Molecular Detection of *Eimeria Zuernii* in Cattle in Malang, East Java, Indonesia by Nested-PCR

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Abstract. Coccidiosis continues to be one of the most important issues in animal health and generates significant economic losses to the livestock industry in Indonesia, specifically due to *Eimeria* spp. Infections. Eimeria are specific to a particular host Eimeria and multi-species infections are more prevalent than infections with only one species. *Eimeria zuernii* is one of the highly pathogenic coccidia that is a major cause of clinical eimeriosis in livestock. This study's objective was to identify *E. zuernii* infections in Malang, East Java, Indonesian farms. The oocysts were concentrated and purified using a fecal harvesting method. The genomic DNA is extracted according to the instructions included with the kit. Internal transcribed spacer-1 (ITS1) nested polymerase chain reaction (nPCR) was used to analyze a total of 102 stool samples. Primer pairs specific to *E. zuernii*, as well as a standard optimum annealing temperature of 55 °C for the species, were discovered. The samples were amplified DNA approximately 334 bp. The results indicated that the prevalence of *Eimeria* spp. Was57.8% (59/102) by floatation method and specific species, *E. zuernii* was 35.3% (36/102) by nPCR of cattle fecal samples in Malang, East Java. When Eimeria species are available, the parasite spreads widely through the group inside a couple of life cycles. A pathogenic *Eimeria species* can infect cattle, thus species identification is critical. Low-infective portions of highly pathogenic species can cause illness, and the productive idea of the parasite quickly prompts high re-contamination. To increase cattle production, eimeriosis management should be considered.

Keywords: Eimeriosis · pathogenic · identification · molecular · livestock

1 Introduction

Coccidiosis continues to be one of the most important issues in animal health and generates significant economic losses to the livestock industry in Indonesia, particularly due to *Eimeria* spp. Infections. *Eimeria zuernii* is one of the highly pathogenic coccidia that is a

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major cause of clinical eimeriosis in livestock. The disease caused by the genus Eimeria is called eimeriosis or formerly often known as coccidiosis. Eimeria in cattle regularly affects the strength of the host, bringing about decreased meat production in beef cattle [1, 2]. Many studies have shown that under natural conditions, mixed infections of *Eimeria* spp. In this case, there are 13 species of Eimeria that can infect cattle, but the most pathogenic ones are *Eimeria zuernii* and *Eimeria bovis.* However, *E. zuernii* is not well known in Indonesia, while in Europe *E. zuernii* is the main parasite that causes losses in livestock [3].

E. bovis is a common parasite also found in cattle and has a worldwide distribution, but *E. zuernii* has a more important economic significance. *E. zuernii* is more pathogenic than *E. bovis* because it causes severe diarrhea in livestock, destroys intestinal epithelium and results in the presence of fragments of intestinal epithelial tissue found in feces. Infections in calves can be caused by an abundance of *E. zuernii* oocysts, which can result in severe diarrhea with stools containing blood, fibrin, and intestinal tissue, decreased appetite, decreased body weight, impaired feed conversion, dysentery, anaemia, and increased susceptibility to other diseases [4, 5]. Chronic infection with *E. zuernii* can develop in older animals, although *E. zuernii* mostly attack young cows. Livestock suffer greatly from diarrhea even though the stool contains little or no blood. If in these conditions the animal can survive, it will look very thin and dehydrated. But unfortunately, these animals are still able to produce oocysts and act as carriers of disease [6].

The incidence of bovine eimeriosis in Indonesia has been reported in several areas in Indonesia [2, 3, 7, 8]. East Java is a province with the highest beef cattle population in Indonesia, namely as many as 4.657.457 individuals or 27.32% of the population national [9]. Malang Regency as one of the areas in East Java has the potential large enough for livestock population development beef cattle. Apart from agro-climatic factors, markets, and appropriate community culture, Malang regency also has a large population of beef cattle compared to other districts [9]. Bovine eimeriosis in east java, especially Malang regency is need to be considered and conducted a survey of Eimeria infection in the context of early control [10]. Ekawasti et al. [3] reported 41.5% of *Eimeria* spp. Infection in East Java (Banyuwangi) by morphological using the sugar flotation method and 1.04% *E. zuernii* infection by PCR. Hastutiek et al. [11] reported that *Eimeria* spp. Oocysts were detected 32 in 50 samples (27.3%) by sugar flotation method, and 80.8% *E. zuernii* infection in cattle in Madura, east java by PCR.

