Identification of bovine *Eimeria* species pathogenic using PCR ITS-1 in Indonesia

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Abstract. *Nurcahyo RW, Ekawasti F, Firdausy LW, Ninditya VI, Nasrulloh MF, Kurniawati DA, Haryadi FR, Prastowo J, Priyowidodo D. 2023. Identification of bovine* Eimeria *species pathogenic using PCR ITS-1 in Indonesia. Biodiversitas 24: 4684-4689.* Bovine eimeriosis is one of Indonesia's cattle's most significant health issues caused by the intestinal protozoan parasite *Eimeria* spp. *Eimeria* spp., *E. zuernii, E. alabamensis, E. aburnensis*, and *E. bovis* are recognized as most pathogenic since they cause clinical symptoms. This study aimed to identify pathogenic *Eimeria* spp. and explore their evolutionary relationships using the molecular marker, the internal transcribed spacer-1 (ITS-1). A total of 171 fecal samples were collected from the provinces of Banten, Lampung, and West Java, and 43 (25.1%) were infected with oocysts of *Eimeria* spp. by microscopic examination. The positive samples were further confirmed for different *Eimeria* species by amplifying and sequencing the ITS-1 gene. After editing and alignment, the sequences were considered for analyses, and the phylogenetic analysis confirmed four species of *Eimeria* such as *E. bovis* 39 (22.8%), *E. zuernii* 23 (13.5%), *E. alabamensis* 7 (4.09%), and *E. aburnensis* 25 (14.6%). In this study, several *Eimeria* species were related to overseas species; therefore, additional risk analysis is needed to control the spread of bovine eimeriosis. It is possible to learn important details regarding the ecology and population genetics of *Eimeria* spp. by employing sequencing and phylogenetic analysis to categorize all positive samples of the eimeriosis as pathogenic *Eimeria* spp.

Keywords: Bovine, *Eimeria*, Java, pathogenic, phylogenetic, Sumatra

INTRODUCTION

Developing an Indonesian program to achieve food self-sufficiency in the cattle industry is a strategic endeavor. Livestock are the primary source of income and subsistence for the majority of Indonesia's rural people. According to the MoARI (2018), there are more than 16.5 million beef cattle in Indonesia, and the primary regions for beef cattle production are Lampung, Banten, and West Java. In rural areas, the small commercial system is operated by family units, and due to the farmer-dependent management method, there are fewer than 50 cows per farm (Ekawasti et al. 2021). However, the production is greatly affected by gastrointestinal (GI) parasitism (Dey et al. 2021). There are currently few data available regarding the spread of GI parasites in Indonesia, particularly the species of *Eimeria* in cattle. *Eimeria* spp., the intestinal protozoan parasite, causes eimeriosis in cattle and has a significant economic impact on the livestock sector, especially cattle (Ekawasti et al. 2021).

Coccidia is a protozoan parasite from the Apicomplexa phylum that can infect all vertebrate species and is highly diverse, with estimates of the genus being more than a thousand species (Blake 2015). According to Girard et al. (2016), the *Eimeria* is a monoxenous coccidian that typically infects a single host during its life cycle. *Eimeria* are obligate intracellular parasites that play important roles in medical and veterinary care (Wiedmer et al. 2020). Watery or bloody diarrhea is the most common clinical symptom of coccidiosis in cattle, followed by a lack of appetite, depression, dehydration, and weight loss, which results in stunted growth. The digestive tract is disrupted by eimeriosis, which delays growth and lowers meat quality in beef cattle (Ekawasti et al. 2021; Zefanya et al. 2021). Few *Eimeria* species can induce clinical signs, and less than 12 different species of *Eimeria* have been found in cattle (Sánchez et al. 2008). The clinical indications listed above are caused by *E. bovis, E. zuernii, E. alabamensis,* and *E. aburnensis* (Li et al. 2021). According to Matjila and Penzhorn (2002) and Bruhn et al. (2011), the global economic cost of eimeriosis in cattle is estimated at approximately USD 400 million. The annual economic losses from both clinical and subclinical coccidiosis have gradually increased and are estimated to be over USD 723 million worldwide, according to Koutny et al. (2021). The risk of environmental pollution can be decreased, and

subclinical production losses can be avoided by preventing eimeriosis (Keeton and Navarre 2018).

