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Host Specific *Eimeria* Genus Diagnosis and QPCR Development in Sheeps and Goats

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Abstract

Coccidiosis of sheep and goats is caused by protozoa in the genus *Eimeria*. These protozoa mainly affect young animals, causing a decrease in production and consequent economic losses. Routine diagnosis is made through morphological observation of the oocysts, which has several limitations. The objective of the present study was to develop a real-time PCR (qPCR) technique for the diagnosis of *Eimeria spp*. in sheep and goats. For this purpose, the 18S rRNA region of the DNA of these parasites was selected because it is a region with low variability among *Eimeria spp*. The qPCR technique was developed using SYBR Green, resulting in a PCR with high sensitivity, and the ability to amplify samples containing only one oocyst of an *Eimeria spp*. There was no cross-reaction with other intestinal protozoa, such as *Blastocystis*, *Microsporidia, Cryptosporidium,* and *Giardia duodenum* . The repeatability test showed that the coefficient of variation was less than 2%. This indicated that this method has good sensitivity, specificity, and reproducibility. Thus, the feasibility of using qPCR in the diagnosis of the genus *Eimeria* was demonstrated in this study. This technique was less laborious and required less skill and diagnostic training compared with the micromorphometry technique conventionally used for this diagnosis put .

Keywords: 18S rRNA; Sheep; Goat; Coccidiosis; *Eimeria*; qPCR

Introduction

Coccidiosis is an important parasitic disease of ruminant livestock caused by protozoan parasites of the genus *Eimeria*, with lambs and kids between 1 and 6 months of age being most susceptible [1]. The occurrence of *Eimeria spp*. varies among countries from 48.7% to 100% [2-5]. In China, the prevalence of *Eimeria spp*. in sheep and goats was 92.9% and 78.7%, respectively [6,7].

Eimeria spp. are host specific, meaning that an *Eimeria spp*. that infects goats does not infect sheep, and vice versa [8]. Thus far, 12 intestinal and one abomasal *Eimeria* species have been observed in sheep with 10 intestinal species recently found in goats [9-12]. *Eimeria ovinoidalis* is the most pathogenic species in sheep. Among those found in goats, *Eimeria ninakohlyakimovae* and *Eimeria caprina* are the most pathogenic [1]. Others are either less pathogenic or nonpathogenic. Some *Eimeria spp*. absorb nutrients from their hosts without causing any detectable clinical signs. However, they could be responsible for a decrease in yield, seen in infected animals.

A few studies have developed PCR assays to diagnose *Eimeria spp*. For example, a primer was obtained for the genus *Eimeria* based on the 18S rRNA site, and was used to construct a phylogenetic tree comprising the main species in cattle [13]. Yang et al. developed a qPCR technique using primers designed at the 18S rRNA region to quantify the production impacts of *Eimeria* in sheep [14].

Concerning technological advancements, new assays, such as real-time PCR (qPCR), are now available and capable of developing a more sensitive and specific diagnosis compared with conventional PCR. Hence, the goal of the present study was to develop and standardize a qPCR technique to diagnose *Eimeria spp*. in sheep and goats.

Materials and Methods

Sample

Positive fecal samples of *Eimeria spp*. were selected from naturally infected animals and stored in 2.0 mL microtubes at -20°C in the Veterinary Parasitology Laboratory of Henan Agricultural University.

Positive fecal samples of *Giardia duodenalis, Blastocystis, Microsporidia* , and *Cryptosporidium parvum*, detected by PCR, were used to evaluate the specificity of the primers developed in this study [15-18].

The primers were designed from the 18S rRNA gene sequences of *Eimeria spp*. and deposited at *Gen Bank* with the following accession numbers: MW512853.1 (*Eimeria spp*. strain 20Q68-2), MN473507.1 (*Eimeria parva* isolate IQ-Deer No.36), MN473485.1 (*E. parva* isolate IQ-Deer No.14), MN473478.1 (*E. parva* isolate IQ-Deer No.7), MN473490.1 (*Eimeria intricata* isolate IQ-Deer No.19), LC507796.1 (*Eimeria hirci*), LC507795.1 (*E. hirci*), MW577425.1 (*Eimeria arloingi strain* 10), MW577427.1 (*Eimeria christenseni strain* 12), KX519412.1 (*E. ninakohlyakimovae*), MW512852.1 (*Eimeria* sp. isolate 20Q68-1), MT337428.1 (*Eimeria sp*. isolate 5-12), MN149906.1 (*Eimeria sp*. isolate TW4), MT801036.1 (*Eimeria sp*. voucher SX2), MT801027.1 (*Eimeria sp*. voucher LX1), and MT801017.1 (*Eimeria sp*. voucher DJY4). After the selection of the sequences, the primers were aligned using the MEGA7 program (www.megasoftware.net). Following the alignment, a specific site was selected and used for primer design, which was performed with Primer Premier 5.0 (www.

