Molecular detection of *Eimeria stiedae* in an Angora rabbit

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Abstract: Hepatic coccidiosis caused by *Eimeria stiedae,* results in severe infection, outbreaks and deaths in young rabbits. Disease is diagnosed by time consuming methods like fecal examination, oocyst sporulation and necropsy. The aim of this study was to detect *E.stiedae* with species specific Polymerase Chain Reaction (PCR), without waiting oocyst sporulation and necropsy. Impression smears were prepared from liver nodules and stained with giemsa. Fecal samples were collected from the intestines and examined by centrifugal flotation with salt solution to detect *Eimeria* spp. oocysts. Then oocysts were sporulated in 2.5% (w/v) aqueous potassium dichromate ($K_2Cr_20_7$). Primers specific to *E.stiedae* were used in PCR reaction. *Eimeria* spp. oocysts were detected after the examination of impression smears and bowel content. *E.stiedae* was diagnosed after the measurement of sporulated oocyst and PCR. At the end of the study, we think that; PCR will be very valuable for the rapid and true diagnosis of hepatic coccidiosis in rabbits.

Key words: Angora rabbit, *Eimeria stiedae*, Molecular detection, Turkey.

Bir Ankara tavşanında *Eimeria stiedae*'nin moleküler tanısı

Özet: *Eimeria stiedae*'nin neden olduğu karaciğer coccidiosisi, genç tavşanlarda ciddi enfeksiyon, salgın ve ölümlerle sonuçlanır. Hastalık dışkı muayenesi, oocyst sporlandırma ve nekropsi gibi zaman alıcı yöntemlerle teşhis edilir. Bu çalışmanın amacı, *E.stiedae*'yı oocyst sporlandırma ve nekropsiyi beklemeden türe özgü Poimeraz Zincir Reaksiyonu (PZR) ile teşhis etmekti. Organ tuşeleri, karaciğer nodüllerinden hazırlanarak giemsa ile boyandı. Dışkı örnekleri bağırsaktan alınarak *Eimeria* spp. oocystlerini belirlemek için tuzlu su-flotasyon yöntemi ile incelendi. Elde edilen oocystler %2.5'lik potasyum dikromat ($K_2Cr_20_7$) çözeltisinde sporlandırıldı. PZR'de ise *E.stiedae* için tür spesifik primerler kullanıldı. Organ tuşeleri ve bağırsak içeriğinin incelenmesi neticesinde *Eimeria* spp. oocystleri, PZR ve sporlandırılmış oocystlerin ölçümü sonucunda *E.stiedae* tespit edildi. Çalışma sonucunda PZR'nin tavşanlarda karaciğer coccidiosisinin hızlı ve doğru bir şekilde teşhisinde etkili bir yöntem olduğu kanısına varıldı.

Anahtar Sözcükler: Ankara tavşanı, *Eimeria stiedae*, Moleküler tanı.

Introduction

Rabbits are raised for various commercial purposes such as wool, fur and meat production. Additionally, they are important laboratory animals. Coccidiosis is an important parasitic disease of rabbits and causes high incidence of morbidity and mortality. The disease occurs in hepatic and intestinal forms [1].

Hepatic coccidiosis caused by *Eimeria stiedae*, results in severe infection, outbreaks and deaths in young rabbits. The parasite completes its development in bile ducts of liver. By time, bile ducts thicken and disrupt the liver function. Eventually, liver becomes markedly enlarged and irregular yellowish and white nodules or cords develop on it, which later on tend to become integrated. Diarrhea and icterus can be seen in infected animals [1,2].

To date, a few studies about microscopic and histopathologic diagnosis of rabbit coccidiosis have been published in Turkey [3,4,5,6,7]. The aim this study was to detect *E.stiedae* with species-specific Polymerase Chain Reaction (PCR), without waiting oocyst sporulation and necropsy.

Material and Method

Liver and bowels of a 4-month old dead Angora rabbit were sent to the Parasitology Department from a rabbit farm. At the anamnesis, we learned

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that 50 rabbits were in the flock and infected 20 of them died.

At macroscopic examination, there were a lot of yellow-white nodules on the liver and many of these nodules contained caseous material (Fig. 1).

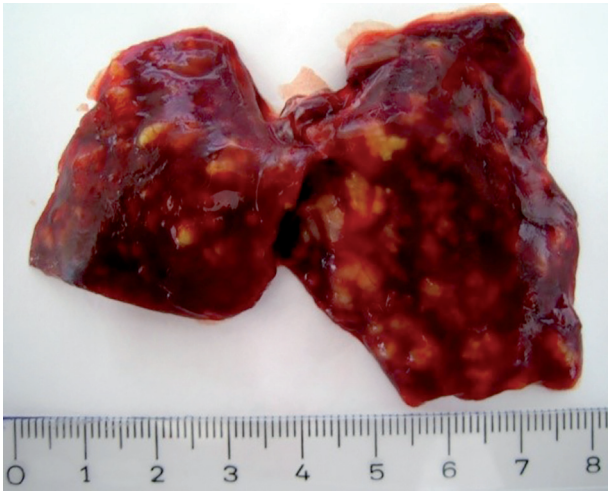

Fig. 1: Yellow nodules throughout the liver parenchyma.