Eimeria species can be identified by observing sporulated oocyst morphology, morphometry, and oocysts in feces during the active stage of infection under a microscope (Chapman et al. 2013; Alam et al. 2020). When four sporocysts containing two sporozoites are present, the sporulated oocysts of *Eimeria* species may typically be distinguished from those of other coccidias. The shape and size of the sporulated oocysts of *Eimeria* spp. are the key morphological features for the identification (Daughschies and Najdrowski 2005). ITS-1 can be an important DNA marker for identifying many diseases, including *Eimeria* infection (Alam et al. 2021). Because it is easy to amplify small amounts of DNA and there is significant variation between closely related species, the ITS region is often employed for molecular phylogeny and taxonomy (Ekawasti et al. 2023). Therefore, the present study was designed to identify pathogenic *Eimeria* spp. in beef cattle in major cow breeding regions, including Lampung, Banten, and West Java of Indonesia. It also aimed to explore their evolutionary relationships using a molecular marker, ITS-1.

MATERIALS AND METHODS

Ethical approval

Research Ethics Committee, Faculty of Veterinary Medicine, University of Gadjah Mada, Indonesia (No.: 00032/EC-FKH/Int./2020) authorized the study.

Study area and samples

We selected Lampung, Banten, and West Java Provinces in Indonesia, the primary beef cattle producers of the country. A total of 171 fecal samples of beef cattle were collected from two districts in Banten (Serang City and Tangerang Selatan City), one in West Java (Bogor District) and one in Lampung (Lampung Tengah District), each with more than two farms (Figure 1). Every sampling site had a year-round average temperature of about 25ºC. Samples were collected from the rectum of cattle, kept at 4°C in distinct plastic bags, and shifted in the laboratory. The samples were separated into the three categories of host's age: calves (under 1 year old), between 1 and 2 years old, and over 2 years old.

Examination of samples

Feces samples were examined microscopically for the presence of oocysts. Oocysts were separated by flotation methods for screening using sugar centrifugation modified based on a previous report (Matsubayashi et al. 2005; Fujino et al. 2006; Ekawasti et al. 2019). Briefly, 1 g of fecal sample was diluted in 9 mL of distilled water and centrifuged at 800 g for 5 min. The supernatant was discarded, and 10 mL of sugar solution with a specific gravity of 1.2 was added to the sediment. Then, the test tube was centrifuged at 800 g for 5 minutes, and added sugar solution to make the test tube full to the brim. A coverslip was placed onto the test tube, waited 3 minutes to float the oocysts, and examined under a microscope.

Using the Whitlock method, a quantitative examination was performed by counting the number of oocysts per milliliter. Purification of oocysts from mixed isolates containing each *Eimeria* spp. with 10.000 oocysts/mL was used in the PCR assays with species-specific primer sets (Ekawasti et al. 2019).