premierbiosoft.com/primerdesign). The primers obtained were named *Eimeria* 18S-F (5′-CAGGCTTGTCGCCCTGA-3′) and *Eimeria* 18S-R (5′-TTCGCAGTAGTTCGTCTTT-3′), with an amplicon of 168 bp.

DNA extraction steps were performed in accordance with product instructions of the Stool DNA Kit (E.Z.N.A Stool DNA Kit, OMEGA, Bozeman, MT, USA).

Endpoint PCR was used with primer sets to obtain the PCR product of *Eimeria spp*. The reaction mixture (25 μL) contained 2.5 μL PCR buffer (10x), 2.0 μL dNTPs (2 mM each), 1.0 μL MgCl^2 (25 mM), 1.0 μL forward and reverse primers (10 μM), 0.2 μL DNA polymerase (1 U•μL-1), 1.0 μL of genomic DNA purified from *Eimeria spp*. and 17.3 μL of PCR double-distilled water. PCR was carried out under the following conditions: an initial denaturation at 94°C for 4 min, followed by 35 cycles of 45 s at 94°C, 35 s at 55°C, and 1 min at 72°C. The final extension step was set for 7 min at 72°C. The PCR amplicon was purified from agarose gel, cloned into a pMD18-T vector, and transformed into *Escherichia coli* DH5α. Recombinant clones were selected by blue/white screening. The recombinant plasmid DNA was extracted using the SanPrep column plasmid DNA small amount extraction kit (Sangon Biotech, Shanghai, China) and sent to Shanghai Sangon Biotech for sequencing. Recombinant plasmids with the correct sequence of the *Eimeria spp*. amplicon (hereafter pMD18T-168) was used as the standard in qPCR.

The pMD18T-168 plasmid was used to generate standard dilution series and the development of the qPCR test. The qPCR instrument used in this work was produced by Analytikjena (jean, Germany). The concentration of the plasmid standard solution was measured using Nano Drop 1000 (Thermo Fisher Scientific, Waltham, MA, USA), and the corresponding copy number was calculated. A tenfold dilution series of pMD18T-168, ranging from 3.43×10^3 to 3.43×10^8 copies/μL, was made and used to construct a standard curve. The reaction mixture contained 1.0 μL of plasmid DNA dilution, 1.0 μL *Eimeria* 18S-F (500 nmol/L), 1.0 μL The results of tests revealed18S-R (500 nmol/L), 10.0 μL of THUNDERBIRD® Next SYBR® qPCR Mix (TOYOBO, Osaka, Japan), and PCR double-distilled water to a final volume of 20 μL. An initial denaturation at 95°C for 30 s was followed by 40 cycles of 5 s at 95ºC, 10 s at 56°C and 15 s at 72ºC. Threshold cycle (Ct) values in dilutions were measured in triplicate and plotted against the logarithm of their initial copy number. Each standard curve was generated by linear regression of the plotted points, and standard curve parameters were obtained. Ct was calculated under default settings for the qPCR soft system Software (ver. 4.0, Analytik).

Each assay designed included negative and positive controls and the standard curve. The negative control was the PCR reaction without template DNA. The positive control was a PCR reaction containing DNA of *Eimeria spp*. All controls and samples were assayed at least three times. The specificity of *Eimeria spp*. qPCR was evaluated by performing the method using gDNA from*G. duodenalis, Blastocystis, Microsporidia,* and *C. parvum* simultaneously. The analytical sensitivity (i.e., limit of detection) was established using eight replicates of serially diluted pMD18T-168 plasmid at 3.43×10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 100 copies/reaction. gDNA was extracted from 10,000 coccidia oocysts, and diluted into five gradients of 10,000, 1,000, 100, 10, and 1 oocyst(s) according to the tenfold dilution series. The established qPCR was used to detect gDNA to determine the sensitivity of the method. The interassay precision of the qPCR was defined as the coefficient of variation (CV) of Ct values obtained for each copy number/reaction in three different assays performed on 3 different days.

