Parasitology International 63 (2014) 527–532

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Detection of four important *Eimeria* species by multiplex PCR in a single assay

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ARTICLE INFO

Article history:
Received 13 August 2013
Received in revised form 24 January 2014
Accepted 25 January 2014
Available online 2 February 2014

Keywords:
Multiplex PCR
Detection
Eimeria

ABSTRACT

The oocysts of some of the recognized species of chicken coccidiosis are difficult to distinguish morphologically. Diagnostic laboratories are increasingly utilizing DNA-based technologies for the specific identification of *Eimeria* species. This study reports a multiplex polymerase chain reaction (PCR) assay based on internal transcribed spacer-1 (ITS-1) for the simultaneous diagnosis of the *Eimeria tenella*, *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria necatrix* species, which infect domestic fowl. Primer pairs specific to each species were designed in order to generate a ladder of amplification products ranging from 20 to 25 bp, and a common optimum annealing temperature for these species was determined to be 52.5 °C. Sensitivity tests were performed for each species, showing a detection threshold of 1–5 pg. All the species were amplified homogeneously, and a homogenous band ladder was observed, indicating that the assay permitted the simultaneous detection of all the species in a single-tube reaction. In the phylogenic study, there was a clear species clustering, which was irrespective of geographical location, for all the ITS-1 sequences used. This multiplex PCR assay represents a rapid and potential cost-effective diagnostic method for the detection of some key *Eimeria* species that infect domestic fowl.

1. Introduction

Coccidiosis induced by *Eimeria* species infection is an important parasitic disease of poultry [1]. It causes production losses, interruptions of feeding, digestion and nutrient absorption processes, blood loss, dehydration, and increased susceptibility to other disease agents, ultimately resulting in high morbidity and mortality rates [2,3]. Tissue damage and changes in intestinal tract function may allow colonization by various harmful bacteria, such as *Clostridium perfringens*, leading to necrotic enteritis [4,5] or *Salmonella typhimurium* [6,7].

Coccidiosis in chicken is caused by seven species of *Eimeria*: *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *Eimeria brunetti*, *Eimeria mitis*, and *Eimeria praecox* [8]. They all have different pathogenicity and parasitic sites, being active from the duodenum to the rectum, and concurrent infection with two or more species of coccidia is common [9].

Eimeria tenella, *E. acervulina*, *E. maxima*, and *E. necatrix* occur frequently in Korea [10]. Therefore, identification of these species in the infected chicken has important implications for disease management, as well as for studying the epidemiology and population biology.

Species identification has been classically based on observation of oocyst shape, size and color, prepatent period, location of the lesions in the intestine, sporulation time, and characteristics of the endogenous stages in the intestinal mucosa. Hence, coccidiosis can be best diagnosed through immediate necropsy, as attempts to identify characteristic lesions in birds that have been dead for one hour or longer are confounded by postmortem changes in intestinal mucosa. The entire intestinal tract should be examined microscopically for special diagnostic characteristics, such as clusters of the large schizonts of *E. necatrix*. However, oocyst size and shape are less useful diagnostic characteristics in chickens than once thought because of the extensive overlapping in size. Lesion Scoring [11], droppings Score [12] and histopathology methods (H & E or other common histologic stains) require highly-trained personnel and are also limited by further overlapping of characteristics among different species. In recent years, more emphasis has been placed on biochemical and physiologic identification, including electrophoresis of metabolic enzymes and PCR [13].

Recently, ribosomal internal transcribed spacer-1 (ITS-1) has been used to aid in coccidiosis diagnosis [14–18]. Eukaryotic cells contain ITS, a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. Read from 5′ to 3′, this polycistronic rRNA precursor transcript contains the 5′ external transcribed sequence (5′ ETS), 18S rRNA, ITS1, 5.8S, rRNA, ITS2, 26S rRNA, and finally the 3′ ETS.

The ITS region is widely used in molecular phylogeny and taxonomy because it is easy to amplify from small quantities of DNA and has a high degree of variation between closely related species [15,19,20]. The present study describes a cost-effective and simple multiplex PCR assay that permits the simultaneous discrimination of the four *Eimeria* species (*E. tenella*, *E. acervulina*, *E. maxima*, and *E. necatrix*) that infect domestic fowl in Korea.

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http://dx.doi.org/10.1016/j.parint.2014.01.006

Table 1
List of ITS-1 sequences.

No.	GenBank accession no.	Isolate ID	Origin
01	AF446061	*Eimeria maxima* isolate C	Australia
02	AF446060	*Eimeria maxima* isolate B	Australia
03	AF446059	*Eimeria maxima* isolate B	Australia
04	AF065095	*Eimeria maxima* Europe	Sweden
05	AF065094	*Eimeria maxima* Europe	Sweden
06	AF446056	*Eimeria acervulina* isolate C	Australia
07	AF446055	*Eimeria acervulina* isolate A	Australia
08	AF026384	*Eimeria acervulina* isolate M1	Sweden
09	FJ447467	*Eimeria acervulina* isolate Jeonju	Korea
10	FJ447468	*Eimeria tenella* isolate Jeonju	Korea
11	EU931579	*Eimeria tenella* isolate WB17	India
12	EU586057	*Eimeria tenella* isolate WB1	India
13	AF446075	*Eimeria tenella* isolate Houghton	United Kingdom
14	AF446074	*Eimeria tenella* isolate D	Australia
15	AF446070	*Eimeria necatrix* isolate C	Australia
16	AF446069	*Eimeria necatrix* isolate A	Australia
17	AF446068	*Eimeria necatrix* isolate A	Australia
18	AF446067	*Eimeria necatrix* isolate A	Australia
19	AF446066	*Eimeria necatrix* isolate A	Australia
20	AF026385	*Eimeria necatrix* isolate Ene-Sw1	Sweden

2. Materials and methods

2.1. Parasites

Twenty field strains comprising the four *Eimeria* species that infect domestic fowl were obtained from the Jeollabuk-do province in Korea. The correct species assignment and purity of all samples were confirmed by singular ITS-PCR. A commercial vaccine (Coccivac D, Schering-Plough Animal Health Corporation, US) was employed as a mixed sample.

