Sains Malaysiana 51(6)(2022): 1677-1686
http://doi.org/10.17576/jsm-2022-5106-06

Isolation and Establishment of *Eimeria tenella* Populations from Local Broiler Chicken Farms

(Pemencilan dan Penghasilan Populasi *Eimeria tenella* daripada Ladang Ayam Pedaging Tempatan)

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Received: 20 March 2021/Accepted: 29 November 2021

ABSTRACT

Coccidiosis is a major intestinal disease in the chicken production industry that is caused by one or more of the seven *Eimeria* species known to infect chickens. Development of effective diagnostic methods and controls requires comprehensive knowledge on the *Eimeria* species in the local population, particularly the pathogenic *E. tenella*. In this study, 35 faecal samples were collected from local chicken farms, and through microscopic observation, nine samples (26%) were found to contain *Eimeria* oocysts. Subsequently, two positive samples, namely NSN6 and SGR6, were selected and propagated via passage in coccidian-free chickens. Species identification analyses based on oocyst morphological characterisation suggested the presence of small-sized oocysts (*E. acervulina* and/or *E. mitis*) and medium-sized oocysts (*E. necatrix, E. tenella,* and/or *E. praecox*) in NSN6 while in SGR6, the large-sized oocysts (*E. brunetti* and/or *E. maxima*) were also present in addition to the small and medium-sized oocysts. Subsequently, species identification with PCR using species-specific primers was successful in determining the presence of specific *Eimeria* species, which are *E. acervulina* and *E. tenella* in NSN6, and *E. acervulina, E. tenella,* and *E. maxima* in SGR6. The *E. tenella* populations of NSN6 and SGR6, namely EtNSN6 and EtSGR6, respectively, were established via passage in coccidian-free chickens and oocyst recovery from the caeca. The purity of EtNSN6 and EtSGR6 populations were confirmed based on oocyst morphological characterisation and PCR. The established EtNSN6 and EtSGR6 populations would be useful as research models for local strains in the development of more effective control methods.

Keywords: Coccidiosis; local strains; oocyst morphology

ABSTRAK

Koksidiosis merupakan penyakit saluran usus utama dalam industri penternakan ayam yang disebabkan oleh satu atau lebih daripada tujuh spesies *Eimeria* yang diketahui menjangkiti ayam. Pembangunan kaedah diagnostik dan kawalan yang berkesan memerlukan pengetahuan tentang spesies *Eimeria* dalam populasi tempatan, khususnya *E. tenella* yang patogenik. Dalam kajian ini, sejumlah 35 sampel tinja ayam diperoleh daripada ladang ayam tempatan dan melalui pemerhatian mikroskop, didapati sembilan sampel (26%) mengandungi oosista *Eimeria*. Seterusnya, dua sampel positif, iaitu NSN6 dan SGR6, dipilih dan dibiakkan secara pasaj dalam ayam bebas koksidia. Analisis pengenalpastian spesies berdasarkan pencirian morfologi oosista mencadangkan kehadiran oosista yang bersaiz kecil (*E. acervulina* dan/atau *E. mitis*) dan oosista yang bersaiz sederhana (*E. necatrix, E. tenella* dan/atau *Eimeria praecox*) dalam NSN6 manakala dalam SGR6, oosista yang bersaiz besar (*E. brunetti* dan/atau *E. maxima*) juga hadir di samping oosista yang bersaiz kecil dan sederhana. Selanjutnya, pengenalpastian spesies dengan PCR menggunakan pencetus khusus terhadap spesies

telah berjaya mengenal pasti kehadiran spesies *Eimeria* secara khusus, iaitu *E. acervulina* dan *E. tenella* dalam NSN6 serta *E. acervulina, E. tenella* dan *E. maxima* dalam SGR6. Populasi *E. tenella* NSN6 dan SGR6, iaitu masing-masing EtNSN6 serta EtSGR6 dihasilkan melalui pasaj dalam ayam bebas koksidia dan perolehan oosista daripada bahagian sekum. Ketulenan populasi EtNSN6 dan EtSGR6 kemudian disahkan secara pencirian morfologi oosista dan PCR. Populasi EtNSN6 dan EtSGR6 adalah berguna sebagai model penyelidikan untuk strain tempatan dalam pembangunan kaedah pengawalan yang lebih berkesan.

