Research Project

Coccidiosis is a gastrointestinal disease with a major economic impact on the poultry industry. Poultry coccidiosis is caused by the protozoa of *Eimeria species*. There are seven known *Eimeria* spp. infecting chickens, and five of them were detected in this study. This study was conducted to determine the species of *Eimeria* spp. oocysts in broiler chicken in Kelantan. A total of 30 fecal samples were collected from three broiler farms of different management in Kelantan. The modified McMaster method was used to quantify the number of oocysts per gram (OPG) of *Eimeria* spp. oocysts in the samples. DNA of the oocysts were extracted for *Eimeria* species identification using Polymerase Chain Reaction (PCR). Results showed that all samples were positive for *Eimeria* spp. microscopically. Samples collected from farms practicing the intensive system revealed a higher OPG count than samples from extensive system farms. The five *Eimeria* spp. detected are *Eimeria tenella, Eimeria maxima, Eimeria acervulina, Eimeria mitis* and *Eimeria praecox.* Identity of the five detected *Eimeria* species were confirmed by comparing the nucleotide sequences revealed using BTSeq with genomic database from National Center for Biotechnology Information (NCBI) with percentage similarity of more than 99%. In short, this study revealed the prominent *Eimeria* spp. infecting local broiler chicken. DNA sequences generated from *Eimeria* spp. in this study may facilitate development of local vaccine to provide better immunity compared with imported vaccines.

Keywords: *Broiler, Coccidiosis, Eimeria species, Kelantan*

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 5436 – Projek Penyelidikan.

Koksidiosis merupakan penyakit saluran usus yang mempunyai impak yang besar terhadap industri ternakan ayam. Protozoa spesis *Eimeria* merupakan penyebab koksidiosis pada industri ternakan ayam. Terdapat tujuh spesis *Eimeria* yang menjangkiti ayam, dan lima daripada spesis tersebut telah dekesan dalam kajian ini. Kajian ini telah dilaksana untuk mengenalpasti *Eimeria* spesis ookista dalam ayan pedaging di Kelantan. Sejumlah 30 sampel najis telah dikumpulkan daripada tiga ladang ternakan ayam pedaging dengan pengurusan yang berbeza di Kelantan. Kaedah McMaster yang diubahsuaikan telah digunakan untuk megukur kuantiti ookista setiap gram (OPG) untuk spesis *Emeria* yang terdapat dalam sampel-sampel tersebut. DNA daripada ookista telah diekstrak untuk pengenalan spesis *Eimeria* dengan menggunakan kaedah tindak balas rantai (PCR). Hasil kajian ini menunjukkan kesemua sampel adalah positive untuk *Eimeria* spp. secara mikroskopik. Sampel daripada ladang ayam yang mengamalkan sistem intensif mencatatkan kuantiti ookista setiap gram (OPG) yang lebih tinggi daripada ladang sistem ekstensif. Lima *Eimeria* spp. yang dikesan merupakan *Eimeria tenella*, *Eimeria maxima*, *Eimeria acervulina*, *Eimeria mitis* dan *Eimeria praecox.* Identiti kelima-lima spesis *Eimeria* yang dikesan telah disahkan dengan membandingkan urutan nukleotida yang dikesan menggunakan BTSeq dengan pengkalan data genomik Pusat Nasional Informasi Bioteknologi (NCBI) dengan peratusan persamaan melebihi 99%. Secara ringkasnya, kajian ini

telah mengesan spesis *Eimeria* yang menjangkiti ayam pedaging tempatan. Urutan nukleotida yang dikenalpasti dalam kajian ini bakal memudahkan penciptaan vaksin tempatan untuk memperlengkapkan imuniti yang lebih baik berbanding dengan vaksin yang diimport.

Kata kunci: *Ayam pedaging,Koksidiosis, Spesis Eimeria, Kelantan*

1.0 Introduction

Broiler farming is Malaysia's most significant livestock industry as broiler meat has become the staple meal for locals (Bahri et al., 2019). This is because broiler meat has a unique position among Malaysia's multi-ethnic population, where there is no religious restriction exists in comparison to other meat such as beef and pork, which are nonetheless unsuitable for eating by certain races or religious practioners of the population (Abdurofi et al., 2017) . In Malaysia, poultry meat has recorded a self-sufficiency level of more than 100% between the years 2013 to 2019 (Department Of Veterinary Service, 2021). Although poultry meat is self-sufficient in Malaysia, relevant issues such as disease control and prevention should not be overlooked.

Poultry coccidiosis is a major impediment to effective commercial and backyard poultry production in Malaysia (Wan Norulhuda et al., 2017). The economic relevance of the disease arises from its high morbidity and death rates in young and adult chickens, and poor feed conversion efficiency and egg production, particularly in subclinical cases (Dakpogana & Salifou, 2013). Poultry coccidiosis is caused by the protozoa of *Eimeria* species, of the phylum *Apicomplexa*, family *Eimeriidae* that infect various location in the gastrointestinal tract of the chicken (Aiello & Moses, 2016).

Currently, there are seven *Eimeria* spp. with different pathogenicity recognized to infect chicken globally (Lopez-Osorio et al., 2020). The seven species are *Eimeria acervulina, Eimeria brunetti, Eimeria maxima, Eimeria mitis, Eimeria necatrix, Eimeria praecox* as well as *Eimeria tenella.* Among the seven species, *Eimeria brunetti*, *Eimeria tenella*, *Eimeria maxima* and *Eimeria necatrix* are considered highly pathogenic, whereas *Eimeria acervuline* and *Eimeria mitis* are categorized as

moderate pathogenic while *Eimeria praecox* is reviewed as the least pathogenic species (Al-Natour et al., 2002).

These seven species of *Eimeria* infecting chicken are of different importance due to the variation of pathogenicity, prevalence, location, and extend of lesion caused in the chicken's gastrointestinal tract (Gussem, 2007). The species variation is meaningful for diagnosis as according to Swayne et al. (2020), identification of *Eimeria* can be made based on their biological characteristics including location and gross lesion in the intestine, the morphology of *Eimeria* oocysts, size of endogenous tissue stages, location of the protozoa in the tissues as well as length of prepatent period.

However, taxonomic difficulties are encountered in differentiating species of *Eimeria* of similar oocysts morphology or overlapping tissue specificity in the gastrointestinal tract of affected chicken (Swayne et al., 2020). Therefore, alternative species-specific diagnostic approach such as *Eimeria* species-specific polymerase chain reaction (PCR) with species specific primers targeting distinct genomic regions of each *Eimeria* was shown to be capable of discriminating different *Eimeria* spp. (Saroj et al., 2014).

In conclusion, *Eimeria* spp. can lead to coccidiosis in broilers and is assumed to be constantly threatening the industry. A conventional method of identifying *Eimeria* spp. base on biological characteristics has its limitations, thus, PCR has become a practical approach for detection and discrimination. The study aims to detect and differentiate the species of *Eimeria* oocysts found in broiler chickens in Kelantan, Malaysia.