Purification of *Eimeria* oocyst

The first step involved diluting the feces in distillate water and filtering them through steel mesh. A sugar solution was added to the sediments after spinning at 800 g for 5 min, and then the sediments were covered with distilled water and centrifuged at 1,200 g for 10 min. Using a Pasteur pipette, the oocysts that floated on the sugar solution's surface were retrieved and cleaned three times with distilled water. The isolated oocysts were kept at 4ºC after being resolved with 1-2 mL of PBS.
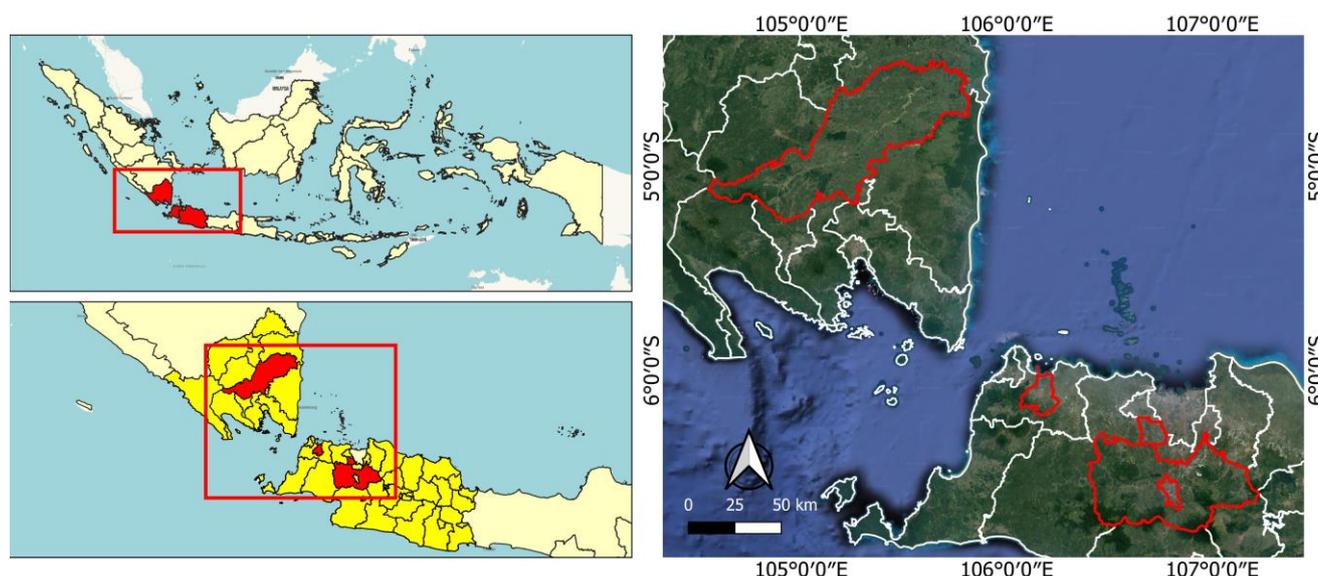

Figure 1. The locations of study area on Sumatra and Java Islands, Indonesia

DNA extraction, PCR, and sequencing

Moreover, 400 µL of previously purified *Eimeria* oocysts were re-suspended in DNAzol for DNA extraction. Five freeze-thaw cycles were performed on them to liberate the genomic DNA (Ekawasti et al. 2019). According to the DNAzol protocol, 200 µL of the supernatant was used after centrifugation at 5400×g for 3 min. Internal transcribed spacer 1 (ITS-1) was used as a target gene to identify four bovine pathogenic *Eimeria* spp species (*E. bovis, E. zuernii, E. alabamensis*, and *E. auburnensis*), as previously reported (Ekawasti et al. 2019; Ekawasti et al. 2022).

A 25 µL reaction mixture including 5 µL of DNA template, 12.5 µL of master mix (My Taq™ Mix, Bioline), 1 µL of each primer at a concentration of 10 pmol, and 5.5 µL of ddH2O was used for the experiment. Furthermore, for 35 cycles, the amplification reaction was maintained at 94ºC for 10 sec, 55ºC for 20 sec, and 72ºC for 20 sec. Following amplification, 10 µL of PCR product were electrophoretically separated on a 1.5% agarose gel using fluorosafe and a marker 100 bp DNA ladder from Geneaid and visualized on a UV transilluminator.

The representative sample location for subsequent sequence analysis in both directions by Macrogen was then determined to be sampled with bands ranging in size from 180 to 350 bp during electrophoresis.

Analysis data

The result of the stool examination was analyzed descriptively, while CLC was also used to assess the top replacement models and optional parameter settings. The Jukes Canter was used as the substitution model and an optional parameter to build the phylogenetic tree.

RESULTS AND DISCUSSION

Eimeriosis was detected in all three provinces' sampling locations' districts by microscopic examination with an overall prevalence of 25.1%. The Bogor District in West Java had the lowest infection rate (6%), whereas Lempuyang Bandar District in Lampung had the highest eimeriosis rate (36%). Additionally, we determined four species of bovine *Eimeria*, namely *E. auburnensis, E. alabamensis, E. zuernii,* and *E. bovis. Eimeria bovis* was found frequently (22.8%), followed by *E. auburnensis* (14.6%), *E. zuernii* (13.5%), and *E. alabamensis* (4.1%). All four species of *Eimeria* were found in the study area except Bogor District of West Java, where only two species of *Eimeria,* including *E. bovis* and *E. zuernii,* were found. Overall, mixed infection of pathogenic bovine *Eimeria* species dominated the occurrence of bovine eimeriosis in this study. The mixed infections in each province were varied; the details are summarized in Table 1.

