

**APPLICATION OF NONINVASIVE MOLECULAR – BIOLOGICAL METHODS
FOR DIAGNOSTICS OF EIMERIOSIS IN DOMESTIC RABBITS
(ORYCTOLAGUS CUNICULUS)**

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ABSTRACT

The parasites of the genus *Eimeria* (Apicomplexa: Eimeriidae) are among the most common in rabbits. We use non-invasive molecular biological methods to determine the species of the genus *Eimeria* and confirmed the existence of five species in experimental infected domestic rabbits (*Oryctolagus cuniculus*). We consider that the obtained results could be used for species identification of parasites of the genus *Eimeria* in mixed populations.

Key words: *Eimeria* spp., *Oryctolagus cuniculus*, diagnostic, PCR, ITS

The species of the genus *Eimeria* (Apicomplexa: Eimeriidae) are the most common parasites in rabbits. In world literature there are described eleven species *Eimeria* spp. found at domestic rabbits (*Oryctolagus cuniculus*) with clearly established morphological differences. In our country, we have scarce data about the diversity of species causing eimeriosis in rabbits. There are some studies on Burgas region (Meshkov, 1981, 1982) in which eight types of *Eimeria* spp. causing coccidiosis in domestic rabbit have been described: *E. magna*, *E. perforans*, *E. irresidua*, *E. media*, *E. stiedai*, *E. intestinalis*, *E. piriformis* and *E. exigua*.

In her PhD thesis (Kostova, 1989) after coproscopic examination of 1750 fecal samples from eight different regions in Bulgaria, she determined seven species *Eimeria* spp. in domestic rabbits by (Levine, 1973), including morphological characteristics as well.

There is some variability in biometric data of the oocysts, which can be explained completely by the effect of various external factors, such as intensity of infection, temperature, diet of the host, the endogenous period for development of the parasite, etc. (E. M. Cheissin, 1967; Milyauskaite & Arnastauskene, 1978). Even though oocyst morphology allows a good differentiation of *Eimeria* in rabbit, it is practically impossible to evaluate carefully thousands of oocysts just to assess if a given strain is really pure. Moreover, a small portion of oocysts may differ from the typically expected morphology and present a challenge to a correct diagnosis. In addition, all these biological features may present a variable level of overlap, hampering in some cases an accurate *Eimeria* species identification (Long and Joyner, 1984). The exact identification of the eimeriosis in rabbits is complicated in mixed populations, therefore more accurate species-specific methods for determining species of the genus *Eimeria* should be applied.

PCR-based molecular methods have been widely used in the diagnosis and characterization of *Eimeria* spp. (Lew et al., 2003; Oliveira et al., 2011). 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 *Eimeria* species in the same host in the field (Wang et al., 2014)

Internal transcribed spacer (ITS) refers to the DNA segment located between the genes of the small ribosomal subunit RNA and a large ribosomal subunit in the chromosome rRNA. In eukaryotes there are two sections ITS. ITS1 is situated between the 18S rRNA genes and 5.8S. ITS2 unit is between the genes of the 5.8S and 28S rRNA.

A comparison of the ITS region sequences is widely used in taxonomy and molecular phylogeny. This is because it can be easily amplified, even small amounts of DNA, and because there is a high degree of variation even between closely related species.

The species of the genus *Eimeria* are host species-specific and localization-specific. In most cases they are present in mixed invasion with different pathogenicity.

Oliveira et al. (2011) describe molecular diagnostic methods for the differentiation of the eleven species of *Eimeria* in domestic rabbits. They determined the nucleotide sequences of ITS1 ribosomal DNA and model species- specific primers for each species, which we used in this study.

Aim

The purpose of this study is to implement new noninvasive molecular-biological methods for an accurate determination of the species from genus *Eimeria* based on ITS1 region.

To achieve the aim, we used oocysts from naturally infected with *Eimeria* domestic rabbits.

Material and methods

Parasite collection: Examination of 30 rabbit's fecal samples and collection of *Eimeria* spp. were conducted.

Coprological examination: Fecal samples were collected in polyethylene plastic labeled bags, and were examined during the same day of collection by the concentration floatation technique according to (Pritchard, M. H. and Kruse, G. O., 1982) and collected in 2% Potassium dichromate (K2Cr2O7) solution.