Impression smears were prepared and stained with giemsa. Fecal samples were collected from the intestines and examined by centrifugal flotation with salt solution to detect *Eimeria* spp. oocysts. Fecal samples were put into a solution of 2.5% (w/v) aqueous potassium dichromate ($K_2Cr_20_7$) and were allowed to sporulate. Oocysts were concentrated by zinc sulphate flotation method [8]. 20 sporulated oocysts were measured by oil immersion lenses on a microscope. All measurements were done as µm. Oocyst identification was done according to related literatures [9,10,11].

Genomic DNA extraction was done from the nodules on the liver tissue and oocysts, using DNeasy TM Tissue Kit (Qiagen, Hilden, Germany) by following the manufacturer's instructions. Before the extraction, liver tissue was broken into small pieces with a scalpel. Then, broken tissue and oocysts were washed with PBS for five times. Esti-ITS1-F (GTGGGTTTTCTGTGCCCTC) and Esti-ITS1-R (AAGGCTGCTGCTTTGCTTC) primer pair, species specific for *E.stiedae*, was used to amplify partial sequence of Internal Transcribed Spacer (ITS-1) fragment of ribosomal DNA (rDNA) repeats [12].

PCR reaction volumes of 50 µl contained 5 µl of template DNA (50 ng), 80 µM of dNTP mix, 2.5 mM of MgCl2, 50 pmol of each primer, 5 µl of 10× PCR buffer and 1.25 U of TaqDNA polymerase (MBI, Fermentas, Lithuania) and 26 µl DNase, RNase free water (Biobasic, Inc). The PCR conditions were: 2 min at 95 °C (initial denaturation), 35 cycles of 1 min at 95 °C, 1 min at 50 °C and 1 min at 72 ◦C and finally 5 min at 72 °C (final extension). The PCR products were separated on agarose gels (1.5%), stained with ethidium bromide and visualized on an UV transilluminator. DNase, RNase free steril distilled water (Biobasic, Inc), used as negative control.

Results

As a result of the microscopic examination of impression smears of liver and bowel content, we detected *Eimeria* spp. oocysts (Fig. 2). Oocysts, left in $K_2Cr_20_7$ for sporulation, were oval-ellipsoid; there was no oocyst residuum, but there was a micropil. Sizes of oocysts were 19.7 X 36.1 (17-21 X 34-38) µm. Sporocysts were ellipsoid; there was a stidea body and a sporocyst residuum. Sizes of sporocysts were 8.9 x 15.0 (8-10 X 12-17) µm. (Fig. 3). At the end of the PCR, we amplified 217 bp of ITS-1 of the ribosomal gene, specific for *E.stiedae* (Fig. 4).

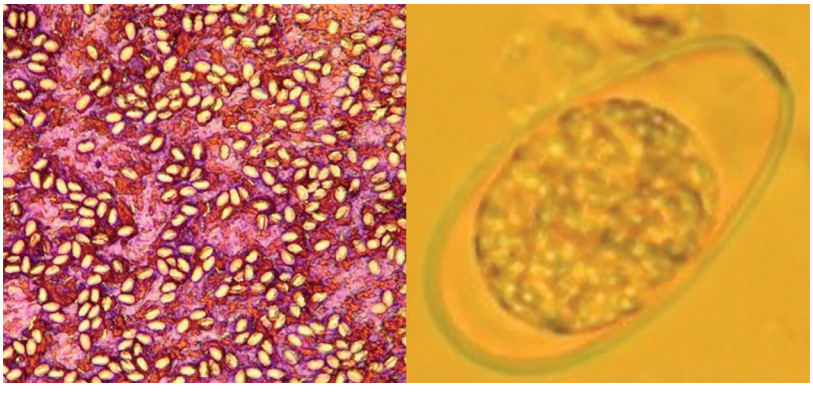

Fig. 2: Impression smear of liver nodules (left) and unsporulated *Eimeria* spp. oocsyt from bowels content (right).

