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Molecular diagnosis of *Eimeria* species affecting naturally infected *Gallus gallus*

F.S. Carvalho[1], A.A. Wenceslau[1], M. Teixeira[2] and G.R. Albuquerque[1]

[1]Programa de Pós-Graduação em Ciência Animal,
Departamento de Ciências Agrárias e Ambientais,
Universidade Estadual de Santa Cruz, Campus Universitário, Ilhéus, BA, Brasil
[2]Embrapa Caprinos e Ovinos, Sobral, CE, Brasil

Corresponding author: A.A. Wenceslau
E-mail: wenceslau@uesc.br

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ABSTRACT. We used PCR to test various protocols and define a technique for DNA extraction directly from chicken-shed stool samples for the identification of *Eimeria* species that parasitize birds. It was possible to extract and amplify DNA of seven *Eimeria* species from field stool samples, using both protocols tested; extractions made with phenol/chloroform protocols gave the best results. The primers were specific and sensitive, allowing amplification of samples containing as few as 20 oocysts, both in individual and in a multiplex PCR. Individualized PCR with the phenol/chloroform DNA extraction protocol detected a larger number of *Eimeria* species. Molecular diagnosis was found to be practical and precise, and can be used for monitoring and epidemiological studies of *Eimeria*.

Key words: Eimeriosis; Multiplex PCR; Broilers

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INTRODUCTION

Coccidiosis is an important parasitic disease of poultry, especially in confined birds. It is caused by protozoa of the genus *Eimeria*, which includes seven species: *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, *and E. tenella*. Infections - usually mixed - occur due to ingestion of oocysts (Fayer, 1980). These infections lead to digestive disorders resulting from damage to the intestinal epithelium, malabsorption of nutrients, changes in protein metabolism after absorption, reduced efficiency of feed conversion, and reduction in weight gain (Conway et al., 1993; Shirley et al., 2005). Mortality and economic losses, especially in cases of outbreaks, are frequent (Morris and Gasser, 2006).

The development of molecular techniques has allowed precise diagnosis of *Eimeria* species, investigation of the genetic variability of these pathogens, and a search for molecular characteristics associated with phenotypical characteristics that may constitute the use of molecular markers (Schnitzler et al., 1998; Costa et al., 2001). Molecular techniques may also contribute to the development of new vaccines and selection of anticoccidial drugs to be used in control programs (Morris and Gasser, 2006; Sun et al., 2009; Lee et al., 2010).

Analysis of internal transcribed spacers (ITS-1 and ITS-2) permits discrimination of all species of the genus *Eimeria*, using polymerase chain reaction (PCR) or real-time PCR (Tsuji et al., 1997; Woods et al., 2000; Fernandez et al., 2003a; Morris and Gasser, 2006; Kawahara et al., 2008). Another marker that has also been used is the sequence-characterized amplified region, which was proposed by Fernandez et al. (2003b); it allows distinguishing *Eimeria* species in individual or in multiplex PCR. Primers that amplify fragments under the same annealing temperature are used.

For successful identification, it is necessary to have an efficient procedure for DNA extraction from oocysts; protocols with phenol/chloroform and commercial extraction kits are commonly used to break the rigid oocyst wall (Fernandez et al., 2003b; Meireles et al., 2004; Haug et al., 2007).

We used PCR to evaluate several protocols and in order to develop a technique for DNA extraction directly from stool samples of broilers, for the identification of *Eimeria* species.

MATERIAL AND METHODS

Sample collection was carried out on poultry farms in the microregion of Feira de Santana, Bahia, Brazil, located at latitude 12° 26'S and longitude 38° 96'W. The climate in this region is sub-humid tropical, with rainfall ranging from annual 848 to 1200 mm; the mean temperature is 24°C, and mean relative humidity ranges from 70 and 75%.

Chickens 3-6 weeks old were sampled from 30 broiler farms administered by companies in this region, as along with chickens reared by independent producers.

Oocysts were isolated from fresh stool samples collected from several sheds of each property. Four handfuls of feces were collected every meter, along the length of each poultry shed. Samples of each house were packed together in plastic bottle with screw cap, homogenized by shaking for 10 min and a sub-sample of approximately 200 g was selected. Then, all samples from each shed were mixed and homogenized and a single 200-g sample representing the entire property was selected. The material was packed in plastic bags, placed under refrigeration, identified and brought to the laboratory for processing.

