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Comparison of Molecular Methods for the Detection of *Eimeria* in Domestic Chickens in Malaysia

(Perbandingan Kaedah Molekul untuk Pengenalpastian *Eimeria* dalam Ayam Ternakan di Malaysia)

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ABSTRACT

Coccidiosis, caused by the Eimeria *species, greatly affects the poultry industry. Severity of the disease varies depending on the identity of the infecting parasites, encouraging identification of* Eimeria *species circulating on a farm as a valuable component of chicken management. Conventional methods of* Eimeria *species identification are time consuming and can be subjective in nature. Given these limitations, molecular approaches have been developed for specific detection of* Eimeria *species. In this study, faecal samples were collected from commercial broiler farms and subjected to microscopic examination for* Eimeria *occurrence.* Eimeria *species were putatively identified by morphological characterisation and grouped into three categories based on oocyst size. Molecular detection of* Eimeria *species occurrence in these samples was then performed using two published PCR assays (the individual components of a SCAR-based multiplex PCR, and assays developed for quantitative PCR, termed PCR-SCAR 1 and PCR-SCAR 2 here) and a LAMP assay. Comparison of the results obtained demonstrated that the three molecular methods were capable of detecting all* Eimeria *species of the reference Houghton strain, but showed varying efficiencies in detecting Malaysian field isolates. PCR-SCAR 2 was found to be the most effective, detecting all seven* Eimeria *species and indicating the presence of* Eimeria *parasites in most flocks. Differences in the ability of the molecular methods to detect* Eimeria *may be a consequence of sequence divergence between isolates from different regions, implying that development of region-specific methods using local* Eimeria *strains may be required to improve the efficiency of molecular assays for* Eimeria *detection.*

Keywords: Coccidiosis; LAMP; protozoan parasite; SCAR

ABSTRAK

Koksidiosis yang disebabkan oleh spesies Eimeria*, memberikan kesan yang besar terhadap industri penternakan ayam. Keparahan penyakit ini bergantung kepada identiti parasit yang menjangkit dan ini menggalakkan usaha pengenalpastian spesies* Eimeria *yang hadir dalam ladang ayam sebagai komponen yang penting dalam pengurusan ayam. Kaedah konvensional pengenalpastian spesies* Eimeria *memakan masa dan didapati bersifat subjektif. Disebabkan oleh kekangan kaedah ini, pendekatan molekul telah dibangunkan untuk pengenalpastian spesies* Eimeria *secara spesifik. Dalam kajian ini, sampel tinja telah dikumpul dari ladang ayam pedaging komersial dan disaring melalui pemerhatian mikroskop untuk mengesan kehadiran* Eimeria. *Spesies* Eimeria *telah dikenal pasti secara pencirian morfologi dan dikelompokkan kepada tiga kategori berdasarkan saiz oosista. Pengesanan molekul terhadap kehadiran spesies* Eimeria *dalam sampel tersebut kemudiannya telah dilakukan dengan menggunakan dua asai PCR yang telah diterbitkan (komponen individu PCR multipleks berasaskan SCAR, dan asai yang dibangunkan untuk PCR kuantitatif yang dikenali sebagai PCR-SCAR 1 dan PCR-SCAR 2) dan asai LAMP. Perbandingan hasil yang diperoleh menunjukkan bahawa ketiga-tiga kaedah molekul ini mampu mengesan kesemua spesies* Eimeria *daripada strain rujukan Houghton, tetapi menunjukkan kecekapan yang berbeza dalam mengesan pencilan lapangan dari Malaysia. PCR-SCAR 2 didapati paling berkesan dan berupaya mengesan kesemua tujuh spesies* Eimeria *serta menunjukkan kehadiran parasit* Eimeria *dalam kebanyakan populasi ayam. Perbezaan dalam keupayaan kaedah molekul untuk mengesan* Eimeria *mungkin disebabkan oleh perbezaan jujukan antara pencilan dari kawasan yang berbeza, dan ini mencadangkan bahawa pembangunan kaedah berasaskan kawasan yang khusus menggunakan strain* Eimeria *tempatan adalah diperlukan untuk meningkatkan kecekapan asai molekul untuk pengesanan* Eimeria.