Kata kunci: Koksidiosis; morfologi oosista; strain tempatan

Introduction

The chicken production industry is one of the fastest growing industries in the agricultural sector and has become an important economic activity in most countries including Malaysia. According to Agrofood Statistics 2018 (MOA 2018), Malaysia produced 13.4 billion eggs and 1.58 million metric tons of meat, indicating an increase of about 20 and 22%, respectively, compared to 2013. The annual per capita consumption of chicken meat in 2018 was 49.1 kg, which was about 6.7% increase compared to 2013, while the local chicken production activities accounted for approximately 86% of the country's total meat supply. The increasing demand for chicken meat is expected to continue in the future as it is the choice of cheaper source of protein compared to other livestock such as cattle, goats, and pigs (Loh 2017).

However, the chicken production industry faces several disease challenges including coccidiosis, which is a parasitic disease caused by the apicomplexan protozoa of the genus *Eimeria*. This intestinal disease is caused by infection of one or more of the seven *Eimeria* species known to infect chickens, namely *E. tenella*, *E. necatrix*, *E. acervulina*, *E. brunetti*, *E. praecox*, *E. maxima,* and *E. mitis* (Chapman 2014). The pathology of coccidiosis is caused by the destruction of epithelial cells and blood capillaries due to the release of merozoites from schizonts that in turn result in debris and blood clots causing blockages that lead to necrosis. In serious cases, necrosis can result in the deaths of infected chickens (Dalloul & Lillehoj 2006). The economic losses worldwide due to coccidiosis is estimated to be at least US$ 13.6 billion (Blake et al. 2020). These losses are associated with the drop in egg production, poor weight gain or feed conversion, failure to thrive, and costs of prevention and treatment (Blake & Tomley 2014). Globally, the prevalence of clinical coccidiosis infections is estimated to occur at 5% while subclinical infections are as high as 20% (Shirley et al. 2005). Additionally, other studies reported higher prevalence of subclinical infections of 75% in Iran (Shirzad et al. 2011) and 92% in Romania (Györke et al. 2013). In Malaysia, a study of 135 village chicken faecal samples from Kelantan showed the prevalence of coccidiosis infection to be 7.4% (Norulhuda et al. 2017), while a study of 240 village chicken faecal samples from Penang and Perak reported a higher prevalence of coccidiosis infection of 27.1% (Haziqah & Irwan-Izzauddin 2019).

The control of coccidiosis is largely achieved through chemotherapy and vaccination. However, the emergence of drug-resistant strains due to persistent use of anticoccidial drugs complicates their application (Karavolias et al. 2018). Together with the pressure to reduce drug use in the food chain, this has made the application of live oocyst vaccines to be increasingly popular in some countries. Live oocyst vaccines comprising species of non-attenuated and attenuated parasites are well established. However, the uptake remains limited by production costs and capacity as the vaccine lines need to be grown in chicken hosts (Chapman & Jeffers 2014). Thus, development of more effective control strategies is needed, and this will require various efforts, including studies on *Eimeria* strains from local field populations.

In this study, chicken faecal droppings were sampled from local chicken farms located in several states of Peninsular Malaysia. Species identification was performed by morphological characterisation, complimented with molecular characterisation using polymerase chain reaction (PCR). This study reports the pioneering efforts on the isolation and establishment of *E. tenella* populations from local broiler chicken farms.

Materials and Methods

Sample Collection

Samples were collected from local chicken farms located in four states of Peninsular Malaysia, namely Selangor, Negeri Sembilan, Pahang and Johor. Prior to the sampling, 50 mL polypropylene conical tubes were added with 1 g glass beads (1 mm diameter) and 4 mL potassium dichromate solution (2% w/v). Collection of single faecal droppings in triplicate tubes per farm was performed from randomly selected corner in an approximate 'W' shape across the pen. Each tube was filled up to the 10 mL line, capped and inverted five times. After sampling, the tubes were transported back to the laboratory and refrigerated at 4 °C until processed.

Subsequently, the samples were enriched by flotation in saturated saline solution to separate oocysts from faecal debris (Kumar et al. 2014). All samples were inspected microscopically to detect the presence of *Eimeria* oocysts.