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Laboratory processing

The samples were initially filtered using sieves covered with folded gauze and centrifuged at 250 *g*. After that, all the material was suspended in a solution of 2.5% potassium dichromate ($K_2Cr_2O_7$) on Petri dishes to sporulate at room temperature for seven days (Duszynski and Wilber, 1997). The oocysts were recovered by centrifugation in a saturated aqueous solution of NaCl, followed by washing with distilled water. Finally, the material was concentrated using centrifugation and stored in potassium dichromate solution, concentration measured in a Neubauer chamber, and after that, stored at 4°C for later analysis.

Comparison of two protocols for DNA extraction

DNA from 11 samples was extracted with phenol/chloroform (Fernandez et al., 2003b) or with a commercial Kit DNAzol (Invitrogen®), in order to define which is the most suitable and thereafter apply to all reactions.

Extraction with phenol/chloroform

Six milliliters of the samples was twice washed using distilled water and centrifuged for 10 min at 14,000 *g* for the removal of potassium dichromate. Subsequently, the pellet was washed with a 5% solution of sodium hypochlorite and was held at 4°C for 10 min, followed by two washes with distilled water. The pellet was eluted with TE (10 mM Tris-HCl, pH 8.0; 200 mM EDTA, pH 8.0) buffer. In order to rupture the walls of the oocysts, 0.350 g 425-600-µM glass beads (Sigma Aldrich Corp.) was added to each tube, followed by vortex stirring for 5 min and centrifugation at 11,500 *g* for 5 min for the removal of cell residues. The beads were washed again with TE buffer, stirred and centrifuged. The volume was transferred into a new tube. Digestion with RNAse A (20 µg/mL) was then conducted at 37°C for 1 h, followed by digestion with proteinase K (120 µg/mL) plus SDS (0.5%) at 50°C for 1 h. DNA was extracted with phenol/chloroform/isoamyl alcohol and chloroform, and was precipitated with 1/10 ammonium acetate in ethanol. The pellet was washed with 85% ethanol and suspended in 10 mM Tris-HCl, pH 8.0, and DNA was quantified using a spectrophotometer with absorbance at 260 and 280 nm.

Extraction using a commercial kit

Six milliliters of the purified material was centrifuged for 10 min at 14,000 *g* and twice washed with distilled water for the extraction of potassium dichromate. Subsequently, the pellet was washed with a 5% solution of sodium hypochlorite and kept at 4°C for 10 min, followed by two washes with distilled water. The pellet was eluted in 350 µL of the extraction kit solution and homogenized for 1 min. To rupture the walls of the oocysts, 0.350 g 425-600-µm glass beads was added to the tubes; the procedures described in the previous protocol were followed. The beads were washed again with 150 µL of the extraction kit solution, stirred and again centrifuged. The volume was transferred to a new tube and 500 µL of the extraction kit plus RNAse A (20 g/mL) was added and conserved at 37°C for 1 h. The tubes were afterwards centrifuged at 10,000 *g* for 5 min and the supernatant transferred. DNA was precipitated with 100% ethanol and centrifuged for 10 min at 10,000 *g*, discarding the supernatant with a pi-

pette; approximately 150 µL remained in the tube. The DNA was precipitated again with 85% ethanol and centrifuged at 14,000 *g* for 10 min. The pellet was eluted in TE (10 mM Tris-HCl, pH 8.0) and quantified in a spectrophotometer using absorbance at 260 and 280 nm.

Polymerase chain reaction

DNA samples extracted by the two previously described protocols were submitted to PCR. The PCR amplifications were conducted separately for each primer pair (Table 1); 200 µM dNTP, 5.0 mM $MgCl_2$, 2 U Taq DNA Polymerase (Invitrogen®), and 1.6X amplification buffer (supplied by the manufacturer) in a final volume of 25 µL were used (Fernandez et al., 2003b).

Table 1. Relationship of primers and their respective concentrations used at PCR.