2.0 Research problem

Coccidiosis in poultry can lead to severe outbreaks due to the direct life cycle and high reproductive potential of *Eimeria* spp. Coccidiosis remains the most common subclinical disease in broilers due to the ubiquitous presence of highly durable *Eimeria* spp. oocysts in the farm environment. However, there is insufficient information on the *Eimeria* spp. oocysts from broiler chicken in Kelantan.

3.0 Research questions

3.1 What is/are the species of *Eimeria* spp. oocysts in broiler chicken from Kelantan?

4.0 Research hypothesis

4.1 There is presence of oocysts of high pathogenic *Eimeria* spp. which are *Eimeria brunetti*, *Eimeria tenella*, *Eimeria maxima* and *Eimeria necatrix* in broiler chicken from Kelantan.

5.0 Objectives

5.1 To determine the species of *Eimeria* spp. oocysts from broiler chicken in Kelantan.

6.0 Literature review

6.1 Transmission Of *Eimeria* Spp.

Transmission of *Eimeria* spp. occurs by ingestion of environmental material such as food and water contaminated by sporulated oocysts (Blake & Tomley, 2014). Infected chicken shed the unsporulated oocysts into the environment in feces. Under favourable condition of humidity, temperature, and oxygen, the oocysts would become infectious through sporulation (Quiroz-Castaneda & Dantan-Gonzalez, 2015). There is no natural intermediate host for *Eimeria* spp. but vectors such as insects, contaminated equipment, wild bird and dust can spread the oocysts mechanically (Swayne et al., 2020). Tough multilayered walls of Eimeria oocysts render them relatively resistant to most disinfectants and may survive up to weeks in the environment (Attree et al., 2021).

6.2 Life Cycle Of *Eimeria* Spp.

Life cycle of *Eimeria* spp. consists of two stages: the exogenous phases of sporogony and the endogenous phase of schizogony and gametogon (Lopez-Osorio et al., 2020). Following the breakage of the oocysts wall in the ventriculus, motile sporozoites are released and enter the mucosal cells of the intestinal tract (Swayne et al., 2020). Inside the parasitophorus vacuole, sporozoites develop into trophozoite which becomes a meront during the first generation of merogony. Merozoite I formed undergo second meront stage, forming merozoite II. Merozoite II undergoes sexual gamogony where female macrogamete and male microgametes are formed. Zygotes are produced following the fertilization of female macrogametes by male microgamates. A resistant oocyst wall is formed by converging of eosinophilic granules and the Eimeria oocysts

are released into the intestinal lumen, and excreted into the environment with the feces (Lopez-Osorio et al., 2020).

6.3 Host Specificity Of *Eimeria* Spp.

Eimeria spp. infecting poultry showed strict host specificity. The chicken is the only host of the 7 species of *Eimeria*, which are *E. acervulina*, *E. brunetti*, *E maxima*, *E mitis*, *E necatrix*, *E. praecox* and *E. tenella* (Swayne et al., 2020). According to Lopez-Osorio et al. (2020), the host specificity of *Eimeria* spp. in chicken may be related to the parasite antigens such as 22, 31 and 37 kDa that binds to molecules present on host cell surface including membrane glycoconjugates as well as epitopes of host cells and sporozoites.

6.4 Site Specificity Of *Eimeria* Spp.

Eimeria spp. exhibit high degrees of site specificity, which explains why a a number of *Eimeria* spp. can infect a chicken. Concurrently, each with a particular site in the intestine (Lopez-Osorio et al., 2020). Research from Li et al. (2020) revealed that it is due to the interaction between microneme proteins (MICs) and their receptor on the surface of the target cell in which the *E. tenella* MICs (EtMICs) could only bind to the caecum and did not bind to any other intestinal tissues.

Schizogony of *E. necatrix* occurs in the anterior and middle lobe of the intestine and subsequent sporozoites are introduced to the caeca, where gametogony takes place (Horton-Smitfa & Long, 1965). *Eimeria tenella* has its specificity towards caecum of chicken, however, in cecatomized chicken, the parasites are found in the proximal large intestine and gametocytogony occurs in the small intestine just above the caecal diverticulum (Leathem, 1969). On the other hand, *E. brunetti* has its site-specificity towards the distal part of the small intestine (Hein, 1974). The jejunum is the

predilection site of *E. maxima*, however, in heavy infestation, the lesion can extend throughout the small intestine (Dubey & Jenkins, 2018).

Research from Novilla et al. (1987) revealed that the preferred site of *E. mitis* development is the ileum. However, limited development of the protozoa also occurs in the jejunum, cecal pouches, cloaca and bursa of Fabricius. Duodenum is the primary site of infection of *E. praecox.* The jejunum and immature asexual stages of the protozoa were also observed at the beginning of the yolk sac diverticulum (Salisch, 1990). *E. acervulina* showed its site-specificity in the epithelial cells of the duodenal villi. However, the lesion may extend through some distance in the small intestine in heavy infections (Warren & Ball, 1967).

6.5 Problems In The Identification Of Eimeria Species

Identifying *Eimeria* oocysts can be challenging as the oocysts size is not necessary constant, especially *E. tenella* and *E. brunetti* (Long & Joyner, 1984). Morphologically similar oocysts with overlapping tissue specificity further complicates the species identification (Swayne et al., 2020). Subclinically affected chickens may not produce sufficient lesions on the intestine, thus complicating species identification. Serum antibodies demonstrated in certain infections have not been proved sufficient specific to be value of identification purpose (Long & Joyner, 1984).

7.0 Materials and methods

7.1 Sample collection

Between 19th to 29th March 2022, a total of 30 fecal samples were collected from three broiler farms located in different districts namely, Bachok, Pengkalan Chepa and Tawang. The farms which were located in Bachok, Pengkalan Chepa and Tawang were managed intensively with deep litter system, extensive with free rangesystem and intensively with raised floor system, respectively. Sampling at the farm was conducted through convenient selection. Pooled fecal droppings were collected into a 50 ml polypropylene tube filled with 10ml of 2% potassium dichromate solution. The pooled fecal droppings were collected starting from one corner of the unit and followed a "W" pathway across the unit, until the tube was filled to the 20 ml mark, as suggested by Kumar et al. (2014). The polypropylene tube was capped and shaken vigorously to mix the content thoroughly. The polypropylene tube was then labelled according to the location and housing of the chicken. The samples were then transported to Parasitology Laboratory, Faculty of Veterinary MeCdicine, University Malaysia Kelantan in an icebox and kept at 4°C until further process. Potassium dichromate solution can maintain the refrigerated oocysts by preventing putrefaction by microoragnism without killing the oocysts (Williams et al., 2010).