PARASITE PROPAGATION AND ISOLATION

Samples in saturated saline solution were diluted with equal volume of water and followed by centrifugation at ~750 g for 10 min to pellet the oocysts. This step was repeated twice to remove salt residues from the samples (Johan et al. 2011). After the final wash, cleaned oocysts were re-suspended in phosphate buffered saline and enumerated using a modified-Fuchs Rosenthal counting chamber (Shirley 1995). The oocysts were subsequently diluted with 2.5% w/v potassium dichromate in water to a final concentration of 100,000-150,000 oocysts/mL. Sporulation was performed in a flask attached with airline tubing to an air pump on a magnetic stirrer, which was set at low speed. Sporulated oocysts of mixed species were orally inoculated into ten 2-week-old coccidian-free chickens with 5,000 oocysts per chicken as recommended by Shirley (1995). Faecal collection was performed at day 5 to 10 post infection (D5pi-D10pi), which coincided with the duration of oocyst production by chicken *Eimeria* species (Marquardt et al. 2000). Purification and sporulation were carried out using the procedures as described previously. This study was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) (Approval number: FST/2016/WAN/28-SEPT./791-SEPT.-2016-APR.-2017).

SPECIES IDENTIFICATION BY OOCYST MORPHOLOGY

A total of 30 randomly selected sporulated oocysts for each sampling day were viewed using the BA210 microscope (Motic, USA) under 40X phase contrast objective lens. The images were captured with the Moticam 5 camera (Motic, USA) and measured using the Image Plus software (Motic, USA). Subsequently, the oocysts were assigned putative species identity based on oocyst size (Haug et al. 2008). The samples were assigned into three categories (according to the initial letters in the species name) in which the small oocysts were categorised as *E. acervulina* and/or *E. mitis* (group AM, ≤18.8 µm long), medium sized oocysts as *E. necatrix*, *E. tenella* and/or *E. praecox* (group NTP, 18.9-23.8 µm long) and the larger oocysts as *E. brunetti* and/or *E. maxima* (group BM, ≥23.9 µm long).

ISOLATION OF GENOMIC DNA

Genomic DNA was isolated based on the protocol previously described by Fernandez et al. (2003a, 2003b) with minor modifications. Purified oocysts were suspended in extraction buffer (10mM Tris-HCl, pH 8.0; 50mM EDTA, pH 8.0) and an equal volume of glass beads (0.5 mm diameter) was added. The mixture was homogenised by vortexing followed by centrifugation at ~750 g for 10 min. The supernatant was treated with proteinase K (100 µg/mL) and SDS (0.5%) at 55 °C for 2 h, and subsequently with RNAse A (20 µg/mL) at 37 °C for 1 h. The genomic DNA was then isolated using phenol/chloroform extraction followed by ethanol precipitation and dissolution in deionised water.

SPECIES IDENTIFICATION BY PCR

PCR assays were performed using Sequence Characterised Amplified Region (SCAR) primers as described by Fernandez et al. (2003a, 2003b). The SCAR primers are capable of producing distinct PCR product size that is specific to each of the seven *Eimeria* species infecting chickens. Each PCR reaction was performed with the pooled genomic DNA extracted from samples D5pi-D10pi. The concentration of the samples was made uniform with the final concentration of ~50 ng genomic DNA. The species-specific assays for all seven *Eimeria* species were performed separately. Briefly, amplification was carried out in 20 µL reaction volumes containing ~50 ng of sample DNA, 1 µM of each pair of primers and 2X Phire HS II Master Mix (Thermo Scientific, USA). The PCR assays were performed on a Mastercycler Pro S PCR machine (Eppendorf, Germany) with cycling conditions consisted of an initial denaturation step at 98 °C for 30 s followed by 35 cycles of 98 °C at 30 s, 65 °C at 5 s and 72 °C at 15 s, with a final extension step at 72 °C for 1 min. The PCR products were visualised by electrophoresis on 1.7% (w/v) agarose gels stained with SYBR™ Safe (Thermofisher, USA). Genomic DNA of each *Eimeria* species (MSD Animal Health, USA) were used as positive controls.

Eimeria tenella PARASITE PURIFICATION

Sporulated oocysts of mixed species were orally inoculated into 2-week-old coccidian-free chickens in doses as recommended by Shirley (1995). Caeca collection was performed at day 7 post infection (D7pi), which coincided with the duration of *E. tenella* oocyst production in chickens (Marquardt et al. 2000). In contrast to other *Eimeria* species, the ease of recovery of *E. tenella* oocysts from the blind-ended caecal pouches facilitates the isolation of pure parasites as well as reduces the risk of contamination from the environment (Chapman & Shirley 2003). Oocyst harvest, purification and sporulation were carried out using the procedures as described by Shirley (1995). Species identification by oocyst morphology and PCR was performed using the procedures as described previously.

