Development of nested duplex PCR assays for detection of pathogen *Eimeria* species in cattle in Papua, Indonesia

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\textbf{Abstract.} *Eimeria bovis* and *Eimeria zuernii* are highly pathogenic *Eimeria* species in cattle that are the most prevalent causes of a severe clinical illness characterized by hemorrhagic diarrhea in calves and young cattle with potentially fatal effects over the world. The oocysts of a handful of the known bovine eimeriosis species are difficult to distinguish morphologically. For the specific differentiating evidence of *Eimeria* species, symptomatic research institutions are increasingly relying on DNA-based technologies. This research offers a duplex polymerase chain reaction (PCR) test based on the internal transcribed spacer-1 (ITS-1) gene that may be used to diagnose *E. bovis* and *E. zuernii* in cattle from various locations at the same time. The oocysts were concentrated and purified using a fecal harvesting method. The genomic DNA is extracted according to the instructions included with the kit. Primer pairs specific to each species, as well as a standard optimum annealing temperature of 55°C for these species, were discovered. The samples were amplified in a homogenous way, resulting in a homogeneous band ladder, revealing that the test could distinguish between two highly pathogenic *Eimeria* species in one tube reaction. This duplex PCR assay can detect a high pathogenic bovine *Eimeria* simultaneously in a rapid and low cost.

\textbf{Keywords:} *Eimeria bovis*, *Eimeria zuernii*, gastrointestinal, molecular, protozoa.

1. \textbf{Introduction}

Bovine eimeriosis is a disease caused by the apicomplexa parasite of the genus *Eimeria* in cattle. This disease is one of the important diseases of the digestive tract which is generally called coccidiosis [1]. It is considered one of the top five illnesses in terms of economic impact in the industry of cattle [2]. *Eimeria* spp. are strictly host-specific, and more than 20 species of *Eimeria* spp. are defined in cattle [3]. *Eimeria bovis* and *Eimeria zuernii* are *Eimeria species* that have high pathogenicity that can cause high morbidity and mortality, especially in calves. This parasite can prevent nutrients from being absorbed in the intestines, resulting in diarrheal stools containing blood, fibrin, and intestinal tissue [3, 4]. While the frequency varies by nation or farm management method [5], the economic losses caused by *Eimeria* infections in cattle are estimated to reach USD 400 million worldwide [6].
In 2018, the cattle population in Indonesia was estimated to be about 16.5 million beef cows [7]. Beef cows is one of the sources of the needed food in the form of meat in Indonesia. Indonesia's agricultural ministry has recently begun establishing strategic plans to develop food self-sufficiency initiatives [7]. Papua Island (786,000 km2) is the first largest island in Indonesia and is a breeding ground for Bali cattle (local Indonesian cow). West Papua Province has good environmental conditions for the development of beef cattle and also has the potential to provide sufficient land and feed for livestock needs. Cattle are one of the important sources of meat production in Papua. Cattle farms in Papua are generally maintained on a small farm scale, which is often constrained by the presence of the gastrointestinal parasitic disease coccidiosis infection in cattle which can reduce livestock production [8].

The shape of sporulated oocysts has been used to identify *Eimeria species* in the past, however some species have morphological similarities [9]. However, PCR prevalence of bovine *Eimeria* spp. was reported in fewer papers. PCR is commonly employed in diagnostics to identify the presence of a certain DNA sequence and to establish precise species identifications. PCR technology, which delivers acceptable and specific products, particularly in the field of genetic diversity characterization and conservation [10]. In Java, Indonesia, Ekawasti et al. [5] reported a specific and sensitive PCR test for bovine *Eimeria*. However, this method has not been used to the simultaneous detection of numerous species. We first describe the invention of a nested duplex PCR molecular diagnostic tool for identifying a highly pathogenic *Eimeria* species specific in cattle. Furthermore, compared to regular PCR, duplex PCR designed to detect several targets concurrently saves time and budget [11,12]. The goal of this work was to create a nested duplex PCR test that could simultaneously detect *E. bovis* and *E. zuernii* as a serious pathogen and to report the frequency of bovine eimeriosis in Papua, Indonesia.

2. **Materials and Methods**

2.1. *Ethical approval*

The study was authorized by the Research Ethics Committee of the University of Gadjah Mada's Faculty of Veterinary Medicine (Approval No.: 00032/EC-FKH/Int./2020).