Oocysts were mixed very well in water, and incubated at 15-30°C for 24-120 hours (or 1-5 days). The culture was stirred every day for an aeration.

DNA extraction: The extraction of DNA from oocyst of *Eimeria* spp. was performed according to (Sambrook J. and Russell D. W. 2001).

Oocysts of *Eimeria* spp. were put in a sterile mortar and liquid nitrogen was used to disrupt the cells. The oocyst pellets were used in DNA extraction kit (Qiagen, Germany).

Polymerase chain reaction (PCR) assay: The oligonucleotide primers used in this study were selected from highly conserved sequences encoding rRNA, ITS1sequence of *Eimeria* spp.

For isolation and purification of the DNA was used solution of GeneJET Genomic DNA Purification Kit (Thermo scientific).

This primer set was used in the PCR assay for partially amplification of the DNA, ITS1 of *Eimeria* spp.

Table 1: Primers species-specific sequences used for general amplification of the ITS1 from *Eimeria* spp.

Eimeria species	Primer ITS1 Forward	Primer ITS1 Reverse	Product	Eimeria spp. Product
Eimeria spp. (all species)	GGGAAGTTGCGTAAATAGA	CTGCGTCCTTCATCGAT	Different for different species	
E. magna	TTTACTTATCACCGAGGGTT-GATC	CGAGAAAGGTAAAGCTTACCA	218 bp	457 bp
E. coecicola	AGCTT-GGTTGGTTCTTATTATTGTAC	CTAGTTGCTTCAACAAATCCATATCA	256 bp	573 bp
E. media	GATTTTTTCTTCACTGCGTCC	TTCATAACAGAAAAGTAAAAAAAGC	152 bp	468 bp
E. exigua	GAATAAGTTGCTCAAAGA-GAGCC	TATATAGACCATCCCAACCCAC	280 bp	472 bp
E. perforans	TTTTATTTCATCCCCATT-GCATCC	CTTTTCATAACAGAAAAGTCAA-GCTTC	157 bp	473 bp
E. flavescens	GAATATTGTTGCAGTTAC-CAACAA	CCTCAACAAACCGTTCTTCATAATC	199 bp	463 bp
E. piriformis	AC-GAATACATCCCTGCCTTAC	ATTGTCTCCCCCTGCACAAAC	289 bp	451 bp
E. intestinalis	TGTTTGACAC-CGAGGAAATA	AACATTAAGCTACCCCTCATCC	241 bp	484 bp
E. stiedai	GTTGGTTTCTGTGCCCTC	AAGGCTGCTGCTTGCCTC	217 bp	563 bp
E. irresidua	TTTGGTGGGAAAAGATGATTCTAC	TTTGCATTATTTAACCCATTCA	226 bp	367 bp
E. vejvodskyi	GTGCTGCCACAAAAGTCACC	GCTACAATTCAATTCCGCC	166 bp	406 bp

PCR reaction was carried out in a total volume of 50 µl containing:

25 µl PCR Master Mix 2x (Thermo scientific) containing, (4 mM MgCl₂; 0.4 mM deoxynucleotides triphosphates mixture (dATP, dCTP, dGTP and dTTP); 0.05 µl thermos aquaticus (Taq) polymerase and reaction buffer;

5 µl of extracted parasite genomic DNA

2 µl for each primers and nuclease-free sterile double distilled water 16 µl.

The obtained sample was amplified via precise cycle in Veriti 96 Well Thermal Cycler from Applied Biosystems as follows:

Initial denaturation – 93 °C for 5 minutes, 40 cycles – 93 °C for 1 minutes, 56 °C for 2 minutes and 72 °C for 2 minute, followed by final extension at 72 °C for 7 minutes.

The PCR amplification products (amplicons) were visualized: Received PCR amplicons (10 µl) were analyzed by dint of 1 % agarose gel electrophoresis. The DNA bands were visualized via ultraviolet transillumination (SCIE-PLAS Vision) after gel staining with ethidium bromide (0.5 µg/ml). Images were processed and analyzed with software program GelAnalyzer 2010a.

Results

In our preliminary studies we used genus-specific primers to detect species from genus *Eimeria*. In agarose gel we proved the presence of parasites from this taxon.

