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[BIO38] Establishment and characterisation of local clonal *Eimeria tenella* populations

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Introduction

Eimeria is an obligate intracellular parasite which invades the intestinal tract of fowl and causes coccidiosis. There are seven known species of avian *Eimeria*: *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox* and *E. tenella* (Williams, 1996). For identification of the avian *Eimeria* species, it is a common practice to use morphological and pathological features such as oocyst shape and size, pre-patent period, sporulation time and intestinal site of infection (Joyner and Long, 1974).

At molecular level, several polymerase chain reaction (PCR) assays have been developed in order to discriminate the *Eimeria* spp. such as using 5S rRNA (Stucki et al., 1993), small subunit rRNA (Tsuji et al., 1997), ribosomal transcribed spacer 1 (ITS-1) (Schnitzler et al., 1998, 1999), and ITS-2 (Woods et al., 2000). Fernandez et al. (2003b) converted randomly amplified polymorphic DNA (RAPD) fragments (Fernandez et al., 2003a) into sequence-characterised amplified region (SCAR) markers and utilised them as a tool for molecular diagnosis.

The aim of this study is to isolate *Eimeria tenella* from a local jungle fowl and establish clonal populations from single sporocysts. Preliminary identification of the *Eimeria* species is carried out based on morphological and pathological features, and determination of the species is performed by using a molecular approach.

Materials and methods

Parasite isolation

Eimeria oocysts were isolated from an intestinal tract of a jungle fowl courtesy of Dr. Shaikh Amin Babjee (Universiti Putra Malaysia). The isolation was done according to Shirley (1995).

Parasite propagation

Parasites were given orally to 3- to 4-week old specific pathogen-free (SPF) White Leghorn chickens (Veterinary Research Institute, Ipoh) in doses as recommended by Shirley (1995). Oocysts collection, purification and sporulation were carried out using the procedures described by Shirley (1995). Subsequent passages of the parasites were done following the above method to maintain the population.

Single sporocyst propagation

Propagation using a single sporocyst was done according to the method described by Shirley and Harvey (1996). Up to 5.0 X 10^4 oocysts were broken with glass beads of 0.4-0.6 mm in diameter range and left to cool on ice. The released sporocysts were purified by passage through a short (2-3 cm) column of smaller glass beads of 0.15-0.25 mm in diameter. The column was prepared using a 1 ml plastic syringe with the end of the barrel plugged with glass wool.

Enumeration of purified sporocysts was done and an aliquot containing 250 sporocysts/ml was prepared. Three drops of 3 µl of the aliquot was placed onto alcohol-cleaned microscope slide and examined under microscope. Only drops with a single sporocyst was added with 20 µl of warm 2% low-melting point agarose-phosphate buffer saline (PBS) pH 7.6, and allowed to set. The agarose button was later slid onto a piece of parafilm and given orally to a 3- or 5-week old chicken. Seven days later, the bird was killed and the content of the caeca was examined for oocysts. Any oocysts found were allowed to sporulate and later passaged in SPF chickens to amplify the number for DNA extraction.

Oocysts morphology

Oocysts were measured with calibrated eyepiece micrometer under a 40X objective

lens and images were captured using a Nikon E4500 digital camera. *E. tenella* H strain given are average values of 100 oocysts.

DNA extraction

DNA extraction was done using the method described by Fernandez et al. (2003b) with slight modifications. Around 1 X 10^5 oocysts that have been surface-cleaned with sodium hypochlorite solution (4-6% active chlorine) on ice for 10 min were washed three times with distilled water. The oocysts were later resuspended in extraction buffer (10 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0), and were then completely broken down by adding 0.4-0.6 mm glass beads to the suspension (1:1 ratio) and vortexing the mixture, before centrifugation at 1,000 rpm for 5 min. The lysate was then digested with RNAse A (20 µg/ml) at 37°C for 1 hour, and later treated with SDS (0.5%) and proteinase K (100 µg/ml) at 50°C for 2 hours. The DNA was then extracted with one volume of phenol, followed by extraction with one volume of phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol. DNA precipitation was carried out by adding ammonium acetate (2.5 M), 10 mg glycogen and 100% ethanol. The pellet was washed with 70% ethanol and resuspended in sterile deionised dH_2O. DNA quantification was performed by using agarose gel electrophoresis.

Species identification using molecular approach

Multiplex PCR using SCAR primers as described by Fernandez et al. (2003b) was carried out for species identification in the parasite populations. As a positive control, DNA from the H strain was used. The sequence of SCAR primers used in the PCR and amplicon size generated by each primer pairs are shown in Table 1.