Specimens	Concentration	Primer sequence
Eimeria acervulina	0.70 µM	F- AGTCAGCCACACAATAATGGCAAACATG R- AGTCAGCCACAGCGAAAGACGTATGTG
E. brunetti	0.85 µM	F- TGGTCGCAGAACCTACAGGGCTGT R- TGGTCGCAGACGTATATTAGGGGTCTG
E. tenella	0.55 µM	F- CCGCCCAAACCAGGTGTCACG R- CCGCCCAAACATGCAAGATGGC
E. mitis	0.55 µM	F- AGTCAGCCACCAGTAGAGCCAATATTT R- AGTCAGCCACAAACAAATTCAAACTCTAC
E. praecox	0.70 µM	F- AGTCAGCCACCACCAAATAGAACCTTGG R- GCCTGCTTACTACAAACTTGCAAGCCCT
E. maxima	0.55 µM	F- GGGTAACGCCAACTGCCGGGTATG R- AGCAAACCGTAAAGGCCGAAGTCCTAGA
E. necatrix	0.70 µM	F- TTCATTTCGCTTAACAATATTTGGCCTCA R- ACAACGCCTCATAACCCCAAGAAATTTTG

F = foward primer; R = reverse primer.

Thermocycling conditions for reactions were 30 cycles, with an annealing temperature of 65°C for 2 min. All amplification products were analyzed by separation on 3% agarose gel, followed by staining with ethidium bromide and examined under UV light. Two positive controls were used - one composed of pure DNA samples isolated from the seven *Eimeria* species conceded by Biovet Laboratory, and the other was the commercial vaccine Bio-Coccivet R® (Biovet laboratory).

Identification of species of the *Eimeria* genus found in the 30 farms

Three techniques were tested - an individual PCR with a pair of primers for each *Eimeria* species, a multiplex PCR with four pairs of primers in one tube and three primers in another tube, and a multiplex PCR with all primers in the same reaction. DNA samples were extracted with phenol/chloroform and the individual amplifications were run using previously described individual conditions.

Amplifications were conducted separately in two tubes in the multiplex PCR - tube 1 containing the primers for *E. acervulina*, *E. brunetti*, *E. mitis*, and *E. maxima*, while tube 2 contained primers for *E. tenella*, *E. praecox*, and *E. necatrix*. The concentrations of reagents also varied depending on the final volume of the reaction; 320 µM dNTP, 5.0 mM $MgCl_2$, 4 U Taq DNA polymerase (Invitrogen®), and 1.6X amplification buffer (supplied by the manufacturer) in a final volume of 30 µL in tube 1 and 25 µL in tube 2.

In one of the tubes of the PCR multiplex, the final reaction volumes ranged between 35 and 60 µL; whereas different reagent and DNA concentrations proportional to each volume tested were used (Fernandez et al., 2003b).

Statistical analysis

The results were analyzed by the Wilcoxon test, with a significance level of 5% using the StatPlus® program (2009).

RESULTS

Experiment 1

We were successful at extracting and amplifying DNA from the seven *Eimeria* species in field samples, using all protocols. The mean DNA concentrations were 880 and 454 ng/µL for extractions with phenol/chloroform and the commercial kit, respectively. Purity identified through the ratio of DNA/RNA concentrations was also higher in samples extracted with phenol/chloroform. The protocol using phenol/chloroform was the most accurate and allowed identifying a greater number of *Eimeria* species in comparison with the extractions performed using the commercial kit (Table 2).

Table 2. Comparison of protocols for DNA extraction from oocysts of the *Eimeria* genus affecting broilers.

Samples	Commercial Kit							Phenol/chloroform						
	Ac	Br	Te	Mi	Pr	Ma	Ne	Ac	Br	Te	Mi	Pr	Ma	Ne
1			+	+	+	+	+			+	+	+	+	+
2				+	+	+	+				+	+	+	+
3				+		+	+	+		+	+	+	+	+
4				+	+	+	+	+		+	+	+	+	+
5			+	+	+	+	+			+	+	+	+	+
6					+			+		+	+	+	+	+
7			+	+	+	+	+	+		+	+	+	+	+
8	+		+	+	+	+	+	+		+	+	+	+	+
9						+	+			+	+	+	+	+
10	+			+	+	+	+	+		+	+	+	+	+
11			+	+	+	+	+			+	+	+	+	+

Ac = *E. acervulina*; Br = *E. brunetti*; Te = *E. tenella*; Mi = *E. mitis*; Pr = *E. praecox*; Ma = *E. maxima*; Ne = *E. necatrix*.