The developed qPCR method and McMaster method were used to monitor the *Eimeria spp*. infection quantity of four naturally infected lambs for 4 weeks and to compare the difference between two methods [19-21].

Data are presented as mean (± SD). The pMD18T-168 DNA levels are presented as a log unit. Graph Pad Prism (www.graphpad-prism.cn) was used for statistical analysis.

Results

The PCR amplification product of *Eimeria spp*. 18S rRNA was detected by 1% agarose gel electrophoresis, and the amplified fragment was between 100 bp and 250 bp, which encompassed the expected fragment size (168 bp). The sequencing results of positive clones were compared with sequences (MW512853.1) in GenBank, with a matching rate of 100% (Figure 1 and Table 1).


M: DNA Marker; 1: amplification band
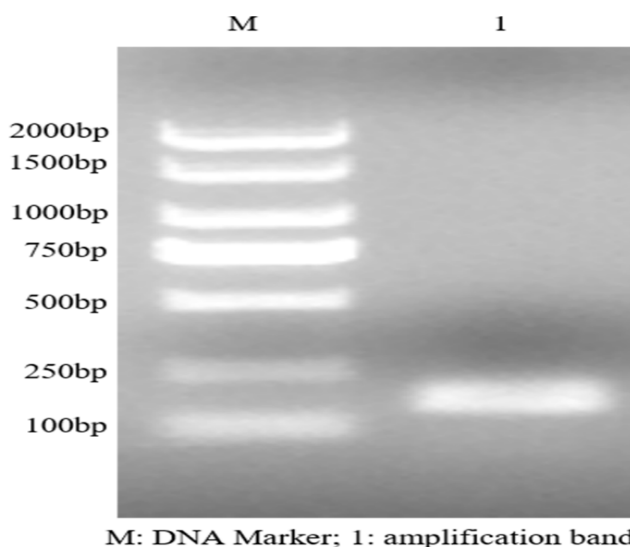
Figure 1: Amplification product. The product was obtained by PCR amplification at the 18S rRNA site of *Eimeria spp*. The expected fragment size was 168 bp, M: DL2000 Marker (Sangon Biotech), 1: amplification band.

| Score	Expect	Identities	Gaps	Strand
311 bits (168)	**2.00E-80**	**168/168(100%)**	**0/168(0%)**	**Plus/Plus**
Query	CAGGCTTGTCGCCCTGAATACTTCAGCATGGAATAATAGGATAGGACCTTGGTTCTATTT	60
Sbjct	CAGGCTTGTCGCCCTGAATACTTCAGCATGGAATAATAGGATAGGACCTTGGTTCTATTT	753
Query	TGTTGGTTTCTAGGACCAAGGTAATGATTAATAGGGACAGTTGGGGGCATTCGTATTTAA	120
Sbjct	TGTTGGTTTCTAGGACCAAGGTAATGATTAATAGGGACAGTTGGGGGCATTCGTATTTAA	813
Query	CTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAA	168
Sbjct	CTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAA	861

Table 1: Sequence alignment. The sequenced sequences were compared with sequences published in *Gen Bank*.