The multiplex amplification was performed by using 200 µM dNTPs, 2.4 mM $MgCl_2$, 5U *Taq* DNA polymerase (Promega, USA), 1.6X PCR buffer and 10 ng of *Eimeria* DNA in a final volume of 35 µl. Different primer concentrations were used as recommended by Fernandez et al. (2003b): 0.85 µM for Br-01 pair; 0.70 µM for Ac-01, Pr-01 and Nc-01 pairs; and 0.55 µM for Tn-01, Mt-01 and Mx-01pairs. Thermal cycling oocysts (the reference strain) were measured with the same technique. The dimensions conditions consisted of 5 min at 96°C as initial denaturation, and 30 cycles of 94°C for 1 min, 65°C for 2 min and 72°C for 2 min, with final extension at 72°C for 7 min. The reactions were performed in a PTC-100/96V thermocycler (MJ Research, USA). The amplification products were analysed by using agarose gel electrophoresis.

Results

Isolation of* Eimeria *species and passages in SPF chicken

Oocysts were successfully recovered from the caeca of the jungle fowl and passaged into a 4-week old SPF chicken. Seven days after infection, the bird was killed and the caeca were examined. The caeca exhibited blood clots and were enlarged, indicating a preliminary diagnosis that the infection was caused by *E. tenella*. Subsequent passages were done by using doses ranged from 1 X 10^3 to 2 X 10^3 and harvested only from the caeca (data not shown). Blood clots and enlarged caeca were observed in birds in all passages. The population (named as AH1 strain) was maintained up to nine passages and stored at 4°C in 2.5% potassium dichromate.

Identification of species by morphology

The mean dimension of 100 oocysts from AH1 strain was measured and compared with H strain (Table 2). Oocysts of the AH1 strain are ovoid in shape with a mean dimension of 22.5 µm (±0.17) x 17.3 µm (±0.14). For *E. tenella* H strain, oocysts were ovoid in shape with a mean dimension of 22.3 µm (±0.17) x 19.1 µm (±0.19) (Figure 1).

Establishment of clonal population

Two attempts were carried out to initiate clonal populations from single sporocysts. The first attempt was carried out with two 3-week old SPF chickens and the second attempt was done using six 5-week old SPF chickens. Each propagation attempt was carried out for 7 days after which ceaca infection and content were examined.

Oocysts were successfully recovered from a bird from each attempt, and the resulting

clonal populations were named as UKM1A and UKM1B strains respectively.

TABLE 1 Sequences of sequence-characterised amplified region (SCAR) primers used in the multiplex PCR assay for discrimination of *Eimeria* species.

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

TABLE 2 Mean dimensions of *E. tenella* strains: H, AH1 and clonal populations UKM1A and UKM1B.

Strain	Shape	Length (μm)	Mean length (μm)	Width (μm)	Mean width (μm)
H	Ovoid	19.2 – 26.4	22.3 (±0.17)	16.8 – 21.6	19.1 (±0.19)
AH1	Ovoid	19.2 – 26.4	22.5 (±0.17)	14.4 – 19.2	17.3 (±0.14)
UKM1A	Ovoid	19.2 – 26.4	22.9 (±0.15)	14.4 – 19.2	17.8 (±0.15)
UKM1B	Ovoid	19.2 – 26.4	23.0 (±0.21)	14.4 – 21.6	18.1 (±0.17)

Approximately 2.6 X 10^4 oocysts were recovered for UKM1A while 1.4 X 10^4 oocysts for UKM1B. Both strains were maintained by passage in SPF chickens.

Oocysts morphology of UKM1A and UKM1B strains

About 100 oocysts from both strains were used for measurement and the mean dimension were calculated (Table 2). Oocysts from the UKM1A strain are ovoid in shape with mean dimension of 22.9 μm (±0.15) x 17.3 μm (±0.15). The oocysts of the UKM1B strain were ovoid in shape with a mean dimension of 23.0 μm (±0.21) x 18.1μm (±0.17).

Confirmation of species with multiplex PCR

Genomic DNA extracted from strains of H, AH1 and clonal populations UKM1A and UKM1B were used as template in multiplex PCR assays. The PCR products for all samples revealed a fragment of 539 bp in length which indicated the presence of *E. tenella*. No other amplicons were observed from the amplification (Figure 2).

Discussion

Jungle fowls are regarded as the ancestor of domestic fowls (Stevens, 1991), and several *Eimeria* spp. isolated from local jungle fowls have been previously described (Long, 1974; Zainal Abidin, 1995). In this study, *Eimeria* oocysts were isolated from the jungle fowl, and when they were passaged in SPF chickens, classical symptoms of caecal

coccidiosis was observed - enlarged caeca containing blood clots. Since these symptoms are commonly associated with infection of *E. tenella*, a series of passages was done where oocysts were recovered only from the caeca at 7 days post-infection as a selective method to obtain *E. tenella*-enriched populations.

For genetic studies where homogenicity of the genome is crucial, a cloned population should ideally be used. Chapman and Rose (1986) developed a method to achieve this purpose by using single sporozoites. However, Shirley and Harvey (1996) showed that the two sporozoites within a sporocyst are genetically identical, thus a clonal population could be established from the propagation of a single sporocyst.