Experiment 2

PCRs were run for samples containing more than 20 oocysts per species in single and multiplex reactions divided into two tubes. The multiplex PCR with seven pairs of primers did not work for the field samples. It was possible to carry out amplifications using up to four pairs of primers in a single reaction (Figure 1).

The PCR by species (Figure 2) - using the phenol/chloroform extraction protocol was significantly ($P < 0.05$) more precise, allowing the identification of a greater number of *Eimeria* species compared to multiplex PCR in two tubes (Table 3). The primers used to amplify the different *Eimeria* species were specific and amplified the previously defined sequences.

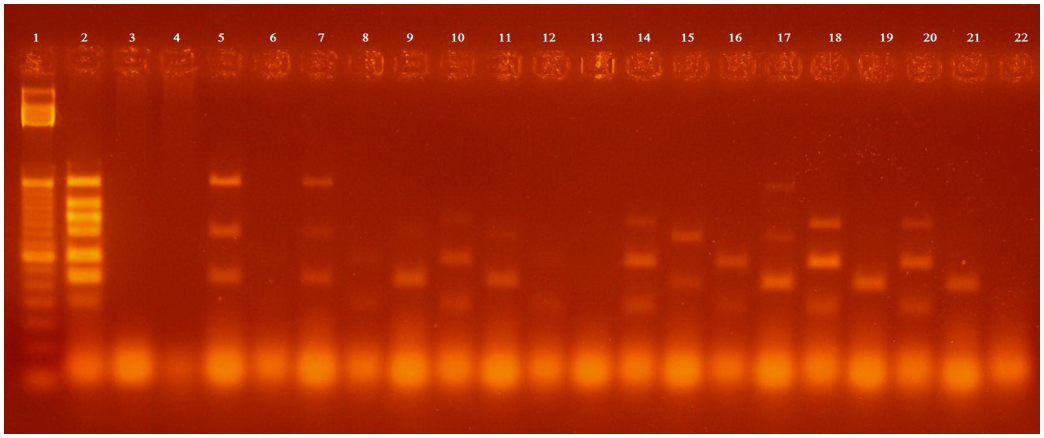

Figure 1. Resolution on 3% agarose gel showing amplification of the seven species of the genus *Eimeria*, conducted in multiplex PCR, in two tubes. *Lane 1* = 50-bp molecular weight marker; *lane 2* = positive control; *lanes 3*, *4*, *6*, and *13* = negative samples; *lane 22* = negative control; *lanes 5*, *7*, *9*, *11*, *15*, *17*, and *19* = samples positive for multiple species - multiplex tube 1 with four pairs of primers; *lanes 8*, *10*, *12*, *14*, *16*, *18*, *20*, and *21* = samples positive for multiple species - multiplex tube 2 with three pairs of primers.