The shape of sporulated oocysts has been used to identify *Eimeria species* [12], however other species demonstrate structural resemblance [13]. In Java, Indonesia, ribosomal internal transcribed spacer-1 (ITS-1) was used to establish a species-specific and precise polymerase chain reaction (PCR) test for bovine Eimeria [3]. *Eimeria* spp. Currently could be identified by PCR, which is more reliable, precise, and time-productive [3]. The ITS region is extensively used for molecular phylogeny and taxonomy because it is simple to amplify from small amounts of DNA and has a serious level of variety across firmly related species [14–16]. The ITS-1 nested PCR examine distinguished more *Eimeria* spp. From a multi-copy genomic target. The necessity for two PCR processes increases the complexity, effort, and value of the nested test, but the enhanced sensitivity was noticeable [17]. Therefore, molecular characterization is essential to

accurately assess the species, particularly using nested PCR. This study aims to identify bovine eimeriosis high pathogen *Eimeria zuernii* in cattle in Malang regency, East Java, Indonesia by nested PCR assay using ITS-1 region.

2 Materials and Methods

2.1 Ethical Approval

The study was approved by Research Ethics Committee, Faculty of Veterinary Medicine, University of Gadjah Mada, Indonesia (Nomor: 00032/EC-FKH/Int./2020).

2.2 Study Areas and Examined Cattle

The study area was Malang district, East Java, Indonesia. A total of 102 fecal samples were collected from beef cattle from subdistricts Karangploso and lawang. On each farm, stool samples were obtained at random. When fecal samples were taken, no animals had clinical signs. All samples were obtained from cow rectums, maintained in individual plastic pouches at 4 °C until laboratory examination.

2.3 Examination of the Feces

The sugar centrifugal flotation technique was used to analyze feces [3]. Briefly, 1 g of fecal samples was used and centrifuged at 800 × g for 5 min. After discarding the supernatant, 10 ml of sugar solution with a dry density of 1.2 was poured to the sediment, then centrifuged at 800 g for 5 min, and the top layer was deposited on a slide. Light microscopy 400X magnification was used to examine the whole smear.

2.4 Eimeria Oocyst Purification

Eimeria oocysts were purified using the sugar flotation technique using remaining feces (about 5–10 g) from positive samples [3]. In a brief, feces were diluted in distilled water and filtered through a metal mesh. After centrifuged at 800 × g for 5 min, sugar solution was added to the sediments, and water was layered on the sediments then centrifuged at 1200 × g for 10 min. The Eimeria oocysts that floated on the top of the sugar solution were retrieved using a Pasteur pipette and rinsed three times with distilled water [3, 7, 8]. The pure oocysts were resolved in 1–2 ml of PBS, the quantity of oocysts counted, and OPG (oocysts per 1-g feces) values estimated. Samples were stored at 4 °C until use in molecular identification [11].

2.5 Genomic DNA Extraction

Only samples containing more than 500 OPG were chosen for analysis as mentioned above [18]. Purified Eimeria oocysts in the amount of 400 μL were utilized. They were frozen and thawed five times in order to extract genomic DNA [3]. Based on the QIAamp DNA Mini Kit, 200 μL of the supernatant was utilized after centrifuged at 5400 g for 3

min (Qiagen, Hilden, Germany). PCR targeting the internal transcribed spacer 1 (ITS-1) region of the ribosomal RNA gene was used to identify *Eimeria zuernii,* as previously described [3, 17, 19]. Thermo Scientific NanoDrop Products were used to purify and concentrate the DNA extracts for DNA quantification, and the extracts were kept at 20 °C until PCR analysis with the extracted as the template [2].

2.6 Nested PCR (nPCR)

The nested PCR procedure with ITS-1 primers was developed to identify *Eimeria species*. In the genus-specific PCR phase, primers amplifying the complete Eimeria ITS-1 sequence were employed, whereas species-specific primers targeting the ITS-1 region were utilized to amplify the distinct *Eimeria species* as previously [17, 19].

Each reaction included 100 ng/μL of templates, 10 M each of genus-specific primers for genus common 348–546 bp in a 25μL reaction mixture (forward-GCA AAA GTC GTA ACA CGG TTT CCG, and reverse-CTG CAA TTC ACA ATG CGT ATC GC). 0.5 μL of MyTaq HS DNA polymerase, 200 μM each of dNTPs,1.5 mM MgCl2 and 5 μL of 5X MyTaq Reaction Buffer (Bioline, UK). An initial denaturing phase at 95 °C for 3 min was followed by 35 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 5 min. Each test contained negative, no-template controls that used distilled water in place of the template. Gel electrophoresis in 1.5% agarose gels (UltraPure™ Agarose product, Invitrogen) stained with SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific, Finland) at 100 V was used to confirm the amplification of certain nested PCR products. The bands were seen using a UV transilluminator. The primary PCR product (1.0 μl in 25.0 μl reaction mixture) was utilized as layout for the nested PCR with species-specific primers for *Eimeria zuernii* were: F: 5´-AAC ATG TTT CTA CCC ACT AC-3´, R: 5´-CGA TAA GGA GGA GGA CAA C-3´ with expected item size 344 bp [3, 19] in individual tubes utilizing a similar enhancement condition depicted above [17].