7.2 Modified McMaster Method

Fecal sample of one gram was weighed and placed into a 100ml beaker. A total of five ml of saturated NaCl solution was then added to the beaker and the feces were broken up using a spatula. Another 10 ml of saturated NaCl was added and the fecal

suspension was stirred using a spatula. The fecal suspension was then filtered though a tea sieve into another 100ml beaker. Filtrate in the second 100ml beaker was stirred and the aliquot was withdrawn using a pipette. The aliquot was then filled into a chamber of McMaster slide. Filtrate in the beaker was stirred again, aliquot was pipetted and another chamber of McMaster slide was filled. If there is presence of bubbles, the aliquot was removed and the chamber was refilled (Zajac et al., 2021). The McMaster slide was then allwed to stand for 3 minutes before examine under a compound microscope (CX21, Olympus, Tokyo Japan).

Upon examination under the compound microscope, the grid lines on the McMaster slide were brought into focus under 4X power objective by adjusting the coarse adjustment knob. The power objective was then turned to 10X objective and the grid lines were focused by adjusting the fine adjustment knob. *Eimeria* spp. Oocysts within two grid lines were identified and calculated. A mechanical tally counter was used to ease the counting process. Oocysts in both McMaster were calculated and the number of oocysts per gram (OPG) was calculated and recorded. Figure 7.1 shows an example of *Eimeria* spp. oocysts (Zajac et al., 2021) while the OPG calculation formula shown below was adapted from Parasitology Laboratory (2022).

$$\text{Oocysts per gram (OPG)} = \frac{\text{Total no. of oocysts counted}}{\text{Weight of feces}} \times \frac{\text{Volume of Nacl solution}}{2\ (0.15)}$$

7.3 Sample Processing

The fecal sample were then proceed for isolation of the *Eimeria* spp oocysts. Firstly, 10 ml of the fecal sample was poured into a clean 50 ml polypropylene conical tube and 1.6 g of sodium chloride was added into each tube. A total of 15 ml saturated NaCl solution was added up to the 25 ml mark. The tube was then capped tightly and shaken

vigorously until the fecal material was completely broken up and mixed well with the salt solution. A total of 25 ml saturated NaCl solution was added to fill up the tube up to 50 ml mark. The tube was capped tightly and shaked to mix thoroughly.

Next, 2 ml of distilled water was gently overlaid using a disposable pipette. The samples were left to stand for ten minutes before being centrifuged (Model 4000, KUBOTA, Japan) at 700 x g for 8 minutes. The layer from the interface between the saturated salt solution and the distilled water of about 5 ml was transferred into a new 50 ml polypropylene conical tube using a Pasteur pipette. The centrifugation and transferring process were repeated for three times until no material was visible at the interface.

The new polypropylene conical tube was filled with distilled water to 50 ml mark and centrifuged at 700 x g for 10 minutes. Using a Pasteur pipette, the supernatant was carefully removed without disturbing the pellet, leaving 4 ml of fluid. The samples were mixed and rinsed the side up to 3 cm from the 50 ml polypropylene tube base before being transferred into 2 ml microfuge tubes.

The microfuge tubes were then centrifuged at 6000 x g for 5 minutes and the supernatant was then discarded. The pelleted oocysts were suspended in 1ml of 2% potassium dichromate solution and allowed to sporulate at 27 ± 2°C for three days (Kumar et al., 2014). The oocysts were examined under a compound microscope for sporulation on day three. Figure 7.2 shows the sporulated oocysts observed. The isolated oocysts suspended in 2% potassium dichromate were then stored at 4°C until further process (Güven et al., 2013).

7.4 DNA Extraction

A total of 14 samples of highest OPG from every housing of each farm were selected to proceed with the DNA extraction step.

Firstly, the oocysts were centrifuged at 3,000 rpm for 5 minutes and the supernatant was removed. The oocysts were resuspended in 1 ml of Tris-EDTA buffer (TE buffer), centrifuged at 3,000 rpm for 5 minutes and the supernatant was discarded to wash the potassium dichromate solution. The washing was repeated twice until the orange-tinged of potassium dichromate solution was not visible in the microfuge tube.

The oocysts were resuspended in 1ml of TE buffer and transferred to a 15 ml polypropylene tube using a 1,000 µL single-channel micropipette (Microyn, USA). The polypropylene tubes were kept on ice and 0.5 ml of ceramic beads (Nucleospin bead tubes type A, 0.6-0.8 mm) were added. The oocysts were smashed by high-speed vortexing for 2 minutes and chilled on ice for at least 5 minutes. The procedure of vortexing and chilling were repeated for another four times to ensure oocysts in every polypropylene tube were smashed for 10 minutes. The oocysts were checked under a compound microscope for breakage of the oocysts wall (Figure 7.3).

The smashed oocysts were centrifuged gently at 90 x g using a tabletop centrifuge machine (Model KA-1000, KUBOTA, Japan) for 5 minutes and the supernatant was collected and transferred to a 15ml polypropylene conical tube.

The ceramic beads with smashed oocysts pellet were resuspended in 2 ml of TE buffer, vortexed for 2 minutes to flush and centrifuged at 90 x g for 5 minutes. The supernatant was collected and added to that collected previously and kept on ice. 0.33 vols, which is 1.32 ml of 10% SDS solution and 80 µL of Proteinase K (100 µg/ml), were added.

The suspension was then incubated at 37°C for 1 hour before being transferred into a 4°C chiller overnight.

The suspension was centrifuged at 750 x g for 10 minutes to pellet oocyst debris. The supernatant was then recovered and transferred into a new 15 ml polypropylene conical tube. To remove contamination protein, an equal volume which is 5 ml of phenol (equilibrated in Tris, pH8.0) was added, mixed on high-speed vortex and centrifuged at 750 x g for 10 minutes. Majority of the aqueous phase was recovered to a new 15ml polypropylene conical tube by using a 1,000 µL single-channel micropipette (Microyn, USA) with disposable tips without touching the transition phase.

Next, an equal volume of 4ml of chloroform was added to the recovered aqueous phase to remove any residual phenol. The suspension was then mixed on high-speed vortex and centrifuge at 750 x g for 10 minutes. Majority of the aqueous phase was recovered and transferred to a new 15 ml polypropylene tube as the previous step until no band was visible at the interface.

A total of 0.1 vol, calculated of 0.2 ml of 3M sodium acetate (pH5.3), 2 vols of 4 ml of 100% ethanol and 3 µL glycogen were added into the recovered aqueous phase, mixed on high-speed vortex and placed at -80°C for 1 hour. After 1 hour, the polypropylene tubes let thaw at room temperature before centrifuged at 1,500 x g for 15 minutes. The supernatant was then removed by using a micropipette.

A total of 1 vols, calculated of 2 ml of 70% ethanol was added and the polypropylene tube was inverted for five times to rock over the pellet and centrifuged at 1,500 x g for 15 minutes. As much as possible, ethanol was removed using a disposable plastic

Pasteur pipette. The suspension was re-centrifuged at 1,500 x g for 10 minutes. The final ethanol was removed with a 100 µL single-channel micropipette (Microyn, USA) with disposable tips. The pellet was then allowed to air dry for at least 30 minutes. 100 µL of denionized water was added to cover the pellet, and resuspension was allowed overnight at 4°C. The extracted DNA was then stored at -20°C until further process.