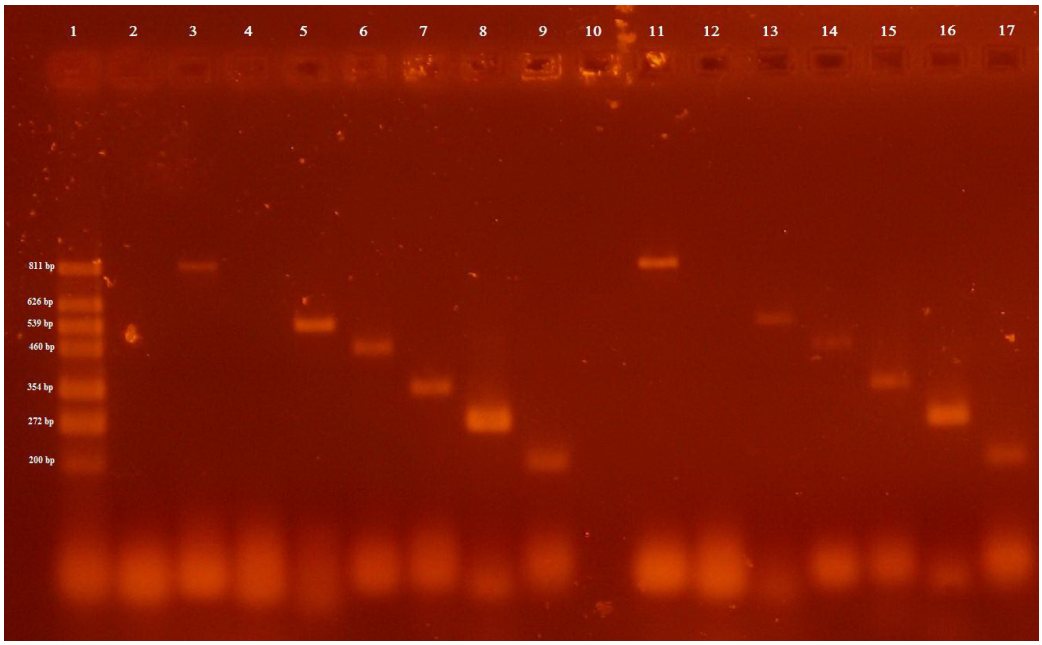

Figure 2. Resolution on 3% agarose gel showing amplification of the seven species of the genus *Eimeria*. *Lane 1* = positive control; *lane 2* = negative control; *lanes 4* and *12* = negative samples; *lane 10* = blank; *lanes 3*, *5*, *6*, *7*, *8*, *9*, and *11* = positive samples for the various *Eimeria* species.

Table 3. Diagnosis and differentiation of seven *Eimeria* species in 30 poultry farms, using the three PCR protocols.

Samples	Individual PCR							Multiplex PCR in two tubes							Multiplex PCR*						
	Ac	Br	Te	Mi	Pr	Ma	Ne	Ac	Br	Te	Mi	Pr	Ma	Ne	Ac	Br	Te	Mi	Pr	Ma	Ne
1			+	+	+	+	+			+	+	+	+	+							
2				+	+	+	+				+	+	+	+							
3	+		+	+	+	+	+				+	+	+	+							
4	+			+	+	+	+				+	+	+	+							
5				+	+	+	+				+	+	+	+							
6	+		+	+	+	+	+			+	+	+	+	+							
7	+			+	+	+															
8	+	+	+	+	+	+	+			+	+	+	+	+							
9	+		+	+	+	+	+	+			+	+	+	+							
10			+	+	+	+	+				+		+	+							
11	+	+	+	+	+	+	+	+			+	+	+	+							
12			+	+	+	+	+				+		+								
13	+		+	+	+	+	+	+			+	+	+	+							
14			+	+	+	+	+			+	+	+	+	+							
15	+	+	+	+	+	+	+						+	+							
16	+			+	+	+	+	+			+	+	+	+							
17			+		+	+															
18	+		+	+	+	+	+	+		+	+	+	+	+							
19	+		+	+	+	+	+	+		+	+		+	+							
20		+		+	+	+	+		+		+		+	+							
21	+		+	+	+	+	+	+			+	+	+	+							
22			+	+	+	+	+					+	+	+							
23	+		+	+	+	+	+	+		+	+	+	+	+							
24	+		+	+	+	+	+				+	+	+	+							
25	+	+	+	+	+	+	+	+	+	+	+	+	+	+							
26			+	+	+	+	+				+	+	+								
27	+		+	+	+	+	+	+	+	+	+	+	+	+							
28					+	+	+					+	+	+							
29			+	+	+	+	+														
30	+		+	+	+	+	+	+		+	+	+	+	+							

Ac = *E. acervulina*; Br = *E. brunetti*; Te = *E. tenella*; Mi = *E. mitis*; Pr = *E. praecox*; Ma = *E. maxima*; Ne = *E. necatrix*.
*No amplification occurred.

DICUSSION

A comparison between the DNA extraction and PCR techniques was made in order to detect and distinguish seven *Eimeria* species that can parasitize broilers. It was possible to extract DNA from samples with both protocols; however, the phenol/chloroform protocol was more efficient when compared to the commercial kit, resulting in samples more concentrated, and pure DNA, and it was able to detect most *Eimeria* species (Table 3). It is likely that the number of washes and the characteristics of the reactants used in each protocol were decisive for the results. According to Sambrook and Fritsch (1989), DNA samples should show a ratio between DNA and RNA ranging from 1.7 to 2.0 in order to be considered free of contaminants. Thus, the protocol with phenol/chloroform was defined as the most suitable based on the analysis of 30 samples.