Development of qPCR for *Eimeria spp*

The qPCR was performed using different copy numbers of the pMD18T-168 plasmid. The linear dynamic range of the assay was 6 logs (Figure 2). Assay linearity was established (R^2=0.99779) between dilutions containing 3.43×10^3-10^8 copies/reaction. The standard curve slope was -3.17, which resulted in a high amplification efficiency of 107%. The results confirmed that, over a wide concentration range, the method had a good linearity and can be used for the detection of target DNA. Gradient dilution of the pMD18T-168 plasmid. The standard curve is Y=-3.17×log(X)+38.97, X-axis represents the copy number of template DNA, and the Y-axis represents the Ct value (Figure 3).
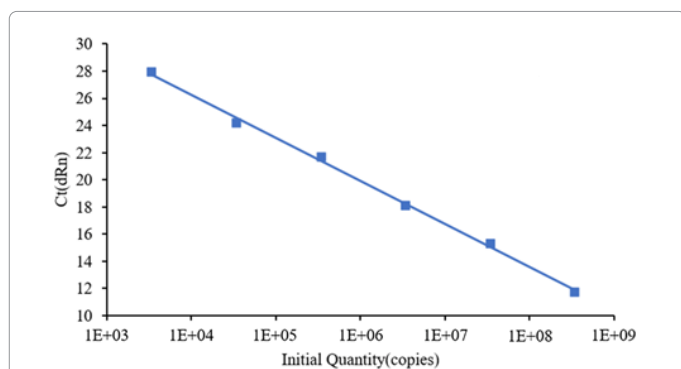
Figure 2: Standard curve. The standard curve was generated by amplification of a tenfold. **Note:** (■) SYBR Standards, RSq=0.99779; (——) SYBR, Y= -3.17 × LOG(X)+38.97, Eff.=107%.
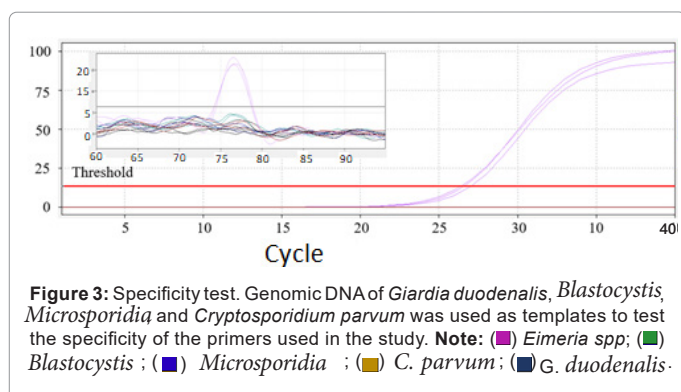

Figure 3: Specificity test. Genomic DNA of *Giardia duodenalis*, *Blastocystis*, *Microsporidia* and *Cryptosporidium parvum* was used as templates to test the specificity of the primers used in the study. **Note:** (■) *Eimeria spp*; (■) *Blastocystis* ; (■) *Microsporidia* ; (■) *C. parvum* ; (■) *G. duodenalis*.

Standardization of qPCR for *Eimeria spp*

The specificity of the qPCR was confirmed because no fluorogenic signal was detected when gDNA of G. *duodenalis, Blastocystis, Microsporidia*, or *C. parvum* were used (Figures 3 and 4). The limit of detection of qPCR was 34 copies/reaction, enabling it to detect samples containing a single oocyst of *Eimeria spp*. The CV of repeatability tests (intra and inter assay) was less than 2%, indicating that the method had good repeatability (Tables 2 and 3).
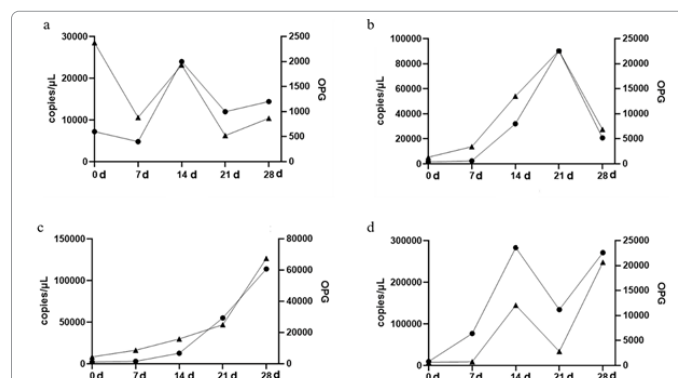
Figure 4: Comparison of infection intensity tests. Coccidia infection intensity of four lambs detected using qPCR and the McMaster method, represented by triangles and circles, respectively. Oocysts per gram (OPG) represents the number of Coccidioides oocysts per gram of feces and is an indicator of the intensity of infection. **Note:** (—●—) McMaster; (—▲—) qPCR.

Plasmid concentration (copies/μL)	Mean Ct value (n=3)
3.43×10^8	11.87 ± 0.75
3.43×10^7	17.04 ± 0.34
3.43×10^6	20.76 ± 0.21
3.43×10^5	25.11 ± 0.32
3.43×10^4	28.1 ± 0.05
3.43×10^3	30.22 ± 0.41
3.43×10^2	30.73 ± 0.66
3.43×10^1	31.87 ± 0.27
3.43×10^0	-

Note: Ct-cycle threshold; "-" - No Ct value

Table 2: Determination of limit of detection for the *Eimeria spp*. qPCR.