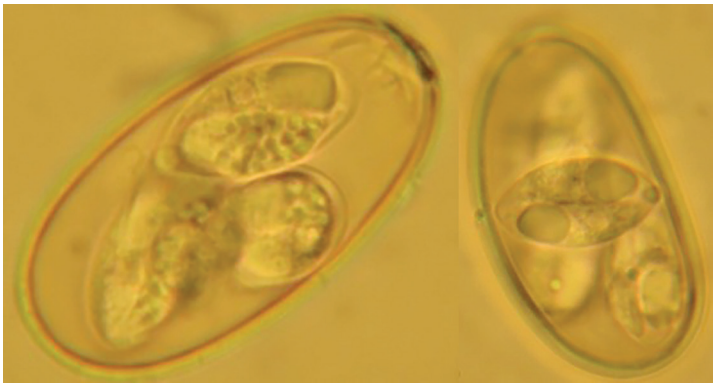

Fig. 3: Sporulated *E.stiedae* oocysts.

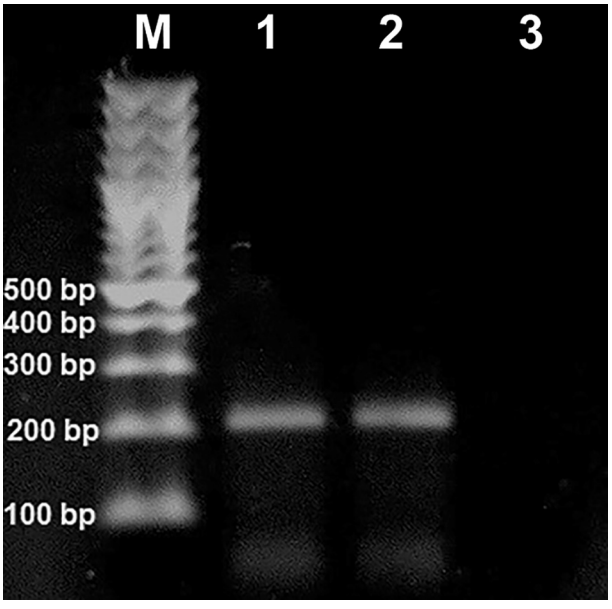

Fig. 4: Amplification products separated by 1.5% agarose gels and stained with ethidium bromide. **M**: Marker, **1**: Template DNA extracted from liver nodules, **2:** Template DNA extracted from bowel content, **3:** Negative control (distilled water).

Discussion

Eleven distinct *Eimeria* species were found in the domestic rabbits. While ten of these species cause intestinal coccidiosis, *E.stiedae* causes hepatic coccidiosis. Agents of intestinal coccidiosis are classified as very pathogenic (*E.intestinalis, E.flavescens*), moderately pathogenic (*E.irresidua, E.magna, E.piriformis*) and slightly pathogenic (*E.media, E.exigua, E.perforans, E.coecicola*). *Eimeria stiedae* can be classified as moderately or very pathogenic [1,13,14]. These species affect the rabbit production and cause reduced growth rate, feed conversion and increased mortality at different rates [1,13]. Efficiency of some anticoccidial drugs has different effects on different *Eimeria* species in rabbits. According to Polozowski [15], robenidine was very effective against intestinal coccidiosis agents at the dose of 66 ppm, but it had weak effect on *E.stiedae* infections. From this point of view species identification may be important in the epidemiology and treatment of rabbit coccidiosis.

Species identification is based on the size of oocysts and sporocysts, morphological features, prepatent period, and site of colonization [9,10,13]. The sporulation time for *E.stiedae* is approximately 2-3 days [16]. Even though oocyst morphology is useful for the diagnosis of *Eimeria* species, it is time consuming and requires specialist personnel. Especially in mix infections, when the number of oocysts of a certain species is not too many, it is very difficult to diagnose at species level [17]. Disease may spread in the flocks, while researchers waiting the sporulation for the diagnosis. The importance of rapid diagnosis becomes prominent in the early administration of medication for the preventation of the disease.

A few studies were published about rabbit coccidiosis in Turkey [3,4,5,6,7]. Most of these studies were about the prevalence of intestinal coccidiosis [3,5,6,7], one of which was focused on the hepatic coccidiosis [4]. Common feature of these studies is that diagnosis of coccidiosis is based on such conventional techniques as oocyst measurement, morphology and histopathology.

Yan et al. [4], developed a multiplex PCR for the simultaneous detection of *E.stiedae, E.intestinalis*, and *E.flavescens*. Oliveira et al. [12], developed 11 species specific PCR primers that discriminate etiological agents of coccidiosis in rabbits.

In this study, we used Esti-ITS1-F and Esti-ITS1-R primer pairs [12] in PCR, addition to oocyst measurement and morphology for the diagnosis of hepatic coccidiosis. According to Oliveira et al. [12], this primer pair was so specific to *E.stiedae*, and no cross-specific bands were observed. They mentioned the detection limit of the PCR as 0.8 sporulated oocyst. With PCR, we got results in one day without waiting oocyst sporulation or histopathology. We concluded that, in case of mix infections and inability to do necropsy, PCR will be very valuable for the rapid and true diagnosis of rabbit coccidiosis.

Acknowledgements

The authors declare that there is no conflict of interests.

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