Results and Discussion

Prevalence of *Eimeria* parasites in local chicken farms

A total of 35 faecal samples were collected from local chicken farms located in four states in Peninsular Malaysia, namely Selangor, Negeri Sembilan, Pahang and Johor (Table 1). Among the 35 samples, 15 samples were obtained from Negeri Sembilan, eight samples were from Pahang, while Selangor and Johor recorded six samples each. All farms practiced raised floor housing system except for NSN1, NSN2, SGR4, and SGR6, which practiced deep litter housing system. The majority (31 farms) of the local farms reared broiler chickens while the rest reared breeder (three farms) and layer (one farm) chickens. All broiler farms reared the Cobb 500 breed except for SGR6, which reared the hybrid village chicken breed. The 35 samples were screened for the presence of *Eimeria* species oocysts with nine samples (26%) exhibited positive results.

TABLE 1. Summary of faecal sampling for *Eimeria* species oocyst detection from local chicken farms

Number	Sample	Housing system	Type of chicken	Chicken breed	State	Detection (+/-)[a]
1	SGR1	Raised floor	Broiler	Cobb 500	Selangor	-
2	SGR2	Raised floor	Broiler	Cobb 500	Selangor	-
3	SGR3	Raised floor	Broiler	Cobb 500	Selangor	-
4	SGR4	Deep litter	Breeder	Cobb 500	Selangor	-
5	SGR5	Raised floor	Layer	Lohmann Brown	Selangor	-
6	SGR6	Deep litter	Broiler	Hybrid village chicken	Selangor	+
7	JHR1	Raised floor	Broiler	Cobb 500	Johor	+
8	JHR2	Raised floor	Broiler	Cobb 500	Johor	-
9	JHR3	Raised floor	Broiler	Cobb 500	Johor	-
10	JHR4	Raised floor	Broiler	Cobb 500	Johor	+
11	JHR5	Raised floor	Broiler	Cobb 500	Johor	+
12	JHR6	Raised floor	Broiler	Cobb 500	Johor	+
13	NSN1	Deep litter	Breeder	Cobb 500	N. Sembilan	-
14	NSN2	Deep litter	Breeder	Cobb 500	N. Sembilan	+
15	NSN3	Raised floor	Broiler	Cobb 500	N. Sembilan	-
16	NSN4	Raised floor	Broiler	Cobb 500	N. Sembilan	-
17	NSN5	Raised floor	Broiler	Cobb 500	N. Sembilan	-
18	NSN6	Raised floor	Broiler	Cobb 500	N. Sembilan	+
19	NSN7	Raised floor	Broiler	Cobb 500	N. Sembilan	-
20	NSN8	Raised floor	Broiler	Cobb 500	N. Sembilan	-
21	NSN9	Raised floor	Broiler	Cobb 500	N. Sembilan	-
22	NSN10	Raised floor	Broiler	Cobb 500	N. Sembilan	-
23	NSN11	Raised floor	Broiler	Cobb 500	N. Sembilan	-
24	NSN12	Raised floor	Broiler	Cobb 500	N. Sembilan	-
25	NSN13	Raised floor	Broiler	Cobb 500	N. Sembilan	+
26	NSN14	Raised floor	Broiler	Cobb 500	N. Sembilan	-
27	NSN15	Raised floor	Broiler	Cobb 500	N. Sembilan	-
28	PHG1	Raised floor	Broiler	Cobb 500	Pahang	+
29	PHG2	Raised floor	Broiler	Cobb 500	Pahang	-
30	PHG3	Raised floor	Broiler	Cobb 500	Pahang	-
31	PHG4	Raised floor	Broiler	Cobb 500	Pahang	-
32	PHG5	Raised floor	Broiler	Cobb 500	Pahang	-
33	PHG6	Raised floor	Broiler	Cobb 500	Pahang	-
34	PHG7	Raised floor	Broiler	Cobb 500	Pahang	-
35	PHG8	Raised floor	Broiler	Cobb 500	Pahang	-

[a] Successful detection is indicated by (+) while no detection is indicated by (-)