2.2. Parasite propagation and purification

Parasites were propagated by passage in two-week-old Ross commercial chicks. The chicks were orally infected with 1×10^4 purified oocysts. Oocyst collection, purification and sporulation followed standard procedures.

2.3. DNA extraction

Oocysts were collected from excrement by the floating method (saturated solution of sugar). The oocysts were soaked in 2.5% potassium dichromate for five days and washed three times with distilled water. Sporulated oocysts were then fully disrupted by vortexing with half the volume of acid-washed glass beads [16–18]. The lysate was centrifuged at 3000 rpm for 10 min to eliminate debris and was then digested with an extraction buffer (0.1 M Tris–HCl, pH 8.0; 1% SDS; 0.1 M NaCl; 1 mM EDTA), protein kinase (10 mg/ml) and distilled water at 37 °C overnight in a hybridization incubator. The DNA was then extracted with phenol:chloroform:isoamyl alcohol (25:24:1) (Fluka, Sigma-Aldrich, US), vortexing for 10 min and centrifuging at 13,000 rpm for 10 min. The upper solution had ethanol added to it (99.5%, Wako, Japan) as well as sterilized sodium chloride to eliminate debris at −20 °C for 1 h. The lysate was then centrifuged at 4 °C and 13,000 rpm for 10 min, and the pellet was washed with 70% ethanol.

2.4. Primer design

Primers were designed based on an alignment of 20 Eimeria species ITS-1 sequences downloaded from Genbank (Table 1); however, the *E. necatrix* reverse primer was based on a known primer [16]. Primers were screened against all *Eimeria* species sequences, which were annotated in the GenBank database, to detect annealing at other sites and to avoid the amplification of undesirable products. Table 2 lists the primers that were used for the multiplex-PCR assay as well as their respective sequences. Primer length varied from 20 to 25 bases.

2.5. PCR tested for individual reactions of each primer pair

Standard PCRs were initially tested for individual reactions of each primer pair in order to obtain a common reaction condition for the four *Eimeria* species. Each PCR was performed in a final volume of 20 μl containing 10 μl of PCR Premix (EmeraldAmp™ PCR Master Mix, Takara, Japan), 20 μM of forward primer and 20 μM of reverse primer (Standard Oligo, Bioneer, Korea), 3 μl of DNA (0.1–1.0 μg, diluted with TE buffer), and 3 μl of distilled water. The PCR program consisted of a denaturation step at 95 °C for 10 min followed by 30 cycles of 98 °C for 10 s, 52.5 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 1 min. Next, 3 μl of PCR product was then visualized in a 2% agarose gel (UltraPure™ Agarose product, Invitrogen, US) by electrophoresis.

2.6. Multiplex PCR

The primer mix of the multiplex PCR assay contained the four primers specific for *E. tenella*, *E. acervulina*, *E. maxima*, and *E. necatrix*. PCR was performed in a final volume of 20 μl containing 10 μl of PCR Premix (EmeraldAmp™ PCR Master Mix, Takara, Japan), 30 μM of forward primer and 15 μM of each reverse primer (Standard Oligo, Bioneer, Korea), and 1 μl of DNA (0.1–1.0 μg, diluted with TE buffer). The PCR program consisted of a denaturation step at 95 °C for 10 min followed by 30 cycles of 98 °C for 10 s, 52.5 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 1 min. Finally, 3 μl of the PCR product was then visualized in a 2% agarose gel (UltraPure™ Agarose product, Invitrogen, US) by electrophoresis.

2.7. Sensitivity and specificity testing

For sensitivity and specificity testing, all isolates were initially identified using conventional methods, such as oocyst size, pre-patent period and lesion mapping, and then confirmed by singular ITS-PCR from feces. For each *Eimeria* species, individual chicks were selected for artificial infection. The samples were extracted and analyzed in parallel.

After 120 h post-infection, fecal samples were collected and oocysts were isolated using the flotation technique. Briefly, chick droppings were mixed in water (1:2 W/V) and filtered by mesh (~1 mm). The suspension was then centrifuged at 1000 g for 10 min. After removing the supernatant, the pellets were re-suspended with 1.27 specific gravity

Table 2
Primers design identification.

Primer	Oligonucleotide sequence(5′-3′)	Amplicon size(bp)	Specificity	Accession no.	Reference
Forward	GTTGCGTAAATAGAGCCCTCT		*Eimeria* spp.		This study
Reverse	ACCAATGCAGAACGCTCCAG	152 bp	*E. maxima*	AF065094	This study
	CAAAAGGTGGCAATGATGCT	281 bp	*E. acervulina*	AF446055	This study
	GATCAGTCTCATCATAATTCTCGCG	450 bp	*E. necatrix*	AF026385	Haug, A. et al.(2007)
	GTTCCAAGCAGCATGTAACG	554 bp	*E. tenella*	AF446074	This study