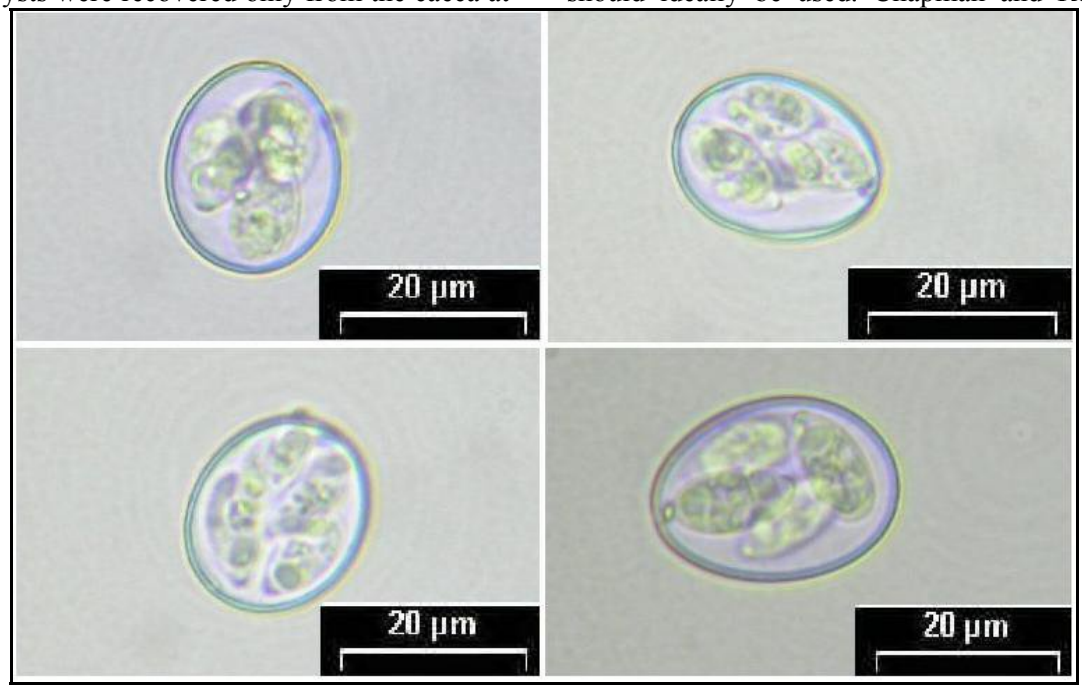

FIGURE 1 Photomicrograph of *E. tenella* (a) H strain, (b) AH1 strain, (c) UKM1A strain and (d) UKM1B strain oocysts under 400X magnification.

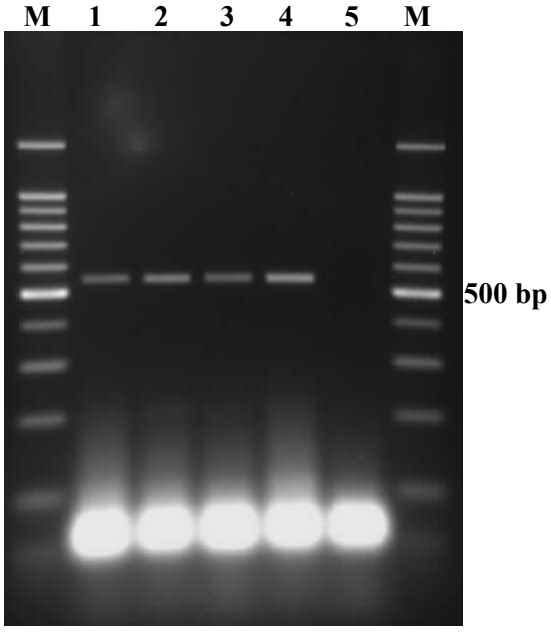

FIGURE 2 The amplification products of multiplex PCR using DNA samples from strains of *E. tenella*: H (lane 1), AH1 (lane 2), and clonal populations UKM1A (lane 3) and UKM1B (Lane 4). Lane 5 is negative control (no DNA) while lane M is 100 bp DNA ladder used as standard markers.

In our attempt to establish clonal populations from single sporocysts, two out of eight birds gave positive results, demonstrating that the technique is reliable. However, the number of oocysts recovered was found to be varied. Several factors could contribute to this variation such as susceptibility of the individual hosts, the discharge of oocysts from the caeca just before the birds were killed, infection from both sporozoites or just one, as well as loss of merozoites from the gut (Shirley and Harvey, 1996).

Characterisation of both clonal populations was carried out utilising a molecular approach based on a recently developed PCR method (Fernandez et al., 2003b). The multiplex PCR assay showed that

a single fragment specific for *E. tenella* was amplified from both clonal populations. This result, in addition to morphological and pathological descriptions, indicated that clonal populations of *E. tenella* from a local jungle fowl were successfully established in this study.

Acknowledgements

The authors wished to thank the Ministry of Science, Technology and Innovation (MOSTI), Malaysia for the National Science Fellowship awarded to Norjoanna Johan. The authors are grateful to Veterinary Research Institute, Ipoh for the supply of SPF chicken and Shaikh Amin Babjee (Universiti Putra Malaysia) for the supply of intestinal tract of jungle fowl.

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