IDENTIFICATION OF *Eimeria* SPECIES IN NSN6 AND SGR6 SAMPLES

The NSN6 and SGR6 samples were among the nine samples that showed the presence of *Eimeria* species oocysts and were selected for propagation and isolation as both samples were the representative of different farming systems. NSN6 was sampled from a farm that practiced raised floor housing system and reared the Cobb 500 breed. In contrast, SGR6 was sourced from a farm that practiced deep litter housing system and reared the hybrid village chicken breed. In order to have a sufficient number of oocysts for characterisation work, both NSN6 and SGR6 samples were propagated in chicken hosts. Production of *Eimeria* parasites required the chicken host as there is no suitable cell line for the *in vitro* replication approach (Barbour et al. 2015). Faecal samples were collected at D5pi-D10pi as this period spans the prepatent time of all seven *Eimeria* species that infect chickens (Shirley 1995). Out of the seven species, *E. praecox*, *E. acervulina,* and *E. mitis* were expected to be produced earlier with the prepatent period of 84 to 99 h, followed by *E. tenella*, *E. brunetti*, *E. maxima,* and *E. necatrix* with the prepatent period of 120 to 138 h (Marquardt et al. 2000). Faecal samples were collected daily throughout this period in order to isolate all possible *Eimeria* species present in the NSN6 and SGR6 samples.

Species identification of the purified and sporulated NSN6 and SGR6 oocysts through morphological characterisation was performed based on oocyst length measurement. Putative *Eimeria* species assignment according to Haug et al. (2008) suggested that NSN6 consisted of the AM group (63.3%) and NTP group (36.7%) while the BM group was not identified. As for SGR6, all groups i.e. AM (43.3%), NTP (37.2%) and BM (19.5%) were detected. The AM group was found to be most prevalent in both samples (Table 2). Previously, Loo et al. (2019) had observed the oocyst morphological size of 18 samples isolated from broiler farms located in Kedah, Perak, Selangor, Negeri Sembilan, Melaka and Pahang in Peninsular Malaysia. All 18 samples were reported to contain *Eimeria* oocysts from the AM group, which were also detected in NSN6 and SGR6. This finding suggested that the AM group, which is categorised as *E. acervulina* and/or *E. mitis,* was prevalent in the broilers farms that were studied. Species identification using morphological characterisation also suggested that all seven *Eimeria* species may be present in SGR6 while only five species may be present in NSN6.

Co-infection of multiple *Eimeria* species that causes coccidiosis disease in the field is common (Williams et al. 1996). Although morphological characterisation could be used to determine the *Eimeria* species, the oocyst size dimension could overlap between species and this requires high level of expertise to distinguish the differences (Long & Joyner 1984). Therefore, the PCR approach is a more practical option as it is able to determine the parasite infection specifically up to the species level. This can be achieved by amplifying the gene of interest from a minimum amount of the parasite genomic DNA (Gasser 1999).

TABLE 2. Species identification using morphological characterisation

Morphology group[a]	NSN6	SGR6
AM	114/180 (63.3%)	78/180 (43.3%)
NTP	66/180 (36.7%)	67/180 (37.2%)
BM	-	35/180 (19.5%)

[a]AM (*E. acervulina*, *E. mitis*); NTP (*E. necatrix*, *E. tenella*, *E. praecox*); BM (*E. brunetti*, *E. maxima*)

Species identification was carried out by PCR and the amplified products were analysed through agarose gel electrophoresis by comparing with the PCR product of positive control for each of the seven *Eimeria* species (Figure 1). The positive controls for both analyses showed seven individual bands that correlated with the PCR products of *E. acervulina* (811 bp), *E. mitis* (460 bp), *E. necatrix* (200 bp), *E. tenella* (539 bp), *E. praecox* (354 bp), *E. brunetti* (626 bp) and *E. maxima* (272 bp) (Fernandez et al. 2003a, 2003b). This showed that all the primer pairs were functional and specific for each *Eimeria* species that infects chickens.

As shown in Figure 1(A), two single bands were observed for the NSN6 sample, which correlated with the PCR product size of *E. acervulina* and *E. tenella*. This result indicated that the NSN6 sample contained two *Eimeria* species i.e. *E. acervulina* and *E. tenella*. In Figure 1(B), three single bands were observed for the SGR6 sample, which correlated with the PCR product size of *E. acervulina*, *E. tenella,* and *E. maxima*. This indicated that the SGR6 sample contained three *Eimeria* species i.e. *E. acervulina*, *E. tenella,* and *E. maxima*. Prevalence studies of chicken coccidiosis infections performed in countries from several continents had reported that both *E. tenella* and *E. acervulina* were the most common species found in chicken farms. Countries such as Czechoslovakia (Kučera 1990), France (Williams et al. 1996) and Korea (Lee et al. 2010) had reported that *E. acervulina* was the most prevalent species, while studies conducted in China (Sun et al. 2009), India (Bhaskaran et al. 2010), Jordan (Al-Natour et al. 2002) and Iran (Hadipour et al. 2013) reported that *E. tenella* had high prevalence. A study by Loo et al. (2019) had used three molecular methods to identify *Eimeria* species in 18 faecal samples from commercial broiler farms located in Kedah, Perak, Selangor, Negeri Sembilan, Melaka and Pahang. The study showed that *E. acervulina* was the most prevalent species followed by *E. tenella* in the six states of Peninsular Malaysia.