The presence of two band in agarose gel gives us a reason to suppose that, there are more than one species in the sample, which proves mixed invasion (Fig. 1).



Figure 1: Agarose gel image with three columns: first is Ladder and two samples with dual bands with genus-specific primers in 451 and 573 bp.

Images of gel-documenting system were made, then they were analyzed by GelAnalyzer2010b. Following log-linear alignment of different columns from the first / weight ladder we differed two separate band in two different samples by 451 and 573 bp. After an alignment of the primers for *Eimeria* spp. with NCBI Nucleotide fragments we received the following products with base pairs in the last column (Table 1).

Used generic specific ITS1 primers could be used for primordial detection in copro samples from rabbit suspected of eimeriosis or for prevention.

To specify the species distinctly we start the test run with each pair of species-specific primers (Fig. 2).

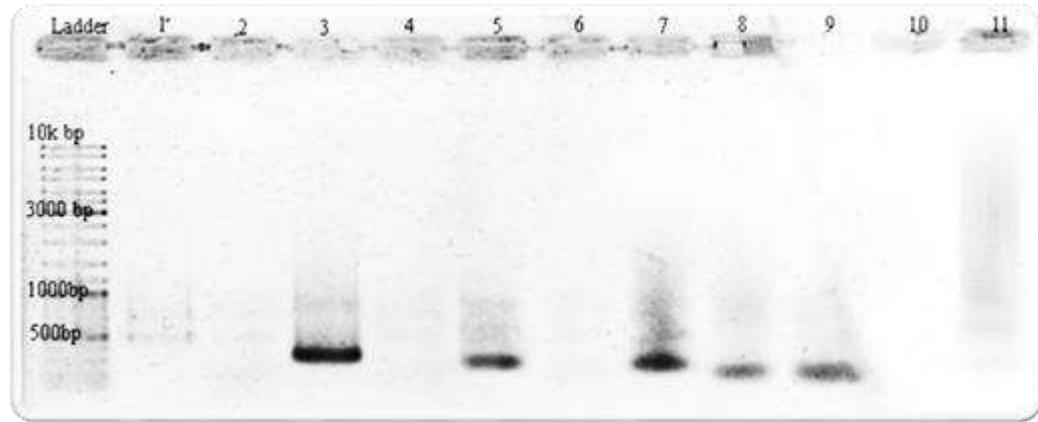


Figure 2: Agarose gel image with twelve column: Lader; 1: *Eimeria stiedai*; 2: *E. coecicola*; 3: *E. exigua*; 4: *E. flavescens*; 5: *E. intestinalis*; 6: *E. irresidua*; 7: *E. magna*; 8: *E. media*; 9: *E. perforans*; 10: *E. piriformis*; 11: *E. vejovski*.

Molecular biological methods make it easy to determine the species composition in the long and labor-intensive morphometric microscopic techniques. They help to overcome the subjective error or technical reasons based mainly on ITS1 conservative species specific gene sequences. Due

to the accumulation of more and more complete databases of genes in every organism on earth and the increasing interest in these methods, their implementation is becoming more available (both) methodically and economic. Undoubtedly they will impose as obligatory methods to prove the taxonomic identity when making diagnosis or choosing biological models for experiments.

We found five of the total eleven identified species of the genus *Eimeria* in domestic rabbits. It was confirmed the presence of *E. exigua*, species with morphometric smallest size, which was not identified by (Kostova, 1989).

There were not confirmed three of the eight morphometric identified species in Bulgaria, probably due to the small number of test animals.

To confirm the molecular biological results, we resorted to the classical histopathological methods.

After the histologic autopsy of the tested animals, we did not find hepatic pathological changes typical for the liver stage of eimeriosis – *E. stiedai*, which has been confirmed by their absence in the noninvasive molecular biological studies.

Discussion

The method is noninvasive and does not put test animals under stress during the co-prosampling.

Through the methods used in this study we were able to confirm the presence of previously identified by (Meshkov, 1981, 1982; Kostova, 1989; Vladov, 2014) *Eimeria* species in domestic rabbits. We consider that the obtained results could be used for species identification of parasites of the genus *Eimeria* in mixed populations.

Conclusion

Conducted tests and analyzes would allow detailed studies with high sensitivity and specificity, that contribute to a better understanding of the epidemiology of this important group of parasites of the genus *Eimeria*.

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