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Research Brief

Molecular analysis of single oocyst of *Eimeria* by whole genome amplification (WGA) based nested PCR

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HIGHLIGHTS

- Whole genome amplification (WGA) is useful for molecular analysis of *Eimeria*.
- WGA-based nested PCR can be applied to single-oocyst amplification of *Eimeria*.
- WGA-based nested PCR can be used for species identification at the single oocyst level.

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ABSTRACT

PCR-based molecular tools are widely used for the identification and characterization of protozoa. Here we report the molecular analysis of *Eimeria* species using combined methods of whole genome amplification (WGA) and nested PCR. Single oocyst of *Eimeria stiedai* or *Eimeria media* was directly used for random amplification of the genomic DNA with either primer extension preamplification (PEP) or multiple displacement amplification (MDA), and then the WGA product was used as template in nested PCR with species-specific primers for ITS-1, 18S rDNA and 23S rDNA of *E. stiedai* and *E. media*. WGA-based PCR was successful for the amplification of these genes from single oocyst. For the species identification of single oocyst isolated from mixed *E. stiedai* or *E. media*, the results from WGA-based PCR were exactly in accordance with those from morphological identification, suggesting the availability of this method in molecular analysis of eimerian parasites at the single oocyst level. WGA-based PCR method can also be applied for the identification and genetic characterization of other protists.

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Eimeria spp. are protozoan pathogens responsible for coccidiosis in poultry and livestock, causing significant economic losses (Shirley et al., 2005). PCR-based molecular tools have been widely used in the diagnosis and characterization of *Eimeria* spp. (Lew et al., 2003; Oliveira et al., 2011; Sun et al., 2009). However, data obtained from regular PCR analyses cannot link species-specific genetic markers with oocyst morphology for species identification due to frequent co-infections of multiple eimerian species in the same host in the field. One way to overcome this problem is either to isolate the single oocyst and passage it in coccidia-free animals or to perform molecular analysis using single oocyst (Auinger et al., 2008). Single-cell PCR was successfully used in the molecular analysis of other apicomplexan parasites such as *Cryptosporidium pavum* and *Plasmodium falciparum* (Chen et al., 1998; Lynn and Pinheiro, 2009; Sunnotel et al., 2006).

For the detection and identification of microorganisms using single-cell PCR, the trace amount of nucleic acids extracted from the single cell makes it a challenge to success. Whole genome amplification (WGA), which can randomly amplify whole DNA in the tested sample and thus increase the copy numbers of target genes, has been increasingly used in pre-PCR amplification for molecular diagnosis (Silander and Saarela, 2008; Zhang et al., 1992).

Here we report the joint application of WGA and nested PCR in the analysis of eimerian parasites at the single oocyst level.

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Oocysts of *Eimeria stiedai* and *Eimeria media*, two *Eimeria* species infecting domestic rabbits, were propagated, sporulated and purified according to established protocols (Long et al., 1976). Purified oocysts were diluted serially in double distilled water to the concentration of approximately one oocyst per microliter. One microliter of oocyst suspension was transferred onto a 5 mm × 2 mm coverslip placed on a slide and checked under the microscope (Leica TCS SP5 II, Leica Microsystems). Once the existence of a single oocyst on the cover slip was confirmed, photographs were recorded for morphological identification. The coverslip was then transferred into a PCR tube containing 10 μl lysis buffer (0.005% SDS in TE solution). After a brief centrifugation, the tube was frozen in liquid nitrogen and thawed in a 55 °C water bath for five rounds to disrupt the oocyst wall. After the addition of 0.5 μl proteinase K (20 mM), the tube was incubated at 55 °C for 2 h and then at 95 °C for 15 min. The whole lysate of the single oocyst was directly used for whole genome amplification.

Two WGA methods, primer extension preamplification (PEP) and multiple displacement amplification (MDA) (Silander and Saarela, 2008; Zhang et al., 1992), were used in this study. Briefly, 25 μl 2× Taqmix (TIANGEN Biotech (Beijing) Co., Ltd.), 5 μl of 400 μM 15-base random oligonucleotides were added into the parasite lysate for the preparation of PEP reaction system (50 μl). Fifty primer-extension cycles were carried out. Each cycle consisted of 1 min at 95 °C, 2 min at 37 °C, a programmed ramping step of 1 degree/10 s to 55 °C, and 4 min at 55 °C. While the MDA reaction system (50 μl) contains 2.5 μl of random hexamer primer (100 μM), 1 μl of 10× phi 29 DNA buffer and the parasite lysate. The mixture was incubated at 95 °C for 3 min and then on ice for 15 min. Subsequently, 0.5 μl of dNTP (10 mM), 0.2 μl of 100× bovine serum albumin (0.2 mg/ml), and 0.5 μl phi 29 DNA polymerase (10 U/μl, New England Biolabs, Beijing) were added to the mixture and incubated overnight at 30 °C. The phi 29 DNA polymerase in the mixture was denatured at 65 °C for 15 min.