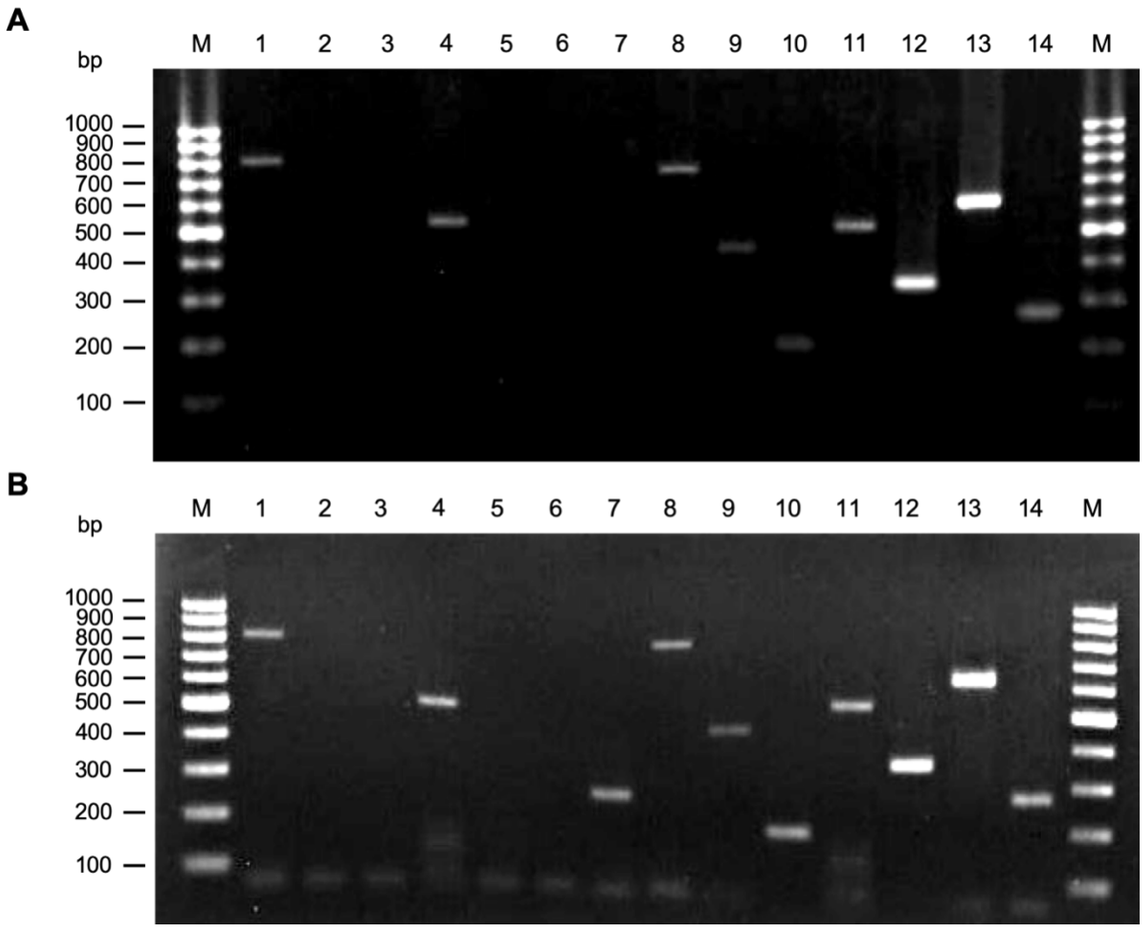

FIGURE 1. Analysis of (A) NSN6 and (B) SGR6 PCR product amplification by agarose gel electrophoresis. M: 100 bp DNA marker; Lanes 1 and 8: *E. acervulina* primers; 2 and 9: *E. mitis* primers; 3 and 10: *E. necatrix* primers; 4 and 11: *E. tenella* primers; 5 and 12: *E. praecox* primers; 6 and 13: *E. brunetti* primers; 7 and 14: *E. maxima* primers; 8-14: Positive control

Species identification of NSN6 and SGR6 through morphological characterisation and PCR showed that *E. acervulina* and *E. tenella* were the prevalent species, and this was consistent with previous findings by Loo et al. (2019), which indicated the prevalence of the two *Eimeria* species in samples from local broiler farms. The identification of *Eimeria* species that are prevalent in chicken populations could determine the impact of coccidiosis in the local chicken farms as each species has its own characteristics with respect to pathogenicity and immunogenicity (Blake & Tomley 2014). Additionally, the information would be useful in the development of anticoccidial vaccines as live oocyst vaccines could be formulated based on the distribution of *Eimeria* species in the local chicken farms. This could bring about an increase in the efficiency and a reduction in the cost of production.