In short, the DNA extraction procedure was firstly done by rupturing the *Eimeria* spp. oocysts wall by grinding with ceramic beads with a vortex mixer. The DNA was then dissolved in TE buffer and the DNA suspended was recovered through standard phenol/chloroform extraction and subsequent precipitation with sodium acetate/ethanol method (Blake et al., 2003). Moreover, the protocol of bead grinding of oocysts combined with classic phenol/chloroform DNA extraction was regarded as the golden standard in the study conducted by Haug et al. (2007).

7.5 Polymerase Chain Reaction (PCR)

The samples were analyzed by monoplex PCR with the sequence characterized amplified region (SCAR) primers to detection of *E. acervulina, E. brunetti, E. tenella. E. mitis, E. praecox, E. maxima and E. nacatrix* (Fernandez et al., 2003). The primers were blasted at the NCBI gene bank (National Center for Biotechnology Information) to check for specificity. The primer used is shown in Table 7.1 below.

Table7.1: Primer sequence of Eimeria* spp. *specific markers

Species	Primer Designation	Primer Sequence (5' → 3')	Amplicon Size (bp)
E. acervulina	Ac-01-F	AGTCAGCCACACAATAATGGCAAACATG	811
	Ac-01-R	AGTCAGCCACAGCGAAAGACGTATGTG	
E. brunetti	Br-01-F	TGGTCGCAGAACCTACAGGGCTGT	626
	Br-01-R	TGGTCGCAGACGTATATTAGGGGTCTG	
E. tenella	Tn-01-F	CCGCCCAAACCAGGTGTCACG	539
	Tn-01-R	CCGCCCAAACATGCAAGATGGC	
E. mitis	Mt-01-F	AGTCAGCCACCAGTAGAGCCAATATTT	460
	Mt-01-R	AGTCAGCCACAAACAAATTCAAACTCTAC	
E. praecox	Pr-01-F	AGTCAGCCACCACCAAATAGAACCTTGG	354
	Pr-01-R	GCCTGCTTACTACAAACTTGCAAGCCCT	
E. maxima	Mx-01-F	GGGTAACGCCAACTGCCGGGTATG	272
	Mx-01-R	AGCAAACCGTAAAGGCCGAAGTCCTAGA	
E. necatrix	Nc-01-F	TTCATTTCGCTTAACAATATTTGGCCTCA	200
	Nc-01-R	AGTCAGCCACACAATAATGGCAAACATG	

**F = forward primer, R = reverse primer*

The PCR reaction was prepared in Table 7.2: Set up for a 50 µL PCR reaction volume as follow.

Table7.2: Set up for a 50µL PCR reaction volume.

Component	Volume	Final Concentration
Nuclease free water	16 µL	-
GoTaq Green Master Mix, 2X (Promega, USA)	25 µL	1X
Forward primer. 10µM	2 µL	0.4 µM
Reverse primer, 10µM	2 µL	0.4 µM
Sample	5 µL	-
Total volume	50 µL	

Next, the PCR reaction mixes were loaded into Nexus Gradient Master Cycler, (Eppendorf, Germany). The cycling instruction was then set for one cycle of initial denaturation at 98°C for 30 seconds, 35 cycles of 5 seconds of denaturation at 98°C, 5 seconds of annealing at 65°C and 15 seconds of extension at 75°C for 15 seconds, followed by final extension at 72°C for 1 minute and hold at 10°C. Re-amplification of the PCR product of positive samples was carried out in order to obtain a thick band for subsequent DNA purification for sequencing.

7.6 Gel Electrophoresis

1.5% agarose gel was prepared by measuring 0.6 g of agarose powder in the weighing boat. The agarose powder was then mixed with 60 ml of 1x TBE (Tris/Borate/EDTA) buffer in a Schott bottle. The mixture was microwaved for 3 minutes until the agarose powder was completely dissolved. The agarose solution was then removed from the microwave and let sit to cool down to about 50°C before 1 µL of Midori Green dye was added to the agarose solution. Next, the agarose solution was poured into the gel

tray with the well comb in place. The gel was then let sit at room temperature for 30 minutes until it had completely solidified.

Once solidified, the agarose gel was placed into the electrophoresis tank. The tank was filled with 1x TBE buffer until the gel was fully covered. 5 µL of 100 bp DNA ladder (Promega, USA) was carefully loaded into the first lane of the gel. Subsequently, 10 µL of the sample PCR products were carefully loaded into the additional wells of the gel.

The gel was then run at 100V for 40 minutes. Once the gel electrophoresis had been completed, the power supply was turned off and the gel was carefully removed from the tank. The DNA fragments were visualized using GelDoc^{TM} EZ Imager (BIORAD, USA). DNA fragment that appear as band on the gel at the expected size was considered as positive.

7.7 DNA Purification

DNA purification of the suspected positive samples of each *Eimeria* spp from each of the farm was performed based on the manufacturer protocol (Geneaid Gel Extraction Protocol). Firstly, gel dissociation was done by excising the agarose gel slice containing relevant DNA fragments and any extra agarose was removed to minimize the size of the gel slice. The gel slice was then transferred to a 1.5 ml microcentrfuge tube and 500 µL of DF buffer was added to the sample and vortexed to mix. Next, the sample was incubated at 60°C for 10 minutes to ensure the gel slice has been completely dissolved. The dissolved sample was then removed from the mini dry bath (Benchmark, China) and let cool to room temperature.

Subsequently, DNA binding was carried out by placing the DF Column in a 2 ml collection tube before the sample mixture was transferred to the DF Column. The DF

Column and Collection Tube was then centrifuged at 14,000 x g for 1 minute. The flow-through was discarded and the DF Column was placed back in the 2 ml Collection Tube.

Next, 600 µL of Wash Buffer was added into the DF Column and let stand for 1 minute. The DF Column together with Collection Tube was then centrifuged at 14,000 x g for 1 minute and the flow-thhough was discarded. The DF Column was placed bacl to the collection tube. The sample was washed again by adding 600 µL of wash buffer, let sit for 1 minute, centrifuged at 14,000 x g for 1 minute and flow through was discarded. The DF Column was placed back in the Collection Tube and centrifuged at 14,000 x g for 3 minutes to dry the column matrix.

Lastly, the DNA elution was dine by transferring the DF Column to a new 15 ml microcentrifuge tube where 50 µL of Elution Buffer was added into the center of the column matrix. The DF Column was let stand for 2 minutes to ensure the elution buffer is completely absorbed and centrifuged at 16,000 x g to elute the purified DNA.

The purified DNA collected in 1.5 ml microcentrifuge tube was labled, sealed and stored at -20°C until being sent for sequencing.

7.8 DNA Sequencing

The purified DNA product was sent to Tree Code Sdn Bhd, Klang, Malaysia for BTSeq (Barcode-Tagged Sequencing). The sequencing result was analysed by using BioEdit software version 7.2 and nucleotide blasting was done on NCBI gene bank (National Center for Biotechnology Information).