Aliquots (1 μl) of the PEP or MDA product were used as template for nested PCR in the analysis of *Eimeria* target genes. Primer pairs used for nested PCR are shown in Table 1. Two successive reactions were carried out for the nested PCR. The first round reaction contains 1 step of predenaturation (94 °C, 2 min), 30 cycles of amplification (30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C) and a final extension step (72 °C for 10 min). The same program was used in the 2nd round amplification while 1 μl product from the 1st round was used as template.

From the single oocyst of *E. stiedai*, ITS-1 region of the ribosomal cistron was successfully amplified by nested PCR from the PEP-amplified template, as confirmed by the observed product size (Fig. 1). The PCR products of ITS-1 region were further confirmed by DNA sequencing (data not shown). Similar results were obtained from PEP-amplified genomic DNA of *E. media* (data not shown). The PEP product derived from *E. stiedai* was also subjected to nested PCR using species-specific ITS-1 primer pairs for the 11 *Eimeria* species of rabbits (Oliveira et al., 2011). PCR products of the expected size was only generated when using *E. stiedai*-specific primers (data not shown), confirming the specificity of the PEP amplification.

The PEP product derived from single oocyst of *E. stiedai* was also used for the nested PCR analysis of 18S rDNA and 23S rDNA regions. These two regions were successfully amplified and sequenced (Fig. 2).

The combination methods of WGA and nested PCR were further used for the detection and differentiation of mixed eimerian parasites. Oocysts of *E. stiedai* and *E. media* were mixed and subjected to single oocyst isolation and PEP-based PCR analysis. Morphological observation indicates that A and B are *E. media* while C–E are *E. stiedai* (Fig. 3). This result is absolutely in accordance with those through the detection of ITS-1 (Fig. 3). Similar results were also obtained in trials performed with MDA-based PCR (data not shown).

A previous study showed that the detection threshold of regular PCR for eimerian parasites of rabbits was approximately 0.8–1.7 sporulated oocysts (Oliveira et al., 2011). This value was calculated through a serial dilutions of genomic DNA extracted from a large number of oocysts, but not from a single oocyst. To avoid the loss of DNA during genomic DNA extraction from a single oocyst, whole lysate of the oocyst should be used in the PCR reaction. In such case, residual PCR inhibitors, such as humic acid derived from the feces, can significantly interfere with the PCR reaction when whole lysate from a single oocyst was used (Wilson, 1997). This factor probably contributed to the failure of detection of single oocyst by direct PCR in this study (data not shown). The introduction of the WGA efficiently overcame such interference and enhanced the sensitivity of single oocyst PCR, allowing the genetic characterization of *Eimeria* spp. at the single oocyst level.

Morphological characterization has long been the choice for species identification of eimerian parasites and even the differentiation of precocious line and parental line of *Eimeria* spp. (Pakandl and Jelinkova, 2006; Poplstein and Vrba, 2011). However, it is often difficult to link the morphological characteristics with DNA sequencing information of DNA markers when the oocysts were derived from mixed infections. As described above, WGA-based PCR analysis of single oocyst effectively solved this problem. It could be a useful tool to study the biological characteristics of

Table 1
Genus- and species-specific primers for nested PCR in *E. stiedai* and *E. media*.

Number	Primer name	Primer sequence (5′–3′)	Product size (bp)	References or GenBank access code
1	E-ITS1-F	GGGAAGTTGCGTAAATAGA	Variable	Oliveira et al., 2010
2	E-ITS1-R	CTGCGTCCTTCATCGAT		
3	Es-ITS1-F	GTGGGTTTTCTGT GCCCTC	217	Oliveira et al., 2010
4	Es-ITS1-R	AAGGCTGCTGCTTTGCTTC		
5	Em-ITS1-F	GATTTTTTTCCACT GCGTCC	152	Oliveira et al., 2010
6	Em-ITS1-R	TTCATAACAGAAAAGGTAAAAAAAGC		
7	Es-18S-eF	TGAGAAACGGCTA CCACA	406	HQ173837.1
8	Es-18S-eR	GAAGTATTCAGGGCGACAA		
9	Es-18S-iF	TCGAGGTAGTGAC GAGAAATA	194	HQ173837.1
10	Es-18S-iR	TGACCACGACAGAAATCCA		
11	Es-23S-eF	CCCGCTAAGTTTA ATCAC	463	HQ173854.1
12	Es-23S-eR	TACACCTCCGTTGCTCTT		
13	Es-23S-iF	CAGAAAGACCCTA TGAAGC	179	HQ173854.1
14	Es-23S-iR	CGTCCCAGTCAAACTACC		

Note: Primers 1 and 2 are genus-specific primers for 11 *Eimeria* species and used here for both *E. stiedai* and *E. media*. For nested PCR, primer pair 1/2 and 3/4 were used for ITS-1 of *E. stiedai*, respectively, primers 7/8 and 9/10 were used for 18S rDNA of *E. stiedai*; primers 11/12 and 13/14 were used for 23S rDNA of *E. stiedai*. Primers 1/2 and 5/6 were used for ITS-1 of *E. media*. Es, *E. stiedai*, Em, *E. media*.

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