2.2. *Collections of fecal samples*

A total of 70 beef cattle fecal samples were collected in October 2020 from two districts in Papua, namely Biak and Serui. This city is located between 0-25 meters above sea level, with an annual temperature range of 23 to 34°C, a relative humidity of 80.0 percent, and an average annual rainfall of 154.9 millimeters. When fecal samples were taken, no animals displayed clinical signs. The samples were obtained from cow rectums, maintained in separate plastic bags, and kept at 4°C until laboratory testing.

2.3. *Sugar floatation method*

Following a recent publication [5], fecal samples were analyzed qualitatively using the sugar flotation technique. In a brief, 1 g of feces was diluted in 9 mL of water and centrifuged at 800 g for 5 minutes. The supernatant was then discarded. The sediment was then treated with 10 mL of sugar solution with a specific gravity of 1.2 (e.g., 100 g of sugar added to 120 mL of distilled water). The sample was then centrifuged at 800 g for 5 minutes before being put on a glass slide. Microscopy was used to examine the whole smear.

2.4. *PCR assay*

The sugar flotation technique was used to purify *Eimeria* oocysts using the remaining feces (about 5–10 g) from positive samples. The feces were diluted in distilled water and then filtered through a steel mesh. Sugar solution was added to the sediments after centrifugation at 800 g for 5 minutes, then distilled water was overlaid and centrifuged at 1200 g for 10 minutes. The *Eimeria* oocysts that floated on top of the sugar solution were collected with a pasteur pipette and rinsed three times with distilled water. Finally, 1–2 ml of PBS was used to dissolve the purified oocysts, which were then kept at 4°C.
2.4.1. DNA extraction
Individual fecal aliquots (200 mg each) were combined with 0.5 mL of DNAzol® (Molecular Research Center, OH, USA) to extract DNA from Eimeria spp. Following that, samples were processed according to the DNAzol® manufacturer’s instructions, with the exception that after being diluted with this reagent, samples were frozen and thawed three times to break the cysts. PCR reactions targeting the ITS1 gene were done using the following primer pairs using the extracted DNA as templates.

2.4.2. PCR Primer and molecular identification of Eimeria spp
Primer sequences of Eimeria (Genus specific) sequence in internal transcribed spacer 1 (ITS-1) region for first PCR were: F: 5’- GCA AAA GTC GTA ACA CGG TTT CCG -3’, R: 5’- CTG CAA TTC ACA ATG CGT ATC GC-3’. Primer species-specific sequences for second nested duplex PCR of E. bovis were: F: 5’-TCA AAC ATC ACC TCC AA-3’, R: 5’-ATA ATT GCG ATA AGG GAG ACA-3’ with expected product size 238 (bp). Primer sequences of E. zuernii were: F: 5’-AAC ATG TTT CTA CCC ACT AC-3’, R: 5’-CGA TAA GGA GGA GGA CAA C-3’ with expected product size 344 bp [5, 13].

All samples were examined using nested-PCR (nPCR) for screening the animals and finding positives and negatives before doing nested duplex PCR in field samples. The nPCR was done with ITS1 primers and was used as a reference to determine if the samples were positive or negative for the molecular testing. This decision was based on evidence from the literature indicating the ITS1 region of the Eimeria genome is the most conserved. The PCR settings for E. bovis included an initial denaturing phase of 30 seconds at 94°C, followed by 35 cycles of 10 seconds at 94°C, 20 seconds at 55°C, 20 seconds at 72°C, and 20 seconds at 72°C, with a final extension of 2 minutes at 72°C. The reaction conditions for E. zuernii included an initial denaturing phase of 30 seconds at 94°C, followed by 35 cycles of 10 seconds at 94°C, 20 seconds at 52°C, 20 seconds at 72°C, and 20 seconds at 72°C, with a final extension of 2 minutes at 72°C. The first and second PCR programs were carried out under the identical circumstances as before. All reactions were carried out at the same time and at the same temperature. 1.5% agarose gel with a 100 bp DNA ladder was used to electrophorese 10 µl of PCR products. UV transilluminator was used to see the enhanced products.