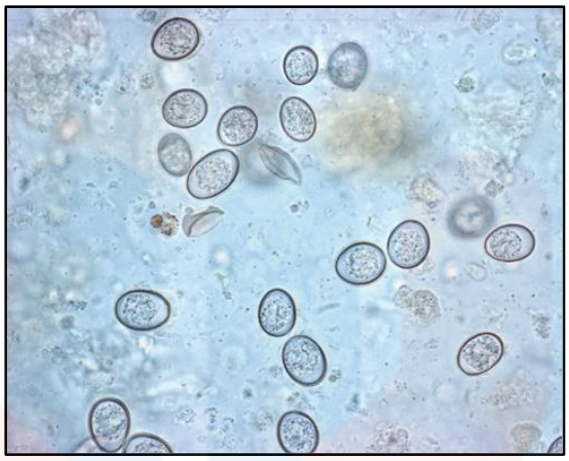

Figure 7.1: *Eimeria* spp. oocysts from a chicken (Zajac et al., 2021).

Figure 7.2: Sporulated *Eimeria* spp. oocysts under 40X magnification.

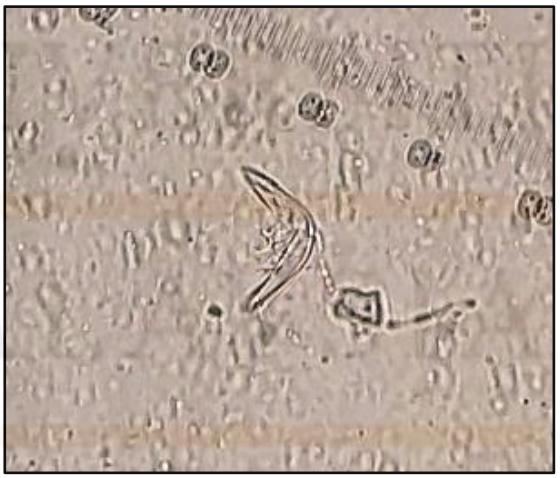

Figure 7.3: The ruptured *Eimeria* spp. oocysts wall under 40X magnification.

8.0 Results

8.1 Fecal Oocysts Count

Fecal oocysts count (FOC) performed by Modified McMaster method revealed all 30 samples collected were positive for *Eimeria* spp. oocysts. Table 8.1 shows the oocysts per gram (OPG) calculated for each sample collected, while Figure 8.1 shows the *Eimeria* spp. oocysts observed under the compound microscope.

The broiler farm managed with deep litter system recorded the highest mean OPG count among the three farms,184,988 OPG. Meanwhile, the farm with free range system reveals the least average oocysts per gram counted with mean OPG of 3,631.

Table8.1: Calculated oocysts per gram (OPG) of the fecal samples.

Districts	Management	Sample	Oocysts per gram(OPG)	Mean (OPG)
Bachok	Intensive, open house with deep litter system	1.1	3,250	184,988
		1.2	18,900	
		2.1	33,100	
		3.1	150,450	
		3.2	34,400	
		4.1	379,250	
		4.2	340,050	
		4.3	325,550	
		4.4	379,940	
Pengkalan Chepa	Extensive, free range system	FR1	1,050	3,631
		FR2	2,100	
		FR3	23,700	
		FR4	250	
		FR5	50	
		FR6	400	
		FR7	800	
		FR8	700	
Tawang	Intensive, open house with raised floor system	twg 1.1	4,200	16,965
		twg 1.2	1,700	
		twg 2.1	3,950	
		twg 2.2	3,950	
		twg 2.3	650	
		twg 2.4	850	
		twg 2.5	4,700	
		twg 3.1	71,250	
		twg 3.2	30,050	
		twg 3.3	24,250	
		twg 3.4	22,250	
		twg 3.5	32,600	
		twg 3.6	20,150	

8.2 Molecular Identification Of *Eimeria* spp. Oocysts

Upon viewing the product of gel electrophoresis shown in Figure 8.2, Figure 8.3, Figure 8.4, Figure 8.5, Figure 8.6, Figure 8.7 and Figure 8.8, the presence of bands at the expected size was taken as a positive result. Two *Eimeria* spp. were detected from the broiler farms in Bachok and Pengkalan Chepa, respectively, while Tawang

recorded the most *Eimeria* spp. detected among the three districts, where a total of 5 different *Eimeria* spp. were detected.

The PCR result also reveals multiple infections with two to three species in samples collected from the Tawang and Pengkalan Chepa districts. A sample from Pengkalan Chepa showed co-infection of two *Eimeria* spp. that are *E. acervulina* and *E. tenella*. On the other hand, all the three samples that showed positive results from Tawang revealed multiple infections of three *Eimeria* spp. with E. acervulina as the predominant infected species. The molecular identification of *Eimeria* spp. in each sample was recorded and tabulated (Table 8.2).

Table 8.2: Result of PCR for species identification of the Eimeria* spp. *oocysts.

District	Sample	*Eimeria species*						
		E. ac	***E. br***	***E. tn***	***E. mt***	***E. pr***	***E. mx***	***E. nc***
Bachok	1.2							
	2.1						+	
	3.1							
	4.1	+						
	4.4	+						
Pengkalan Chepa	FR3	+		+				
	FR6							
	FR7							
	FR8			+				
Tawang	twg 1.1							
	twg 1.2							
	twg 2.5	+		+			+	
	twg 3.1	+			+	+		
	twg 3.5	+		+	+			

****E.ac = E. acervulina, E. br = E. brunetti, E. tn = E. tenella, E. mt = E. mitis, E. pr = E. praecox, E. mx = E. maxima, E. nc = E. necatrix, + = positive***

E. acervuline is the most predominant species infecting broiler chickens in Kelantan, as it is found on every farm in each district. *Eimeria tenella* and *E. maxima* are the second dominant species found in two out of the three farms. Meanwhile, *E. mitis* and *E. praecox* are the least common species where they are only detected from the broiler farm located in Tawang. *Eimeria brunetti* and *E. necatrix* were not detected in any broiler farm.

Table 8.3: Percentage of each Eimeria* spp. *detected

District (management)	**No. of examined samples**	**No. of positive samples (%)**	***No. of positive samples (%)***						
			E. ac	***E. br***	***E. tn***	***E. mt***	***E. pr***	***E. mx***	***E. nc***
Bachok (Intensive, open house with deep litter system)	5	3 (60)	2 (40)	-	-	-	-	1 (20)	-
Pengkalan Chepa (Extensive, free range system)	4	2 (50)	1 (25)	-	2 (50)	-	-	-	-
Tawang (Intensive, open house with raised floor system)	5	3 (60)	3 (60)	-	2 (40)	2 (40)	1 (20)	1 (20)	-

E.ac = E. acervulina, E. br = E. brunetti, E. tn = E. tenella, E. mt = E. mitis, E. pr = E. praecox, E. mx = E. maxima, E. nc = E. necatrix, - = not detected

Although the broiler farm managed with deep litter system and raised floor system showed the same number and percentage of positive samples, the farm managed with raised floor system revealed simultaneous infection of more *Eimeria* spp. compared to the one managed with deep litter system.