Kata kunci: Koksidiosis; LAMP; parasit protozoa; SCAR

INTRODUCTION

Coccidiosis is an enteric disease that causes significant losses to the livestock production industry by reducing animal performance and lowering productivity. Parasites of the protozoan *Eimeria* species are known to be causative agents of avian coccidiosis, a disease that threatens almost all of the ~60 billion chickens reared worldwide annually (Blake & Tomley 2014). Control of the disease is mainly achieved through prophylactic chemotherapy, supplemented by vaccination. However, these control methods have their disadvantages. The emergence of drug-resistant strains due to persistent use of anticoccidial drugs complicates their application (Chapman 1997). Live parasite vaccines are becoming increasingly popular in some regions (Chapman & Jeffers 2014), but uptake remains limited by production costs and capacity. Identification of *Eimeria* species that are present in chicken flocks can improve management of the disease, and aid in the development of more efficient control methods.

Conventionally, detection and identification of *Eimeria* species have been based on comparing clinical features, gut pathology in the host, morphology of sporulated oocysts and/or pre-patent period (Long et al. 1976). Such methods remain widely used, although their subjective nature, low throughput and requirement for trained specialists can prove limiting. Problems in the precise discrimination of species with overlapping morphological characteristics can arise when dealing with mixed populations (Long & Joyner 1984). In response, several molecular approaches have been developed to improve *Eimeria* species identification, including polymerase chain reaction (PCR) assays based on 5S rRNA (Stucki et al. 1993), small subunit rRNA (Tsuji et al. 1997), internal transcribed spacer-1 (ITS-1) (Lew et al. 2003; Schnitzler et al. 1998, 1999; Su et al. 2003), ITS-2 (Gasser et al. 2001; Lien et al. 2007) and Sequence Characterised Amplified Region (SCAR) markers (Fernandez et al. 2003a, 2003b). In addition, other molecular methods such as quantitative PCR (Morgan et al. 2009; Vrba et al. 2010) and Loop-Mediated Isothermal Amplification (LAMP) (Barkway et al. 2011) have also been introduced.

In this study, two PCR assays were employed to detect *Eimeria* species circulating in Malaysian broiler chicken farms. The first PCR assay utilised primers targeting SCAR markers as described by Fernandez et al. (2003a, 2003b), while the second assay was performed using primers originally developed for quantitative PCR that target unique single copy sequences derived from SCAR markers as described by Vrba et al. (2010). In addition, a LAMP assay described by Barkway et al. (2011) was performed for comparison. The detection of *Eimeria* species parasites using molecular methods can aid farmers and relevant authorities in the management of avian coccidiosis, and contribute towards the development of more efficient control strategies.

MATERIALS AND METHODS

SAMPLE COLLECTION AND MORPHOLOGICAL IDENTIFICATION

A total of 18 samples were collected from commercial broiler farms located in Peninsular Malaysia (Figure 1). Faecal samples of ~45 g were collected from the floor of each chicken pen when the birds were between three and four weeks old. Samples were enriched by flotation in saturated saline solution to separate oocysts from faecal debris (Shirley 1995). All samples were inspected microscopically to confirm the presence of *Eimeria* oocysts. The purified oocysts were then sporulated in 2% (w/v) potassium dichromate. Total number of oocysts was determined by enumeration using the Fuchs-Rosenthal haemocytometer counting method (Shirley et al. 2005). Subsequently, oocysts were assigned putative species identity based on microscopic morphology (Long et al. 1976), assigning oocysts to species groups based on oocyst size (Haug et al. 2008). The reference Houghton strains of each *Eimeria* species were used as positive controls.

ISOLATION OF GENOMIC DNA

Genomic DNA was isolated based on the protocol previously described by Fernandez et al. (2003a) with minor modifications. Purified oocysts were suspended in extraction buffer (10mM Tris-HCl, pH 8.0; 50mM EDTA, pH8.0) and an equal volume of glass beads (0.5 mm diameter) was added. The mixture was homogenised using a bead beater for one min followed by centrifugation at ~750 g for 10 min. The supernatant was treated with proteinase K (100 μg/mL) and SDS (0.5%) for 2 h at 55ºC. The genomic DNA was isolated using phenol/chloroform extraction followed by ethanol precipitation and dissolution in deionised water.