ESTABLISHMENT OF LOCAL *Eimeria tenella* POPULATIONS

E. tenella populations from NSN6 and SGR6 samples, namely EtNSN6 and EtSGR6, respectively, were established by orally inoculating sporulated oocysts of mixed species into chicken hosts. Caeca collection was performed at D7pi, which coincides with the duration of oocyst production by the species (Marquardt et al. 2000). In contrast to other *Eimeria* species, the ease of recovery of *E. tenella* oocysts from the blind-ended caecal pouches facilitates the isolation of pure parasites as well as reduces the risk of contamination from the environment (Chapman & Shirley 2003).

The purity of the isolated EtNSN6 and EtSGR6 samples was initially determined by species identification using both morphological characterisation. The morphological characterisation of the purified and sporulated EtNSN6 and EtSGR6 oocysts was performed based on oocyst length measurement. The average dimension (length × width) for EtNSN6 oocysts was 22.25 (±1.24) × 18.10 (±1.73) µm while for EtSGR6 oocysts was 22.66 (±1.60) × 18.66 (±1.19) µm. The putative *Eimeria* species assignment according to Haug et al. (2008) suggested that both EtNSN6 and EtSGR6 were categorised in the NTP group, which could possibly consist of *E. necatrix*, *E. tenella,* and/or *E. praecox*. However, among the three species, only *E. tenella* infection was concentrated at the caecum, while the site for *E. necatrix* infection was at the duodenum and jejunum, and the site for *E. praecox* infection was only at the duodenum (Marquardt et al. 2000). The shape index value (length/width ratio) for EtNSN6 and EtSGR6 oocysts was 1.23 and 1.21, respectively. Based on Joyner and Norton (1969), the dimension for *E. tenella* Houghton strain oocysts was 21.85 × 17.96 µm with a shape index of 1.22, while Long (1973) reported a dimension of 21.29 × 17.84 µm with a shape index of 1.19 for the same strain. Besides that, *E. tenella* Weybridge strain oocysts were reported to have a dimension of 21.81 × 17.86 µm with a shape index of 1.22 (Joyner & Norton 1969) while *E. tenella* Wisconsin strain oocyst dimension was 22.50 × 18.10 µm with a shape index of 1.24 (Jeffers 1978). This showed that the dimension and shape index value for EtNSN6 and EtSGR6 oocysts were similar to other *E. tenella* strains.

The purity of the isolated EtNSN6 and EtSGR6 samples was further assessed by species identification through the PCR method using the SCAR primers. The PCR results were analysed through agarose gel electrophoresis by comparing with the PCR product of positive control for each of the seven *Eimeria* species. In Figure 2, a single band was observed for both EtNSN6 and EtSGR6 samples, which correlated with the PCR product size of *E. tenella*. The results indicated that both EtNSN6 and EtSGR6 samples contained a single *Eimeria* species i.e. *E. tenella*. The results from species identification using the PCR method were consistent with morphological characterisation and suggested that both EtNSN6 and EtSGR6 samples isolated from the chicken caeca were pure *E. tenella* populations.

The establishment of local *E. tenella* populations could serve as research models that provide better representation of the local coccidiosis disease scenario. Previously, researchers have used the *E. tenella* Houghton strain as the research model to study the disease. This is because the Houghton strain has been extensively studied and made as a reference strain by most research laboratories in the world (Chapman & Shirley 2003). Among the studies carried out using the Houghton strain included the characterisation of transcriptomes and genomes (Amiruddin et al. 2012; Ng et al. 2002; Reid et al. 2014; Wan et al. 1999), surface antigens (Chow et al. 2011; Ho et al. 2020; Ramly et al. 2021, 2013) and potential drug targets (Lee et al. 2020; Loo et al. 2010; Yao et al. 2016). With the local *E. tenella* populations established, these studies could be expanded to include comparative analyses between the local strains and strains from other countries and geographical regions.