Upon blasting the nucleotide sequences of the selected samples, all samples revealed percentage identical of more than 99% (Table 8.4). The sequencing result had affirmed that the DNA isolated and detected were *E. acervulina*, *E mitis*, *E. maxima*, *E. praecox* and *E. tenella*, as expected with the respective primers used. Sample MX2.1 and PR3.1 revealed percentage identical of 100% with the genomic sequence of *Eimeria maxima* and *Eimeria praecox* respectively. This provides a strong stance of the methods used in this study are able to detect the *Eimeria* spp.

Table 8.4: Result of nucleotide sequence blasting

District (management)	Sample	Description	Scientific Name	Query Cover	Percentage Identical	Accession
Bachok (Intensive, open house with deep litter system)	AC 4.1	Eimeria acervulina RAPD-SCAR marker Ac-A03-811 genomic sequence	*Eimeria acervulina*	100%	99.88%	AY571520.1
	MX 2.1	Eimeria maxima RAPD-SCAR marker Mx-A09-1008 genomic sequence	*Eimeria maxima*	93%	100%	AY571588.1
Pengkalan Chepa (Extensive, free range system)	AC 3	Eimeria acervulina RAPD-SCAR marker Ac-A03-811 genomic sequence	*Eimeria acervulina*	100%	99.88%	AY571520.1
	TN 8	Eimeria tenella RAPD-SCAR marker Tn-K04-539 genomic sequence	*Eimeria tenella*	96%	99.81%	AY571634.1
Tawang (Intensive, open house with raised floor system)	AC 3.5	Eimeria acervulina RAPD-SCAR marker Ac-A03-811 genomic sequence	*Eimeria acervulina*	100%	99.87%	AY571520.1
	MT 3.1	Eimeria mitis RAPD-SCAR marker Mt-A03-460 genomic sequence	*Eimeria mitis*	93%	99.12%	AY571503.1
	MT 3.5	Eimeria mitis RAPD-SCAR marker Mt-A03-460 genomic sequence	*Eimeria mitis*	95%	99.11%	AY571503.1
	MX 2.5	Eimeria maxima RAPD-SCAR marker Mx-A09-1008 genomic sequence	*Eimeria maxima*	92%	99.62%	AY571588.1
	PR 3.1	Eimeria praecox RAPD-SCAR marker Pr-A03-718 genomic sequence	*Eimeria praecox*	93%	100%	AY571602.1
	TN 2.5	Eimeria tenella RAPD-SCAR marker Tn-K04-539 genomic sequence	*Eimeria tenella*	99%	99.81%	AY571634.1

AC = E. acervulina, TN = E. tenella, MT = E. mitis, PR = E. praecox, MX = E. maxima

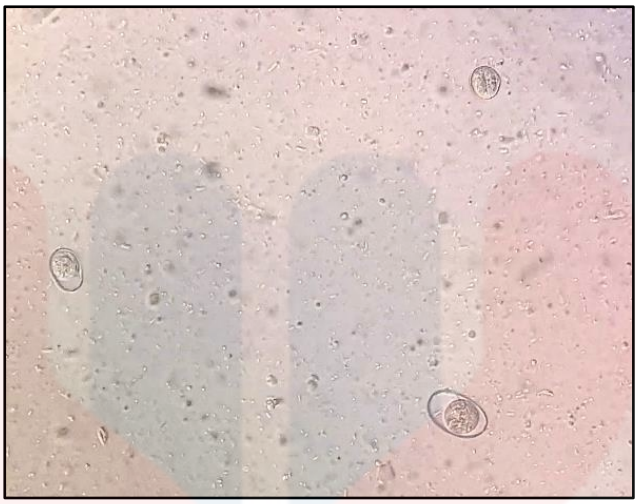

Figure 8.1: *Eimeria* spp. oocysts observed under a compound microscope (40X magnification)