PCR-SCAR 1 ASSAY

PCR-SCAR 1 assays were performed as described by Fernandez et al. (2003a) using primers with a diagnostic sensitivity of 1-5 pg genomic DNA, equivalent to between two and eight sporulated oocysts, as reported in Fernandez et al. (2003b). The species-specific assays for all seven *Eimeria* species were performed separately. Briefly, amplification was carried out in 25 μL reaction volumes containing 2 μL of sample DNA, 1 μM of each pair of SCAR primers, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 1× reaction buffer and 1 U of *Taq* DNA polymerase (New England Biolabs). Cycling conditions comprised of an initial denaturation step at 94ºC for 3 min followed by 30 cycles of 1 min at 94ºC, 30 s at 62ºC and 1.5 min at 72ºC, with a final extension step at 72ºC for 7 min. PCR products were visualised by electrophoresis on 1.8% (w/v) agarose gels stained with ethidium bromide.

PCR-SCAR 2 ASSAY

PCR-SCAR 2 assays were performed using primers with a diagnostic sensitivity of approximately ten copies, equivalent to between one and two sporulated oocysts, as reported by Vrba et al. (2010). The assays were performed in 20 μL reaction volumes containing 2 μL of sample DNA, 0.5 μM of each forward and reverse primers, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 1× reaction buffer and 0.5 U of *Taq* DNA polymerase (New England Biolabs). Cycling conditions comprised of an initial denaturation step at 95ºC for 1 min followed by 35 cycles at 95ºC for 15 s and 60ºC for 30 s. PCR products were visualised by electrophoresis on 1.8% (w/v) agarose gels stained with ethidium bromide.

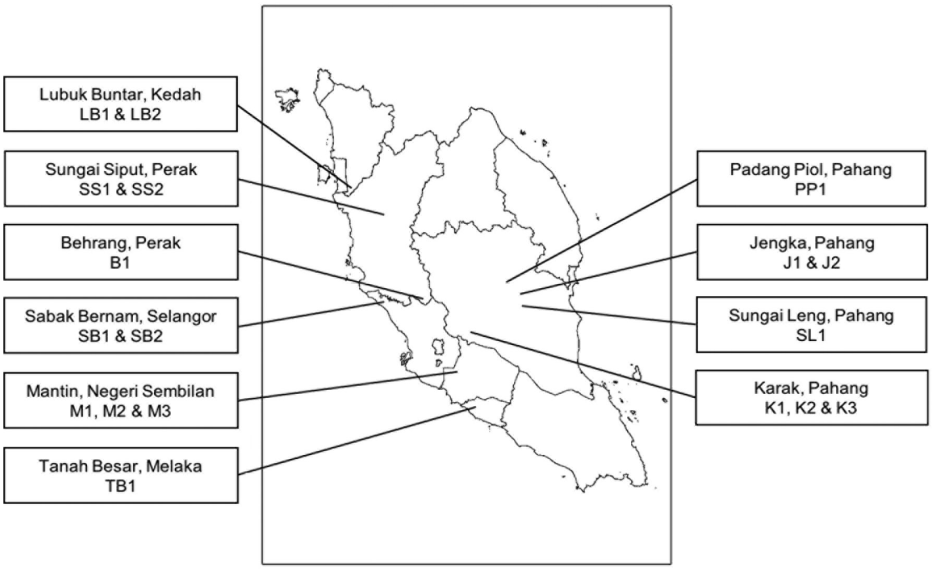

FIGURE 1. Geographical distribution of broiler chicken farms sampled during these studies across six states in Peninsular Malaysia

LAMP ASSAY

LAMP assays were performed as described by Barkway et al. (2011) in a final volume of 25 μL containing 8 U of *Bst* DNA polymerase (large fragment; New England Biolabs) in 1× ThermoPol Reaction buffer (New England Biolabs) supplemented with 2 mM of $MgCl_2$, 1 M of betaine and 0.4 mM of each dNTP. LAMP oligonucleotides FIP and BIP (40 pmol), LB and LF (20 pmol), and F3 and B3 (5 pmol), were added together with 2 μL of sample DNA. Each reaction was incubated at 62ºC for 30 min and then 80ºC for 5 min to terminate the reaction. LAMP products were visualised by electrophoresis on 1.8% (w/v) agarose gels stained with ethidium bromide. The diagnostic sensitivity of the assay has been reported to be approximately ten copies, equivalent to between one and two sporulated oocysts (Barkway et al. 2011).