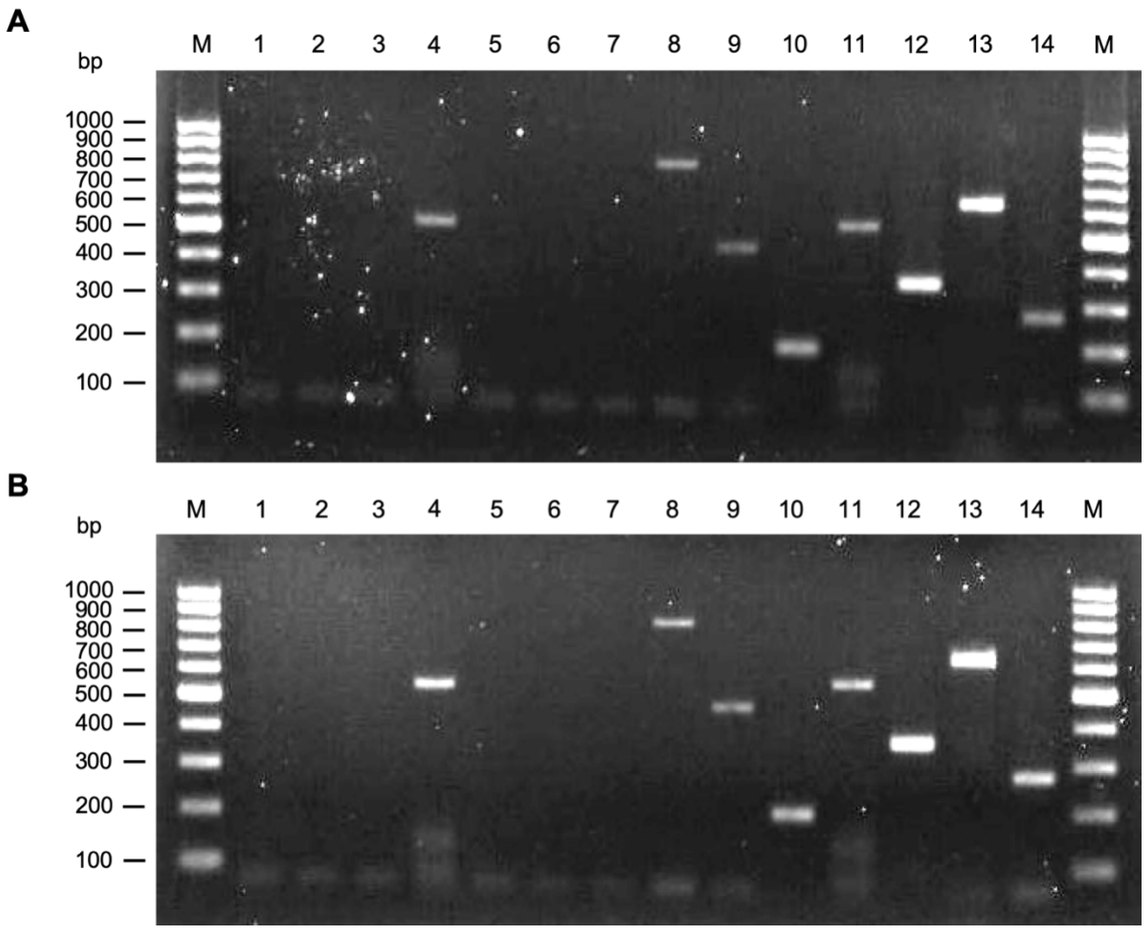

FIGURE 2. Analysis of (A) EtNSN6 and (B) EtSGR6 PCR product amplification by agarose gel electrophoresis. M: 100 bp DNA marker; Lanes 1 and 8: *E. acervulina* primers; 2 and 9: *E. mitis* primers; 3 and 10: *E. necatrix* primers; 4 and 11: *E. tenella* primers; 5 and 12: *E. praecox* primers; 6 and 13: *E. brunetti* primers; 7 and 14: *E. maxima* primers; 8-14: Positive control

CONCLUSIONS

The results of this study showed that both *E. acervulina* and *E. tenella* were detected in samples NSN6 and SGR6, consistent with previous findings that indicated the prevalence of the two *Eimeria* species in a number of local chicken farms in Peninsular Malaysia. The local *E. tenella* populations, EtNSN6 and EtSGR6, were established and could serve as research models to study the coccidiosis disease in the local environment. This could aid efforts in the development of more effective control methods.

ACKNOWLEDGEMENTS

This work was financially supported by Research University Grant from Universiti Kebangsaan Malaysia (ETP-2014-006).

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