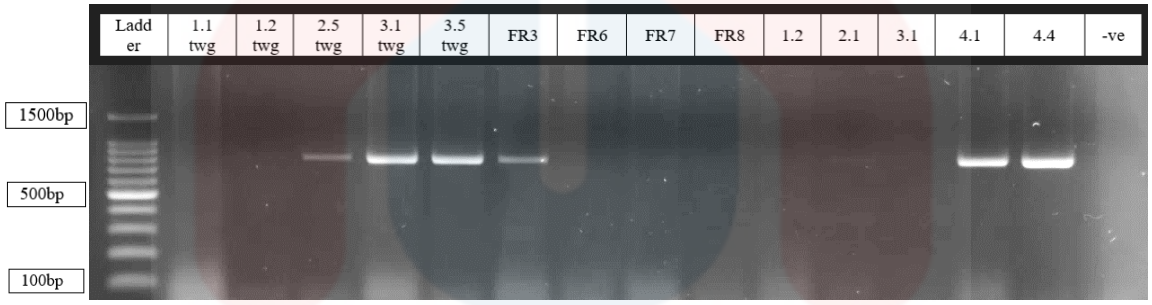

Figure 8.2: Result of molecular identification *Eimeria acervulina*

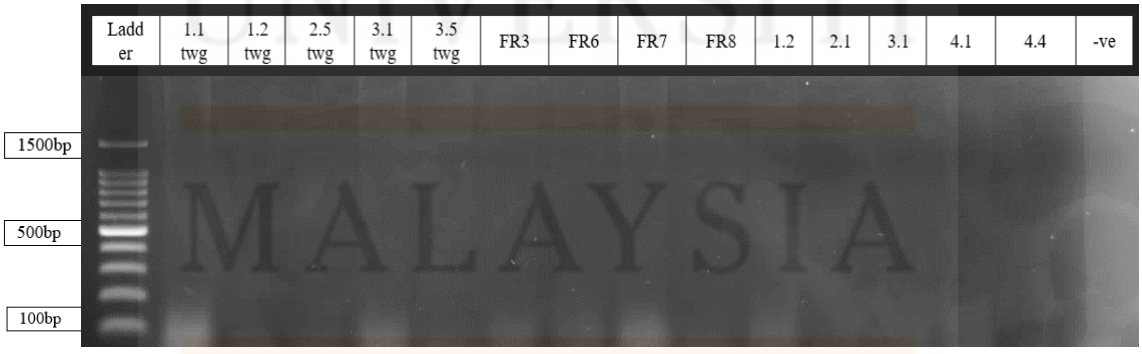

Figure 8.3: Result of molecular identification *Eimeria brunetti*

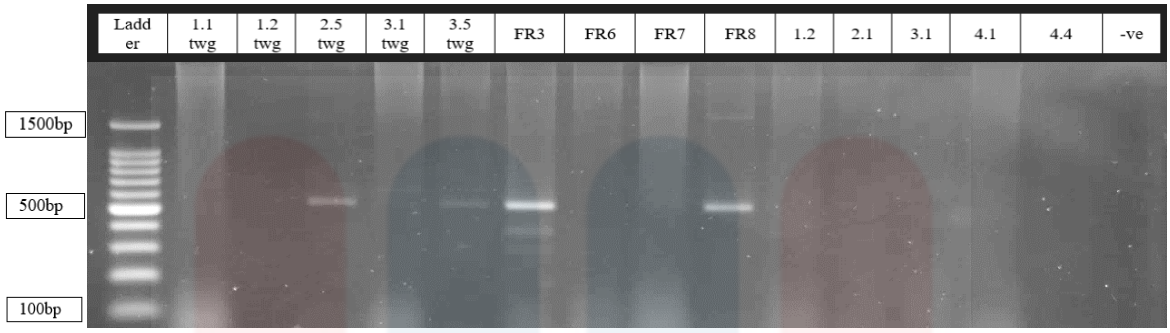

Figure 8.4: Result of molecular identification *Eimeria tenella*

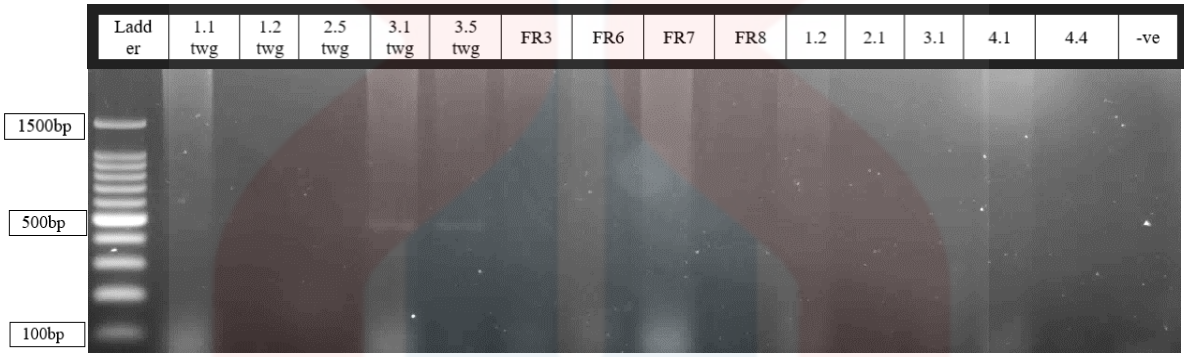

Figure 8.5: Result of molecular identification *Eimeria mitis*

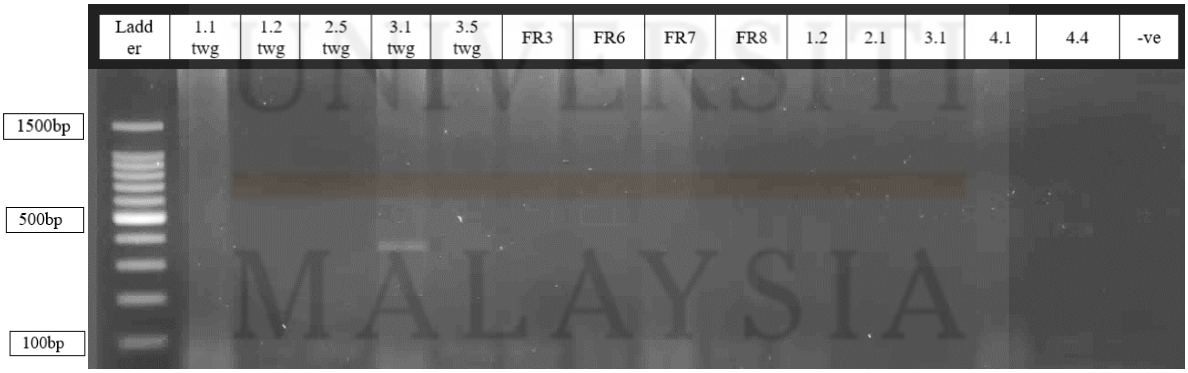

Figure 8.6: Result of molecular identification *Eimeria praecox*

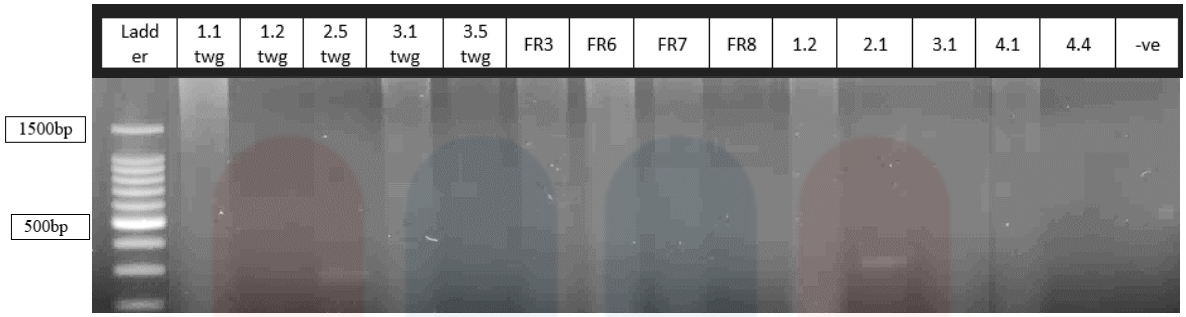

Figure 8.7: Result of molecular identification *Eimeria maxima*

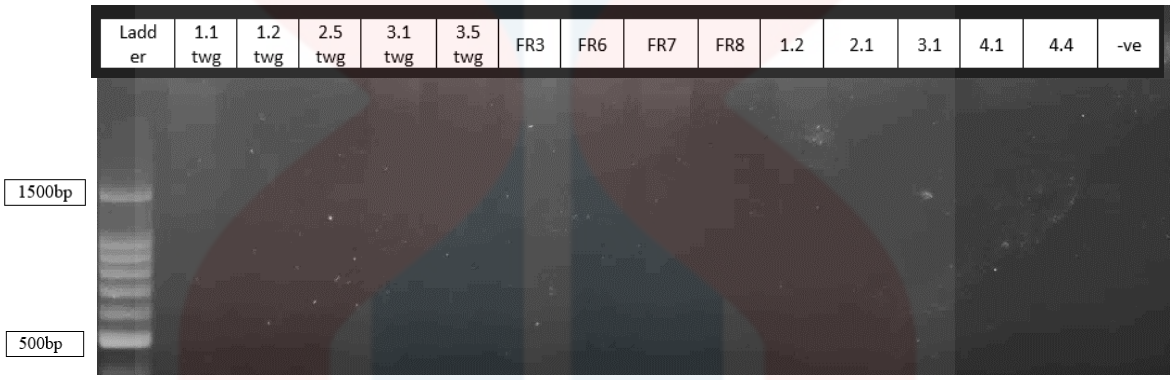

Figure 8.8: Result of molecular identification *Eimeria necatrix*

9.0 Discussion

9.1 Strains of *Eimeria* spp. Detected

In this study, five out of seven *Eimeria* spp. infecting chicken of different pathogenicity was identified. The highly pathogenic species identified are *E. tenella* and *E.maxima*, while *E. acervulina* and *E. mitis* of moderate pathogenicity were identified and *E. praecox* of least pathogenicity was detected. However, the clinical signs manifested by the chickens in each system varied. This is because the manifestation of the clinical signs does not only depend on the pathogenicity of infected *Eimeria* spp. but also the amount of oocysts load, host immunity, and the presence of multiple infection of more than one *Eimeria* spp. (Swayne et al., 2020).