RESULTS AND DISCUSSION

To provide baseline data, *Eimeria* oocysts present in all 18 samples were assigned putative species identity based upon microscopic morphological characterisation. The samples were assigned into three categories; the small oocysts were categorised as *E. acervulina* or *E. mitis* (group AM, ≤18.8 μm long), medium sized oocysts as *E. necatrix*, *E. tenella* or *E. praecox* (group NTP, 18.9-23.8 μm long) and the larger oocysts as *E. brunetti* or *E. maxima* (group BM, ≥23.9 μm long). Small oocysts, representing the AM group, were detected in all 18 samples. Of the 18 samples, 16 were also found to contain medium sized oocysts (NTP group) while 14 samples were found to contain the large sized oocysts (BM group). Previous studies carried out in Czechoslovakia (Kucera 1990), France (Williams et al. 1996) and Korea (Lee et al. 2010) reported *E. acervulina* to be the most highly prevalent species, while studies in China (Sun et al. 2009), India (Bhaskaran et al. 2010), Jordan (Al-Natour et al. 2002) and Iran (Hadipour et al. 2013) showed *E. tenella* to be highly prevalent. These studies conducted in countries from several regions of the world show that both *E. acervulina* and *E. tenella* are common among chicken farms, which is consistent with the occurrence of AM and NTP *Eimeria* species found in the 18 samples.

Subsequently, two PCR assays (PCR-SCAR 1 and PCR-SCAR 2) and a LAMP assay were employed for the detection of *Eimeria* species DNA extracted from the same faecal samples. The three assays could be performed rapidly using a common reaction temperature for all seven *Eimeria* species, whereas other assays such as those based on ITS-1 required independent annealing temperatures for each *Eimeria* species. Therefore, the three assays were chosen based on their practicality as diagnostics methods. The specificity of all three assays has been demonstrated, amplifying only from target *Eimeria* species with no cross-reactivity to other species or the chicken host (Fernandez et al. 2003b; Vrba et al. 2010; Barkway et al. 2011).

The PCR-SCAR 1 assay utilised primers that target species-specific SCAR markers derived from random amplification of polymorphic DNA (RAPD) fragments. During validation, the primers were tested using isolates of all seven *Eimeria* species collected from three geographical regions, namely South America, North America and Europe. More recently the assay has also been tested using isolates collected in Asia (India; Kumar et al. 2014). When PCR-SCAR 1 assays were carried out for the field samples, *Eimeria* species were found to be present in 11 out of 18 (61%) broiler farms. Six species comprising *E. acervulina*, *E. tenella*, *E. mitis*, *E. praecox*, *E. maxima* and *E. necatrix* were identified. The most prevalent species was *E. acervulina* (44%), followed by *E. tenella, E. praecox* and *E. maxima* (all 22%). *Eimeria mitis* and *E. necatrix* were found in only one farm, respectively, while

E. brunetti was not detected in any of the 18 farms that were sampled (Table 1). Out of the 11 farms that were found to be infected by molecular analysis, seven farms (64%) were detected with only one species, while mixed infections with two or more species were found in four farms (36%). Geographical distribution indicated that *E. acervulina* was prevalent in all six states in Peninsular Malaysia while *E. tenella* was present in Selangor and Pahang. Both *E. praecox* and *E. maxima* were detected in Selangor, Pahang and Negeri Sembilan, while *E. mitis* was detected in Selangor and *E. necatrix* in Pahang.

The second assay, PCR-SCAR 2, was performed using primers for each of the seven *Eimeria* species targeting unique single copy sequences derived from SCAR markers as described by Vrba et al. (2010). These markers were previously found to be non-polymorphic following sequencing from multiple strains of each species and appear to be present as single copies per genome. Marker polymorphism and species specificity were assessed using isolates from four geographical regions, namely South America, North America, Europe and Africa, supplemented more recently by samples from Asia (Kumar et al. 2014). When PCR-SCAR 2 assays were carried out for the field samples, *Eimeria* species were found to be present in 16 out of 18 (89%) broiler farms. All seven species comprising *E. acervulina*, *E. brunetti*, *E. tenella, E. mitis*, *E. praecox*, *E. maxima* and *E. necatrix* were identified. The most prevalent species identified was *E. acervulina* (89%), followed by *E. tenella* (33%), *E. praecox* (28%) and *E. mitis* (22%). Both *E. maxima* and *E. brunetti* were found in three farms, while *E. necatrix* was found in only one farm. Out of the 16 farms that were found to be infected, five farms (31%) were detected with only one species, while mixed infection with two or more species were found in 11 farms (69%). *Eimeria acervulina* was detected in all six states in Peninsular Malaysia, while *E. tenella* was present in Selangor and Pahang. Both *E. praecox* and *E. maxima* were detected in Pahang and Negeri Sembilan, with *E. mitis* in Selangor, Pahang and Negeri Sembilan; *E. brunetti* in Kedah, Perak and Pahang, and *E. necatrix* in Pahang.