3 Results

3.1 Fecal Examination

Out of a total of 102 fecal samples for Malang regency examined by the sugar floatation method, 59 samples (35.3%) were positive for *Eimeria* spp. And 36 samples in 59 samples positive *Eimeria* spp. (57.8%) were positive *Eimeria zuernii* infection (Table 1). Although in the positive *Eimeria* spp. Sample there was mixed infection between species, this study only analysed the percentage of infection the most pathogenic bovine eimeria, *E. zuernii.*

3.2 PCR Amplification

Eighteen samples from 36 purified samples containing oocysts of more than 500 oocysts per gram (OPG) were continued to the DNA isolation stage and then performed nested PCR. The initial stage of PCR amplification is to analyze the purity and concentration

Table 1. Research regions and findings for Eimeria spp. In Malang regency, East Java, Indonesia

Subdistric	Jumlah sample	Examination		
		Floatation method	Purification	nPCR* (10mg/ul)
Karangploso	46	32	32	18
Lawang	56	27	27	18
Total	102	59 (57,84%)	59	36 (35.30%)

*Eimeria positive sample purified ≥ 500 OPG for isolation DNA, and ≥ 10 mg/µl for amplification requirement

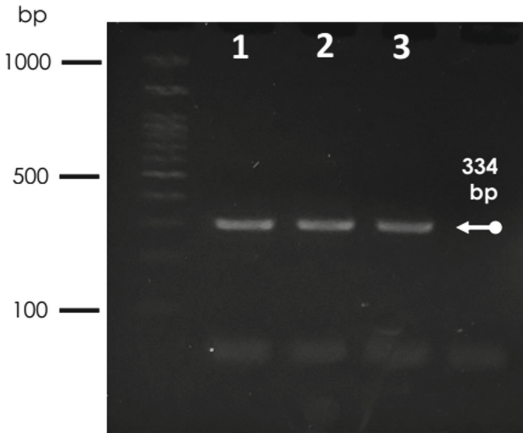

Fig. 1. Nested PCR using ITS-1 primer of *E. zuernii (334 bp)*

of DNA, where the minimum requirement for DNA purity is 1–2 (ideally 1.8–2). Meanwhile, the concentration of DNA for DNA profiling is 20 g/ml [20]. In this study, the results of DNA purity by nanodrop from existing samples showed between 1,069 g/µl and 1,265 g/µl (data not shown), so that the DNA purity requirements to proceed to the PCR amplification stage were met so that it could be used in identification. Pure DNA from *Eimeria zuernii* was used to optimize nested PCR using the ITS-1primer. Specific PCR amplicons of *E. zuernii* (334 bp) were visualised (Fig. 1).

4 Discussion

Bovine eimeriosis is a disease that is very feared by cattle farms because it can cause huge losses. Identification of bovine eimeriosis has been carried out in Indonesia by several researchers in various regions, especially in the areas of beef cattle breeding centers [10]. East Java is a province with the highest population of beef cattle in Indonesia, Malang Regency as one of the regions in East Java has a large potential for developing beef cattle population. Malang Regency also has a large beef cattle population compared to other districts [9]. This potential needs to be supported by various aspects, one of which

is animal health management. The management of young cattle under a year old needs to be monitored and improved so that production declines as a result of high pathogen *Eimeria* spp. Infection, including *Eimeria zuernii*, may be avoided. [10].

In addition to helping with illness diagnosis, accurate identification of *Eimeria* spp. is crucial for managing subclinical infections, creating and implementing efficient control measures, and conducting biological and epidemiological research [10, 21]. The morphological characteristics of oocysts, parasite biology, clinical symptoms in the infected animals, and the typical macroscopic lesions determined after necropsy have historically been used to identify *Eimeria* spp. [22]. However, mixed infections of many *Eimeria* spp. Are frequently seen in natural settings, and morphological traits and pathological alterations may overlap, trying to make correct diagnosis difficult and reducing the ability to identify subclinical illness [3, 17, 22]. Therefore, it has been advised that these techniques shouldn't be employed alone to distinguish between *Eimeria* species. Although recognized as the "gold standard" of detection for many infections, PCR tests capable of recognizing and distinguishing *Eimeria* spp. Have been available for more than 20 years. However, this technique has not yet replaced conventional coccidia diagnostics [23].