According to the age of the host, calves aged less than one year (90.0%) were more prone to *Eimeria* infections than that young (66.7%). Least infections were found in adults over two years old (16.4%). Additionally, we separated the host's age into three categories: calves (under 1 year old), between 1 and 2 years old, and over 2 years old. The most frequent *Eimeria* species in each age range of hosts was *Eimeria bovis*, while the least frequent was *E. alabamensis* (Table 2). The phylogenetic analysis revealed that pathogenic *Eimeria* species from Indonesia were grouped with sequences from *Eimeria* species from various geographic origins in the same clade (Figure 2).
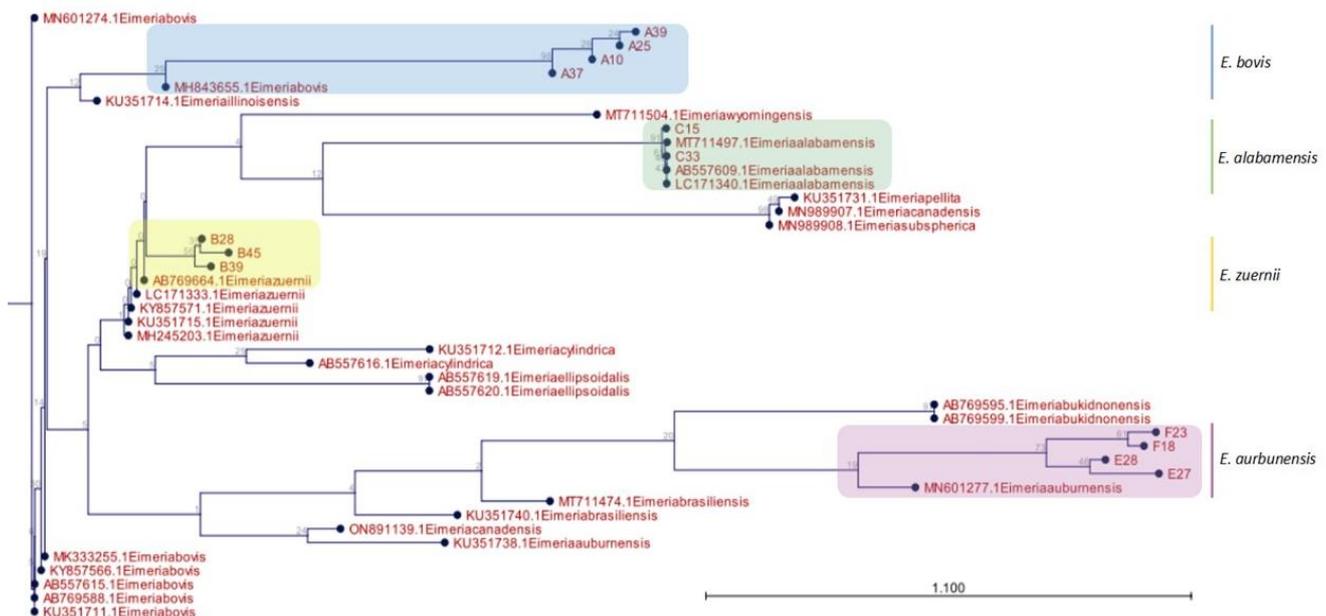
Figure 2. Phylogenetic analyses of pathogenic *Eimeria* spp. Sequences from the present study and other relevant sequences. The phylogenetic tree was constructed based on partial sequences of ITS-1 by CLC sequence viewer version 8.0.