2.4.3. Development of nested duplex PCR assay
The duplex nPCR was done with the same primer concentrations as the previous test after the morphological test and individual nPCR with each pair of primers. The size of diagnostic fragments of PCR results was different for all two high pathogen Eimeria species, according to the nested duplex PCR devised and tested in this work. The species-specific product sizes for E. bovis and E. zuernii were 344 and 238 bp, respectively (Figure 2.). PCR was carried out in a Thermo Scientific™ Arktik™ Thermal Cycler (Thermo Fisher Scientific, Finland). The steps of the nested PCRs and nested duplex PCR assays were performed using 25 µL reactions containing 1 µL of DNA template, 0.25 M each primer, 1.5 mM MgCl2, 0.01 U Taq DNA polymerase, and 0.2 mM dNTP. The PCR conditions for nested duplex included an initial denaturing phase at 94°C for 5 min followed by 35 cycles which at 94°C for 30 sec, 55°C for 1 min, 72°C for 2 min with a final extension at 72°C for 2 min. Electrophoresis for PCR products was performed using 1.5% agarose and SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific, Finland), run at 100 Volt. The bands were visualized using a UV transilluminator.

3. Results and Discussion
The progress of cattle with clinical coccidiosis is mostly influenced by many factors, such as the species of Eimeria, number of consumed oocysts, age of affected animal, and breeding system; besides the management practices. The only practical way to recognize bovine Eimeria spp. is the detection of oocysts’ morphology. Nevertheless, the morphology of oocysts is not comprehensively efficient as numerous Eimeria spp. Have confusing features besides its intraspecies dissimilarity [14]. In addition, fecal inspection in conjunction with morphological identification is very intensive work that
requires a skilled method. *Eimeria* detection and differentiation has a higher sensitivity than the traditional method of identifying oocysts and is regarded as a useful technique for diagnosing bovine eimeriosis infection [13,14]. Therefore, the present study determined the prevalence of *Eimeria* species infecting using oocysts morphological features and developed the nested duplex PCR assay.

### 3.1 Morphological features by the sugar flotation method

Our fecal investigations using the sugar flotation technique revealed that *Eimeria* spp. were present (64.28%). Utilizing the sugar flotation process, the positive results were from 57.14% in Biak and 67.35% in Serui (Table 1.). Species identification of *Eimeria* by morphologically in deep oocysts could not be carried out. *Eimeria* species oocysts have almost the same shape and size overlapping each other [5]. The PCR method was used to identify and discriminate *Eimeria* spp. using the ribosomal 1 (ITS-1) internal transcription spacer [5, 13].

| No. | District | No. of Examined cattle | Examination |
|-----|----------|------------------------|-------------|
|     |          |                        | Floatation method | Nested Single PCR | Nested Duplex PCR |
|     |          |                        | Positive | E. bovis | E. zuernii | E. bovis | E. zuernii | % |
| 1   | Serui    | 49                     | 33       | 67.35 | 15 | 15 | 15 | 45.45 |
| 2   | Biak     | 21                     | 12       | 57.14 | 9  | 9  | 9  | 75  |
| Total |         | 70                     | 45       | 64.28 | 24 | 24 | 24 | 53.33 |

Based on Table 1. It shows that the results of the morphological examination of the sample using the flotation test method are 64.28% *Eimeria* spp. which could not be distinguished by species, while in PCR analysis 53.33% was positive for *E. bovis* and *E. Zuernii* either by nested sPCR or nested dPCR. This shows that both PCR methods using purified DNA have the same sensitivity and specificity. *Eimeria* in PCR analysis might be affected by the number of oocysts in feces or contaminated PCR inhibitors. In the described approach, DNA was isolated from isolates having 10,000 oocysts/ml, and a modest number of oocysts (20 oocysts per sample) had been proven that utilizing PCR to identify *Eimeria* species was inadequate [5,13]. The sampling location is a tiny farm with two to four cows housed in a single enclosure, and the animals often migrate between farms, making them easier to taint [15]. As a result, the surroundings in which cows reside may change the possibility of oocysts and diseases being transmitted.

### 3.2 Nested duplex PCR assay

Detecting and differentiating *E. bovis* and *E. zuernii* in stool samples is critical for determining the real frequency of pathogenic *Eimeria* spp. in the population and for correct diagnosis of eimeriosis. For the reliable identification of *E. bovis* and *E. Zuernii*, many DNA-based molecular approaches have been investigated [5,13]. Because nested PCR enhances sensitivity, it was employed in this investigation. Even after purification, clinical materials such as feces can include PCR inhibitors. The two cycles of PCR may have helped to compensate for the inhibitors seen in clinical sample. The concentration of the initial PCR product may be too low to detect using stained gels and a UV transilluminator. Using a UV transilluminator and agarose gel electrophoresis with stain, the detection limit is approximately 10 ng of DNA [13]. In the nesting process, the first PCR product may be sufficient to provide a suitable template for the synthesis of a second PCR product, allowing to be identified [16].