The third assay, LAMP has been widely recognised as a cost effective diagnostic tool with excellent reproducibility. Assays targeting well-studied apicomplexan parasites have been described for *Cryptosporidium* (Karanis et al. 2007), *Plasmodium falciparum* (Poon et al. 2006) and *Toxoplasma gondii* (Sotiriadou & Karanis 2008). LAMP is an isothermal nucleic acid amplification technique that is performed without the series of alternating temperature steps or cycles required for PCR. Typically, four different primers are used to identify six distinct regions on the target locus, which adds to the specificity compared to PCR. The amount of DNA produced in LAMP is also considerably higher than PCR. Thus, detection of successful amplification can be determined through photometry for turbidity caused by an increasing quantity of magnesium pyrophosphate precipitate in solution as a by-product of amplification. Hence, this allows easy visualisation by the naked eye compared to PCR, which requires gel electrophoresis

TABLE 1. Summary of *Eimeria* species infection in 18 farms located at Kedah, Perak, Selangor, Negeri Sembilan, Melaka and Pahang in Peninsular Malaysia

Eimeria species	PCR-SCAR 1		PCR-SCAR 2		LAMP	
	Prevalence (*n*=18)	Location	Prevalence (*n*=18)	Location	Prevalence (*n*=18)	Location
Ac	8/18 (44%)	KDH (2), PRK (1), SGR (1), NSN (2), MLK (1), PHG (1)	16/18 (89%)	KDH (2), PRK (2), SGR (2), NSN (3), MLK (1), PHG (6)	12/18 (67%)	KDH (2), PRK (2), SGR (2), NSN (2), MLK (1), PHG (3)
Mt	1/18 (6%)	SGR (1)	4/18 (22%)	PHG (1), SGR (2), NSN (1)	-	-
Nc	1/18 (6%)	PHG (1)	1/18 (6%)	PHG (1)	-	-
Tn	4/18 (22%)	SGR (2), PHG (2)	6/18 (33%)	PHG (4), SGR (2)	-	-
Pr	4/18 (22%)	SGR (1), NSN (2), PHG (1)	5/18 (28%)	PHG (3), NSN (2)	3/18 (17%)	PHG (1), NSN (2)
Br	-	-	3/18 (17%)	KDH (1), PRK (1), PHG (1),	-	-
Mx	4/18 (22%)	SGR (1), NSN (1), PHG (2)	3/18 (17%)	PHG (1), NSN (2)	2/18 (11%)	PRK (1), PHG (1),
Total samples with *Eimeria* parasites	13/18 (72%)		16/18 (89%)		15/18 (83%)	

Ac: *E. acervulina*, Mt: *E. mitis*, Nc: *E. necatrix,* Tn: *E. tenella*, Pr: *E. praecox*, Br: *E. brunetti*, Mx: *E. maxima*
KDH: Kedah, PRK: Perak, SGR: Selangor, NSN: Negeri Sembilan, MLK: Melaka, PHG: Pahang

TABLE 2. Summary of *Eimeria* detection based upon oocyst morphological characterisation, PCR and LAMP assay results

Sample	Morphology			PCR-SCAR 1							PCR-SCAR 2							LAMP						
	AM	NTP	BM	Ac	Mt	Nc	Tn	Pr	Br	Mx	Ac	Mt	Nc	Tn	Pr	Br	Mx	Ac	Mt	Nc	Tn	Pr	Br	Mx
Control				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LB1	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
LB2	+	+	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-
SS1	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
SS2	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B1	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-
PP1	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
J1	+	+	+	-	-	-	+	+	-	+	+	+	-	+	+	+	+	-	-	-	-	+	-	+
J2	+	+	+	-	-	+	+	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-
SL1	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-
K1	+	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-
K2	+	+	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-	-
K3	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
SB1	+	+	+	-	-	-	+	-	-	+	+	+	-	+	-	-	-	+	-	-	-	-	-	-
SB2	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	-	-
M1	+	+	+	-	-	-	-	+	-	+	+	-	-	-	+	-	+	+	-	-	-	+	-	-
M2	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-	+	-	-
M3	+	+	+	+	-	-	-	+	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-
TB1	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
Total	18	16	14	8	1	1	4	4	0	4	16	4	1	6	5	3	3	12	0	0	0	3	0	2