Table 1. Summary of study areas and results for *Eimeria* spp.

| No. of region | Name of province | District | No. of sample | Floatation method | PCR method* | | | | Notes |
					E.b 238bp	*E.z* 344bp	*E.alab* 184bp	*E.aubu* 295bp	
I	Banten	Tangerang Selatan	60	18 (30%)	17	7	1	11	Mixed infection with two species (11), three species (2), four species (1)
		Curug Serang	39	14 (35.6%)	11	10	4	10	Mixed infection with two species (2), three species (8), four species (1)
II	Lampung	Lempuyang Bandar	22	8 (36%)	8	3	2	4	Mixed infection with two species (2), three species (2), four species (1)
III	West Java	Bogor	50	3 (6%)	3	3	0	0	Mixed infection with two species (3)
Total			171	43 (25.1%)	39 (22.8%)	23 (13.5%)	7 (4.09%)	25 (14.6%)	Mixed infections were identified in 33 of 43 samples

Note: *E.b: Eimeria bovis, E.z: Eimeria zuernii, E.alab: Eimeria alabamensis, E.aubu: Eimeria auburnensis*

Table 2. Summary of ages and results for *Eimeria* spp.

| No of region | Age (year) | No. of sample | No. of sample positive PCR | PCR analysis* | | | | Notes |
				E.b 238bp	*E.z* 344bp	*E.alab* 184bp	*E.aubu* 295bp	
I	<1	10	9 (90%)	9	3	3	9	Mixed infection with two species (4), three species (1), four species (2)
II	1-2	15	10 (66.7%)	8	6	0	2	Mixed infection with two species (6), three species (1),
III	>2	146	24 (16.4%)	20	14	4	14	Mixed infection with two species (8), three species (10), four species (1)
Total		171	43 (25.1%)	39 (22.8%)	23 (13.5%)	7 (4.09%)	25 (14.6%)	Mixed infection were identified in 33 of 43 samples

Note: *E.b: Eimeria bovis, E.z: Eimeria zuernii, E.alab: Eimeria alabamensis, E.aubu: Eimeria auburnensis*

In this study, *Eimeria bovis* from Banten (A10, A39), Lampung (A25), and West Java (A37) were clustered in the same clade with sequences of *E. bovis* from isolate Yogyakarta, then *Eimeria zuernii* from Banten (B39, B45), Lampung (B28), and Japan were clustered in the same clade with sequences of *E. zuernii*. *Eimeria auburnensis* from Banten (F18); Lampung (E27, E28, F23) were clustered with sequences of *E. auburnensis* from isolate India, as were *Eimeria alabamensis* from Banten (C15) and Lampung (C33). The differences in the specific *Eimeria* detection capabilities of the known molecular techniques may not be sufficient to detect regional *Eimeria* species accurately. A more sensitive primer-based approach based on local *Eimeria* strains is thus necessary to increase the sensitivity of detecting bovine coccidiosis on surrounding farms (Loo et al. 2019).

According to a previous study by Ekawasti et al. (2021), the provinces with the highest percentage of bovine eimeriosis were Lampung, Banten, and West Java Province. Compared to Java Island (Banten and West Java Province), Lampung Province on Sumatera Island has a greater rate of bovine eimeriosis. Season, sampling-related temperature variations, and other risk variables such as livestock upkeep management can all affect the variation in occurrence from year to year (Ekawasti and Wardhana 2019).

Bovine eimeriosis is more common and noticeable in calves than in adult cattle, which includes abdominal pain, fever, watery to bloody diarrhea, and dehydration (Kim et al. 2018). It also typically affects calves that are 3-6 months old or have been weaned (Ayana et al. 2022; Hamid et al. 2016; Taylor et al. 2016; Dong et al. 2012). The calf group (under one year old) also had the highest percentage of infection in this instance, and we again obtained the same conclusion. Besides the *Eimeria* species pathogenicity, adult cattle may acquire immunity from prior exposure, whereas in calves, either the cessation of colostrum feeding after weaning, which affects their passive immunity, or the immaturity of their gut-associated lymphoid tissue (GALT), is likely to be the cause of bovine eimeriosis in calves (Hamid et al. 2016; Li et al. 2021; Malek and Kuraa 2018; Taylor et al. 2016).