9.2 Effect Of Management System On Oocysts Load

Broiler chickens reared in an intensive, deep litter system recorded the highest oocysts load while those kept in an intensive, raised floor system recorded the most strain of *Eimeria* spp. detected. The deep litter system allows accumulation of a higher amount of *Eimeria* spp. oocysts in the environment, putting the chickens at a higher risk of coccidiosis (Dakpogana & Salifou, 2013). There can be persistent shedding of *Eimeria* spp. oocyst into the environment when the sporulated oocyts in the deep litter are consumed and proliferate in the intestinal tract of the infected chicken and release of unsporulated oocyst in feces. Furthermore, the deep litter at the farm was observed not to have been cleared totally and the house was not sanitised after each cycle of production. Thus, this would add on to the parasitic load that could have built up from each production cycle.

The oocyst load recorded in the raised floor system is also relatively high. The presence of lack of concern for the farm sanitation was noticed on the farm, where

droppings were accumulated on the floor and between the flooring gaps instead of falling to the ground. The accumulation of feces created a wet, damp environment favouring sporulation of the *Eimeria* spp. oocysts. The study conducted by Abebe and Amede (2018) indicated that the prevalance of coccidiosis is affected by poor management such as moist litter on the floor encourages sporulation of *Eimeria* spp. oocysts.

The calculated oocysts load is the least in chickens reared under extensive, free-ranging system. The chickens have a lower stocking density, free to roam, and absence of damp litter accumulated in the environment thus contributing to the less susceptibility to coccidiosis, as suggested by Wan Norulhuda et al. (2017).

9.3 Potential Undetected *Eimeria* spp.

From the results of molecular detection of *Eimeria* species, only eight out of fourteen samples showed positive results despite all samples being positive for *Eimeria* spp. oocysts microscopically, indicating the potential of false-negative results in this study. The oocysts load of certain *Eimeria* spp. can be low when multiple infection are occurring in a single chicken, leading to insufficient DNA extracted to be detected via PCR (Hoan et al., 2014). The lowest oocyst load of *Eimeria* spp. detected in this study is 700 OPG; undetected samples may have an oocysts number below 700 OPG.

9.4 Strengths of Molecular Identification of *Eimeria* spp.

The common practice for *Eimeria* spp. identification is the microscopic examination of the intestinal scrappings and pathological lesion examination on the gastrointestinal upon necropsy. However, multiple infections of different strains of *Eimeria* spp. are common. It hinders accurate diagnosis and undermines detection of subclinical disease as the pathological lesions may overlap among the species infecting the avian (Gadelhaq et al., 2015).

The sensitivity and specificity of the molecular method in identifying and discriminating the *Eimeria* spp. infecting the chicken provides the provision of the accurate species identification and detection of subclinical infection of certain species. This would provide valuable information for the operating farm to plan appropriate immunization program against coccidiosis. The molecular method can determine the species composition of the *Eimeria* spp. presence in a broiler farm and able to assist in choosing suitable vaccines to protect the chickens against coccidiosis (Jenkins et al., 2006).

9.5 Potential Of Anticoccidial Drug Resistency

The potential of anticoccidial drug resistance in broiler chickens in Kelantan was noticed while conducting this study. The Modified McMaster technique reveals significant oocyst load in all the 3 sites of sample collection despite the chickens are fed with commercial feed containing anticoccidial drug for coccidiosis control. Moreover, presence of watery diarrhea was noticed during the sample collection process on the farms.

Twenty out of 22 samples collected from the intensive system recorded oocysts per gram(OPG) of more than 1,000 OPG. The mean OPG calculated for samples collected from all the three farms collected are above 1,000 OPG as well. The number of oocysts per count of more than 1,000 is significant in a broiler operation. According to Swayne et al. (2020), oocysts load of 1,000 OPG can manifest mild coccidiosis and reduce weight gain of the affected chickens.

In short, broiler chickens in all the three investigated farms showed moderate to heavy load of Eimeria spp. oocysts load in the dropping despite feed incorporated with anticoccidial medication. The intensive use of anticoccidial drugs as coccidial control

has led to the emergence of resistance to all anticoccidial agent as long-term exposure to any drug will result in loss of sensitivity (Peek & Landman, 2011).

9.6 Potential of *Eimeria* Vaccination Programme

Detection of the five *Eimeria* species infecting broiler chicken in Kelantan in this study provides valuable information for the local farmer on the predominant *Eimeria* spp. infecting the birds. DNA extracted from the positive samples was sent for genomic sequencing and the oocysts isolated from each samples were preserved in the laboratory. If there is a genetic drift of local *Eimeria* spp. strains detected, the local vaccine may be produced from the isolated oocysts. Moreover, the emergence of anticoccidial drug resistance has prompted the farmers to include coccidial vaccination in their herd health management (Soutter et al., 2020).

10.0 Conclusion

In conclusion, five *Eimeria* species are detected in the broiler chicken in Kelantan. The identified *Eimeria species* are *Eimeria tenella*, *Eimeria maxima*, *Eimeria acervulina*, *Eimeria mitis* and *Eimeria praecox*. *E. acervulina* is the predominant species found in all the investigated farms. At least one high pathogenic *Eimeria* spp. of *E. tenella* and *E. maxima* found in each investigated farm. The intensive management systems reveals a higher coccidiosis load than the extensive management system. There is an emergence of anticoccidial control resistance where birds in the inspected farm have moderate to heavy burden of *Eimeria* spp. oocysts in the droppings. Coccidial vaccination can be an effective alternative for coccidiosis control in the industry.

11.0 Recommendations and future work

For future studies, it will be meaningful to have spectrophotometry for check the quality and quantity of the extracted DNA. The limit of detection of minimum nanogram per microlith of the method used in this study can be determined. It will be beneficial for future studies to ensure that minimal detectable DNA concentration can be achieved for a better result.

In addition, the result of the study will be more reliable and conclusive if a positive control can be included in the molecular identification steps. A known sample or a known *Eimeria* spp. vaccine can be used as a positive control to indicate that all reaction ingredients are working.

Moreover, types of the anticoccidial agent incorporated in the feed and its effectiveness can be investigated as well. Resistance to each of the coccidial control drugs can be identified as well. This will be benefit the local industry in planning and selecting suitable control measures for coccidiosis management.

Last but not least, a bigger sample size and more extensive coverage of investigated areas will be beneficial. Prevalence of the *Eimeria* spp. can be calculated and provide a better statistically significance. Risk factors other than management systems such as biosecurity management and herd health program, can be investigated as well.

Appendices

Appendix 1: The housing of the broiler farm from the district Bachok.

Appendix 2: The free-roaming chickens in the broiler farm located in Pengkalan Chepa.

Appendix 3: The internal view of the housing of the broiler farm located in Tawang.

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