Oocyst density	Mean Ct value (n=3)
10,000	15.95
1000	20.6
100	24.47
10	29.64
1	32.76

Note: Ct-cycle threshold

Table 3: Determination of gDNA of oocyst(s) by the *Eimeria spp*. qPCR.

Figure 4 illustrates of the comparison of the established fluorescence-based quantitative method and McMaster method used to measure the infection intensity of four lambs naturally infected with coccidia for four consecutive weeks. According to the results, the trend in changes of oocyst infection intensity in the four lambs detected by the two methods was roughly consistent, indicating that the fluorescence-based quantitative method can be used for quantitative detection of coccidia infection in sheep (Figure 4 and Table 4).

Plasmid concentration (copies/μL)	Intraassay			Interassay		
	Mean Ct value	SD	CV	Mean Ct value	SD	CV
3.43×10^8	11.68	0.22	1.88%	11.77	0.1	0.81%
3.43×10^7	17.4	0.12	0.69%	17.14	0.23	1.33%
3.43×10^6	20.62	0.24	1.16%	20.82	0.23	1.11%
3.43×10^5	24.82	0.29	1.16%	24.96	0.15	0.58%
3.43×10^4	27.24	0.32	1.17%	27.82	0.5	1.80%

Note: Ct-Cycle Threshold; SD-Standard Deviation

Table 4: Intra- and interassay reproducibility tests of qPCR.

Discussion

In the present study, the 18S rRNA site of the DNA from several species of *Eimeria spp*. derived from sheep and goats was used to design primers because this is a highly conserved region among species [22].

A standardized qPCR is expected to provide sensitive, accurate, and reproducible results. One essential requirement for developing a standardized PCR test is the availability of a common standard that is easy to produce in large amounts, is stable, and shows consistent quality across different production batches [23]. Recombinant plasmids are stable and easy to standardize, and are often used as standard materials to construct qPCR standard curves [24,25]. In this study, a plasmid DNA of *Eimeria spp*., pMD18T-168, was constructed and used as a calibrator to develop a reliable absolute quantitative qPCR for quantifying parasite load in biological samples.

The results of tests revealed that the assay could reliably detect 34 copies of the cloned *Eimeria* amplicon per μL of fecal DNA extract, and it was possible to find samples containing a single oocyst of *Eimeria spp*. This demonstrated that the assay showed high sensitivity and could be a useful tool for routine diagnosis. The results resemble those obtained by Kokuzawa, et al. who used a conventional PCR, which was also based on the 18S rRNA site, to amplify a single oocyst of this protozoan, with the goal of subsequent sequencing and evaluation of genetic variability among oocysts from the same species [13]. The method established in this study is more sensitive than the qPCR established to diagnose *Eimeria spp*. in sheep, with a sensitivity of 80 copies per μL of fecal DNA extract reported by Yang, et al. [14]. In addition, the established method is also suitable for goats, because the primers were designed from the 18S rRNA gene sequences of *Eimeria spp*. of both sheep and goats.

Fluorescence-based quantitative PCR is widely used in parasite detection. For example, RT-PCR was used to quantitatively detect the amount of *Cryptosporidium* oocysts in mouse fecal samples to study the ovulation rule of immunosuppressed and non-immunosuppressed mice infected with *Cryptosporidium* mouse [26]. A quantitative RT-PCR was established to study the growth inhibition effect of drugs on *Cryptosporidium parvum* [27].

Conclusion

In this study, the developed qPCR method was used to detect the intensity of coccidia infection in lambs naturally infected with *Eimeria*. The results were similar to those of routine detection, indicating that the established qPCR can be used to detect the intensity of intestinal coccidia infection in lambs. A plasmid DNA of *Eimeria spp*. pMD18T-168, was constructed and used as a calibrator to develop a reliable qPCR for quantifying parasite load in biological samples. The method was shown to be highly sensitive, accurate, and reproducible. The qPCR method was successfully applied for quantifying parasite load of *Eimeria spp*. in lamb feces. The method could be used to evaluate the effect of anticoccidial drugs and growth and reproduction of Coccidia *in vitro* and *in vivo*.

Declarations

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Disclosure

The authors declare no competing interests.

Data availability

All data generated or analyzed during this study are included in this published article.

Ethics approval

All experimental procedures were reviewed and approved by the Henan Agriculture University Animal Care and Use Committee (license number SCXK (Henan) 2013-0001).

Consent to participate

Not applicable.

Consent for publication

All authors consent to be published.

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