At least 20 *Eimeria* species are known to cause bovine eimeriosis, with 12 species being the most prevalent. i.e., *E. alabamensis, E. auburnensis, E. bovis, E. brasiliensis, E. bukidnonensis, E. canadensis, E. cylindrical, E. ellipsoidalis, E. pellita, E. subspherica, E. wyomingensis*, and *E. zuernii* (Taylor et al. 2016). According to their pathogenicity, the species of *Eimeria* in cattle are divided into three groups: highly pathogenic (*E. bovis* and *E. zuernii*), moderately pathogenic (*E. ellipsoidalis, E. alabamensis, E. auburnensis,* and *E. subspherica*), and non-pathogenic (*E. brasiliensis, E. bukidnonensis, E. canadensis, E. cylindrical, E. pellita,* and *E. wyomingensis*) (Lasen et al. 2009). Various morphologically and molecularly identification methods are useful to determine

the *Eimeria* species and their pathogenicity. Here, we differentiate molecularly and present the sequence analysis of bovine eimeriosis from three different locations in Indonesia with the phylogenetic relationship between samples and other *Eimeria* species available in GenBank, which will contribute to the molecular epidemiology and *Eimeria* diversity in cattle populations.

The study uses phylogenetic analysis to show the relationship between studied samples and other *Eimeria* species retrieved from GenBank. Furthermore, we distinguish *Eimeria* species molecularly and present the sequence analysis of bovine eimeriosis from three locations in Indonesia. This work will help with molecular epidemiology and diversity patterns of the *Eimeria* population in cattle.

Previous research has identified six to ten *Eimeria* species that regularly infect cattle in Indonesia.: *E. bovis, E. zuernii, E. alabamensis, E. auburnensis, E. cylindrical, E. ellipsoidalis, E. bukidnonensis, E. canadensis, E. wyomingensis,* and *E. brasiliensis* respectively, in both categories within location and cattle age (Ekawasti et al. 2019; Hamid et al. 2016; Sufi et al. 2017).

Eimeria bovis is known as the most pathogenic and dominant *Eimeria* species causing bovine eimeriosis worldwide, including in tropical areas such as Indonesia (Ekawasti et al. 2022; Hastutiek et al. 2022; Hamid et al. 2016; Sufi et al. 2017), Ethiopia (Ayana et al. 2022), Bangladesh (Deb et al. 2022), or subtropical areas in the Mediteran (Morgoglione et al. 2020) and Brazil (Cardim et al. 2018).

Our result indicates that the pathogenic species of bovine *Eimeria* are widespread in Banten, West Java, and Lampung Province, which is potentially a source of infection, particularly in areas of dense cattle breeding. Control of *Eimeria* cattle can be done by properly managing the stable according to the conditions of each cage and accompanied by regular inspection controls. Control with early detection using appropriate and accurate methods can design an efficient early control strategy.

In addition, in our BLAST of the National Center for Biotechnology Information (NCBI) result, only *Eimeria bovis* has a relationship with the nearest region in Indonesia, Yogyakarta. In contrast, the similarity of the others was close with *Eimeria* spp. from Japan and India. These findings suggest that continuous surveillance and epidemiological study of *Eimeria* spp. are needed to prevent unexpected outbreaks in livestock in Indonesia.

The clinical symptoms may vary depending on the age of the animal, the OPG (oocyst per gram) load, and the pathogenicity of the *Eimeria* species (Enemark et al. 2013; Bangoura et al. 2012; Daughschies and Najdrowski 2005). The bovine eimeriosis in our study shows that mixed infection is more prevalent than single infection (Ekawasti et al. 2022; Hamid et al. 2016; Sufi et al. 2017), but unfortunately, in this study, we did not analyze the correlation between the OPG of each species and the consistency of feces or the clinical sign in the host. Besides the age difference, environmental factors also play a role in the danger of spreading *Eimeria* infection in cattle. In several areas in Indonesia, the pathogenic species *Eimeria*

has been proliferating, with the highest percentage shown by *E. bovis*.

Finally, some *Eimeria* species were found to be related to abroad species in this study, implying that more risk analysis is required in attempts to restrict the spread of bovine eimeriosis It is possible to learn important details about the ecology and population genetics of *Eimeria* spp. by employing sequencing and phylogenetic analysis to categorize all positive samples of the eimeriosis as pathogenic *Eimeria* spp. It is necessary to pay attention to the endemicity of the pathogenic species *Eimeria*, which is related to improving the health and productivity of cattle in Indonesia.

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