Using a commercial kit identical to that used in our study, Meireles et al. (2004) needed to purify DNA samples after the extraction procedure using the Prep-A Gene DNA purification system kit (Bio-Rad®) in order to obtain cleaner samples; also, at least 50 *Eimeria* oocysts were required to obtain good results in individualized PCR. These results show that contaminants in samples complicate the procedures of DNA and PCR extraction. The techniques for recovering oocysts from stool samples should be optimized in order to provide cleaner samples for DNA extraction.

The resistance of oocysts of *Eimeria* species, due to the outer oocyte wall, an important protective barrier, can also be a limiting factor for obtaining DNA. Glass beads were used to rupture the oocyst wall; this procedure was essential for the success of the extraction. The use of glass beads has been the most commonly used procedure for disruption of oocyst walls (Procunier et al., 1993; Molloy et al., 1998; Fernandez et al., 2003b). It is important to have the spheres of correct size, quantify the number of oocysts, take into account the contaminants in the sample and the time spent in disruption procedure (Haug et al., 2007). Other methods to rupture oocysts may also be employed, although the results are variable and large numbers of oocysts are often not affected. Oocysts are known to be highly resistant to chemical products and to some mechanical action (DiFabio, 1984; Tsuji et al., 1997; Zhao et al., 2001).

It was possible to detect the *Eimeria* species in the individual PCR and multiplex reactions in two tubes containing samples from 20 oocysts. The number of oocysts appears to be a limiting factor for identication through PCR. Lee et al. (2010) used 50 oocysts for individual amplifications. Fernandez et al. (2003b) found a minimum limit of 50 oocysts for detection of six species using the PCR multiplex, while the limit for *E. tenella* was found to be 100 oocysts both in individual and multiplex reactions. On the other hand, Haug et al. (2007) managed to amplify individual samples containing between 2 and 10 *E. tenella* oocysts, using phenol/chloroform for DNA extraction. In Japan, Kawahara et al. (2008) used an average of 100 oocysts for the identification of five species of the genus *Eimeria*.

The PCR multiplex used to analyze the seven species at the same time was worked only with pure and isolated DNA. Failure to identify the field samples was possibly related to low-final DNA concentration of *Eimeria* species, associated with possible inhibitors that remained along in the sample after the extraction process. As indicated by Haug et al. (2007), multiplex PCR is a technique that is more difficult to optimize, giving lower sensitivity and reproducibility. Furthermore, extraction of DNA from fecal samples may generate inhibitors of Taq DNA polymerase, negatively affecting the reactions. The results obtained with multiplex PCR in two tubes were probably possible due to a smaller dilution of the DNA sample and consequently fewer contaminants.

When the results of multiplex PCR in two tubes and the individual PCR were compared, an obvious higher efficiency in individual reactions was noted. The number of *Eimeria* species found in the individual reactions was significantly higher than those found in the multiplex PCR in two tubes (Table 3). These results probably have a direct relationship with the final DNA concentration of each species, as well as with contamination in individual reactions.

The primers were highly selective and only allowed the amplification of specific sequences of each species studied, even in samples contaminated with DNA from other microorganisms. Another important factor is the ability of amplification of different species using a single annealing temperature, thereby allowing analysis of a large number of samples at once, as well as permitting the use of multiplex PCR as also found by Fernandez et al. (2003b). Other primers for detection of *Eimeria* species are known, but they require different annealing temperatures, requiring that reactions be performed separately, requiring more time and reagents (Meireles et al., 2004; Prado, 2005; Haug et al., 2007).

Identification and diagnosis by PCR has allowed accurate monitoring of the *Eimeria* species affecting broilers (small quantities of oocysts). This is an important factor, considering that birds with a low parasite load, with no overt clinical signs, can be monitored in order to avoid worsening the condition. According to Conway et al. (1993), only 100 *E. acervulina* and

E. tenella oocysts are needed to cause serious gastrointestinal disorders in birds. Furthermore, molecular diagnosis can accurately reveal the prevalence of *Eimeria* species, thus contributing to the strategic employment of medicines and vaccines (Sun et al., 2009; Lee et al., 2010).

Molecular techniques such as multiplex PCR can be enhanced for fast and accurate diagnosis of *Eimeria* species in field samples.

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