The current work is the first to use nested duplex PCR for simultaneous identification of *Eimeria* species that infect domestic cattle. In the first experiment, the specificity of *E. bovis* and *E. zuernii* markers was determined in individual responses (Figure 1.). According to Fernandez et al. [17], we...
started standardizing the duplex reaction by altering the following parameters: extension and annealing temperature and duration, number of cycles, buffer, MgCl₂, and dNTP concentration, primer and enzyme concentration. Additionally, touchdown protocols and varying ramping periods between the annealing and extension processes were explored. The *E. bovis* and *E. zuernii* markers consistently showed a pattern of amplification bands after extensive testing. This novel collection of markers was put to the test, and the results confirmed their species-specific character. The two primer pairs were proven to be species-specific in a nested duplex PCR utilizing DNA samples from each *Eimeria* species (Figure 1). A sample containing a DNA combination of the two species was also used to test this set of primers. Figure 2 shows a consistent band ladder, suggesting that the assay allowed for the simultaneous detection of all species in one tube reaction.

**Figure 1.** PCR analysis of *Eimeria bovis* and *E. zuernii*: (a) Nested single PCR, (b) Nested duplex PCR; Lane M, molecular size marker; *E. bovis*, 238 bp and *E. zuernii*, 344 bp.

When compared to other tests that simply focus on detecting each species separately, single PCR and duplex PCR findings are similar, meaning that a significant savings in plastics and reagents can be realized. Furthermore, because two species may be identified in a single tube, the test provides for higher throughput, allowing it to be used in large-scale investigations with little human resources. Aside from the benefits of nested duplex PCR, standardization of this type of test is far more challenging than normal individual amplification procedures. Because numerous primers are employed in the same reaction, each with a varied binding efficiency, diverse reaction kinetics occur at the same time, resulting in a variety of amplification patterns [17]. Even intricate chemical cross-interactions that might occur during the cycle can amplify this trait, affecting primer binding kinetics and resulting in different amplification rates for each product. Following the standardization of the reaction conditions, potential variables that could affect the efficiency of the reaction were investigated, including the use of different enzymes and thermocyclers, as well as the impact of a high concentration of one species on the amplification rate of the others. The test proved to be exceedingly robust and trustworthy in the absence of these circumstances. According to Fernandez et al. [17], the DNA yield is not proportional to the number of oocysts, especially when the parasite concentration is low. This observation might be attributed to a reduction in efficiency of oocyst mechanical disruption in low level of concentration solutions. *Eimeria* oocysts have been found to be more chemical and enzymatically resistant. Several techniques based on chemical breakdown of the oocyst shell, as well as long-term incubations and freeze-thawing the oocyst, have been created to overcome this limitation [5,18].

The duplex PCR test described here might be developed into multiplex PCR and transformed to a quantitative assay in the future with the introduction in real-time PCR. This method would be extremely valuable in epizootiological studies since it would allow researchers to evaluate dynamic changes in bovine eimeriosis, treatment rotation, and other factors. The total cost of large-scale surveys remains prohibitively high due to the intricacy of the technology utilized in this test. Nonetheless, this promising approach is expected to gain popularity in the near future, particularly among reference laboratories,
leading to the modification of existing tests. To the best of our knowledge, this is the first publication of a nested duplex PCR test based on ITS1 markers, which might be a novel approach to generating new molecular tools for the diagnosis of bovine eimeriosis.

4. Conclusion
According to the current investigation, infection with *Eimeria* spp. (64.28%) was found. Our findings indicate that *E. bovis* and *E. zuernii* are common in Papua, Indonesia. It is critical to investigate the pathogenic *Eimeria* species’ endemicity on farms. Nested dPCR is an approach for detecting and distinguishing *E. bovis* and *E. zuernii* as pathogen species specificity. The test is extremely specific, sensitive, rapid, and affordable for the simultaneous identification of two highly pathogenic *Eimeria* species. Sensitivity and specificity of nested duplex PCR are identical to those of nested single-PCR.

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