Successful detection is indicated by (+) while no detection is indicated by (-). Control: Houghton strain
AM = Small oocysts (Ac, Mt), NTP = Medium oocysts (Nc, Tn, Pr), BM = Large oocysts (Br, Mx)
Ac: *E. acervulina*, Mt: *E. mitis*, Nc: *E. necatrix,* Tn: *E. tenella*, Pr: *E. praecox*, Br: *E. brunetti*, Mx: *E. maxima*

analysis (Notomi et al. 2000). When LAMP assays were carried out for the field samples, *Eimeria* species were found to be present in 15 out of 18 (83%) broiler farms. Just three species comprising *E. acervulina*, *E. praecox* and *E. maxima* were identified. Overall, the most prevalent species detected was *E. acervulina* (67%), followed by *E. praecox* (17%) and *E. maxima* (11%). *Eimeria brunetti*, *E. tenella, E. mitis* and *E. necatrix* were not detected in any of the 18 farms that were sampled. Out of the 15 farms that were found to be infected, 14 farms (93%) were found to host a single species, while mixed infection with two or more species were found in one farm (7%). The results of the geographical distribution shown that *E. acervulina* was also prevalent in all six states in Peninsular Malaysia as detected by PCR-SCAR 1 and PCR-SCAR 2 methods, while *E. praecox* was present in Pahang and Negeri Sembilan and *E. maxima* in Perak and Pahang.

The results obtained showed that the three molecular methods were capable of detecting *Eimeria* species of the reference Houghton strain. Analysis of the field isolates with all three molecular detection methods also demonstrated results comparable with morphological characterisation in that *E. acervulina* was identified as the most common species. However, these molecular methods showed a high level of variation in detection (Table 2). The PCR-SCAR 2 assay was able to detect all seven *Eimeria* species, as compared to PCR-SCAR 1 (six species) and LAMP (three species). PCR-SCAR 1 did not detect *E. brunetti* while four species, namely *E. brunetti*, *E. tenella, E. mitis* and *E. necatrix* were not detected by LAMP. Both PCR-SCAR 1 and PCR-SCAR 2 assays were performed using similar reagents with slightly different cycling conditions. The conditions were kept as close as possible to those described in the original publications to permit direct comparison. Thus, the difference in efficiency between the two PCR assays could be due to primer design - PCR-SCAR 2 was developed based on samples from four geographically distant regions (Vrba et al. 2010), while PCR-SCAR 1 was developed based on three geographical regions (Fernandez et al. 2003a, 2003b). A wider geographical coverage could provide higher efficiency in the development of PCR primers to detect local *Eimeria* populations and multiple infections. The LAMP assay detected fewer species when compared with both PCR assays. This is not surprising since the LAMP assay involves more primers, with a requirement for greater target sequence similarity, which increases the likelihood of it being affected by sequence divergence between strains. Thus, the efficiency differences observed between the LAMP and the two PCR assays in detecting local *Eimeria* populations may be due, at least in part, to sequence variation in primer annealing sites. The results of this study also suggest that primers from published sources were not able to detect the presence of *Eimeria* species in all of the samples, despite positive confirmation by microscopic inspection. This is likely due to genetic diversity that is demonstrated by *Eimeria* species from different geographical regions. Such diversity may have been intra-species specific (i.e. mutations located within primer annealing sites). Alternatively, it may be that oocysts of the cryptic operational taxonomic units (OTUs) x, y and/or z were recovered from these samples (Clark et al. 2016). Recent studies with DNA extracted from Nigerian OTU samples have suggested that molecular diagnostic tools designed to target the seven recognised *Eimeria* species that infect chickens may not be capable of detecting these cryptic parasites (Jatau et al. 2016).

CONCLUSION

Current available molecular methods may not be sufficient for the specific detection of local *Eimeria* species. Primer design at conserved regions of the target gene sequences should include strains from other geographical regions that were not considered in the original studies including Malaysian isolates. Thus, development of methods based on more sensitive primers using local *Eimeria* strains is required for improved detection of avian coccidiosis in local farms.

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