Use of PCR has been hampered by characteristics of eimerian biology such as the oocyst wall's resilience to all but mechanical destruction, access to template DNA being restricted, and PCR being inhibited by the surrounding feces. Very few research has focused on the usability of these approaches for identifying *Eimeria* spp., despite the fact that various PCR tests have been developed to identify particular *Eimeria species* [13, 24, 25]. Using Eimeria oocysts enriched by flotation in saturated sugar and performing a freeze thaw 5 times to break up the oocyst walls greatly increased the sensitivity of the PCR [3].

Purification of oocysts in positive samples of *Eimeria* spp. to obtain pure oocysts. The number of oocysts was determined after the purified oocysts were resolved in 1–2 ml of phosphate-buffered saline (PBS). DNA was extracted from isolates containing 10,000 oocysts/ml in the referenced method [11]. High accuracy when starting with over 500 oocysts per gram (OPG) in (the equivalent of 25 oocysts per PCR from the beginning of the protocol) [17]. The samples with 500 OPG or less at the start of the procedure were chosen for additional *Eimeria* species identification tests, however those with less than 500 OPG were treated to be less sensitive for PCR-based species identification [18]. It was observed that 20 oocysts of each species were insufficient for PCR-based species identification [3]. Although the test worked well with pure genomic DNA, it decreased a part of its sensitivity and variety of identification of species when used with the typical field samples [25]. Early identification of bovine *Eimeria* species is importance for effective control of clinical and subclinical eimeriosis. This can be done with PCR molecular tests which can detect quickly and accurately, especially nested PCR which is more sensitive and specific [3, 7, 8]. Contrary to standard parasitological methods, these methods need time and knowledge, both of which are becoming more and more costly and limited [24].

In the present PCR analyses, we identified *Eimeria* spp. in 59 samples by floatation method then remaininng sample purified. After counting the number of oocysts, only 36 samples with oocyst content $\geq$ 500 OPG. Hence, PCR was performed on only 36 samples containing oocysts according to amplification requirements to identify *E. zuernii*

by nested PCR analysis. All of 36 sample nested PCR assay were positive for *E. zuernii* (35.30%). No age-dependent tendency could be identified in this data.

Nested-PCR process allows to reduce contamination product during amplification of unneeded primers. Two sets primer is used to support this method, the second set amplifies the target second during the first process. The target DNA sequence from a set primers called inner primers are stored between the target sequences of the second set of the primer which is known as the outer primary. In practice, the first reaction of PCR using the outer primer, then the second PCR reaction was carried out with inner primer or nested primer using the result of the reaction product first as amplification target. Nested primer will blend with the product The first PCR and produces a shorter product than the first product [26]. Applying the ITS-1 nested PCR test previously published by Lew et al. [27] led to the discovery of additional *Eimeria* spp. Multi-copy genomic targets and a nested PCR approach. The nested test is more complicated, takes longer, and more costly because of the two PCR processes required, but the increased sensitivity was noticeable. Prevalence of *Eimeria* spp. in cattle of Indonesia reported in six papers to date of 15.33% to 85.07% [12–17] and in Madura cattle of 66.0% at Socah and 88.2% at Kamal reported by Hastutiek et al. [16] by morphological observation. These differences in prevalence might be attributable to different area, sampling season, detection method, or management strategies of the farms.

To date, there has not been much use of molecular biological techniques to distinguish between different species of bovine eimeria, but by offering protocols that support their economical, reliable, and simple application with an easy-to-interpret output, both developed and developing regions may benefit more from their use. The techniques outlined here may be created and included into standard livestock management and veterinary surveillance as the cost of PCR tools and reagents continues to decline [17]. We demonstrated that samples may be recognized to species level for *Eimeria zuernii* by the molecular approach nested PCR despite the small number of samples in our survey.

The farms in the study locations are typically built on tiny plots of land, and family groups handle management tasks like removing waste and clean enclosures. Cattle are often raised in groups of two to four in stalls that are easily transferable between farms [3, 10]. As a result, there may be an increased risk of infection and oocyst transmission in the area where cattle are kept.

5 Conclusion

The presence of Eimeria oocysts can be, typically, detected through a parasitological test using the floatation method. The floatation method in this study showed that 57,84% (59/102) samples were positive for Eimeria. However, this approach can only be providing information up to genus levels hence requires alternatives that serve more specific identification. Molecular methods such as nested PCR (nPCR) is a method that was able to specifically identify the species in the interspecies mixture of DNA. In this study, nPCR using species-specific primer ITS-1 is seen to be recognizing the species of Eimeria, specifically. This method enables us to demonstrate that up to 35.30% (36 out of 102) of our Malang-origin samples were *E. zuernii* which suggested that *E. zuernii* had spread in Malang. Owing to the high pathogenicity of the *E. zuernii*, detection and evaluation of endemicity of the species among livestock in Indonesia is of paramount importance.

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