

Molecular Detection of *Eimeria bovis* in Indonesian Beef Cattle Using Nested PCR Technique

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ARTICLE INFO

Article history:

Received March 1, 2024

Received in revised form May 13, 2024

Accepted May 27, 2024

KEYWORDS:

Beef cattle,
Coccidiosis,
E. bovis,
ITS-1,
nPCR

ABSTRACT

Eimeria bovis is a pathogenic protozoan that causes cattle digestive tract infections, which can cause economic losses to farmers. It is necessary to develop specific and accurate detection methods to conserve livestock and prevent coccidiosis in Indonesia. This study aims to detect *E. bovis* by nested PCR and determine the relationship with reference sequences. A total of 167 samples of beef cattle feces were taken randomly from community farms spread across 18 provinces in Indonesia. The feces were examined natively, and then the oocysts were purified by the sugar flotation method, extracted by KIT extraction, and amplified by the nPCR technique. Positive samples were followed by sequencing and phylogenetic analysis using MEGA 11 software. This study used two pairs of primers (outer and inner) taken from ITS-1 molecular markers. As many as 96 out of 167 samples (57.5%) were positive for *Eimeria* spp., and 48 of the 96 samples were positive for *Eimeria* spp. (50%) were detected to be positive for *E. bovis* based on the presence of a 238 bp DNA fragment. The results of the phylogenetic analysis showed that the study sample formed a separate cluster from the *E. bovis* cluster from abroad. In conclusion, *E. bovis* was detected in 16 out of 18 provinces in this study, and the nPCR technique proved to have better sensitivity and specificity.

1. Introduction

Bovine coccidiosis, caused by protozoa of the class Coccidia, family Eimeriidae, and genus *Eimeria*, is a prevalent disease affecting the global cattle population, particularly those under one year old (Bruhn *et al.* 2011). This disease results in significant economic losses in the livestock industry due to substantial expenses associated with treatment, leading to increased vulnerability to other diseases, growth delays, and contributing to decreased meat production and quality in beef cattle (Abebe and Wossene 2008; Lassen *et al.* 2014; Lopez-Osorio *et al.* 2020; Ekawasti *et al.* 2021). The estimated economic impact is approximately 400 million dollars on the American market, with over 3.8 million dollars allocated to treatment in Canada (Matjila and Penzhorn 2002; Rehman *et al.* 2011). The annual economic losses associated with both clinical and subclinical coccidiosis experience a gradual rise and

are projected to surpass USD 723 million globally (Koutny *et al.* 2012).

More than 20 species of *Eimeria* have been identified in cattle, among which *Eimeria bovis* and *Eimeria zuernii* are acknowledged as pathogens due to their association with clinical cases of coccidiosis in young animals (Bangoura *et al.* 2011; Florião *et al.* 2016). The predominant clinical symptom of coccidiosis in cattle is watery or bloody diarrhea, followed by loss of appetite, depression, dehydration, and weight loss, ultimately leading to growth inhibition (Ekawasti *et al.* 2021).

Eimeria bovis has been identified as the species with the highest prevalence causing coccidiosis cases in South Korea (79%), Brazil (30.25%), Colombia (33.5%), Iran (23.7%), and several other countries (Heidari and Gharekhani 2014; Lee *et al.* 2019; Cardim *et al.* 2020; Lopez-Osorio *et al.* 2020). In Indonesia, including regions such as Madura, Sumatra, Java, and Sulawesi, *Eimeria bovis* also dominates the prevalence of coccidiosis cases, as indicated by morphological examinations (Hamid *et al.* 2016;

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Ekawasti *et al.* 2021; Hastutiek *et al.* 2022). Given the beef cattle population reaching 18.05 million in 2021 (CAoSRI 2022), this disease poses a significant threat to the Indonesian government's strategic efforts for meat self-sufficiency.

Traditionally, the identification of *Eimeria* spp. relied on morphological characteristics of sporulated oocysts and host specificity. However, the morphological technique is considered to have relatively low sensitivity and practical limitations related to time (Carvalho *et al.* 2011). Additionally, morphological methods are not entirely reliable due to confusing features and the presence of intraspecies variation in form and size between species. Therefore, the diagnostic approach still employed today, which is based on morphology, is not recommended for identifying *Eimeria* spp. (Ekawasti *et al.* 2022).

Various studies have aimed to develop PCR assays for diagnosing *Eimeria* spp. (Kawahara *et al.* 2010); devised a PCR technique using primers designed for the ITS-1 region to diagnose the genus and assess the applicability of the primer design for species diagnosis of this parasite in cattle. Another study produced a primer for the genus *Eimeria* based on the 18S rRNA site, aiming to construct a phylogenetic tree encompassing the main species in cattle (Kokuzawa *et al.* 2013). Due to technological advancements, new assays, such as nested PCR (nPCR), have become available and are capable of offering a more sensitive and specific diagnosis compared to conventional PCR. Therefore, the objective of this study is to identify *Eimeria bovis* in beef cattle across the 18 provinces in Indonesia and to explore their evolutionary relationships using the molecular marker ITS-1.

2. Materials and Methods

2.1. Ethical Approval

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

2.2. Samples and Study Areas

A total of 167 samples were collected from 18 provinces across Indonesia, including Bangka Belitung, Banten, Gorontalo, West Java, Central Java, East Java, West Kalimantan, South Kalimantan, East Kalimantan, Lampung, North Maluku, Nusa Tenggara Barat, Nusa Tenggara Timur, Papua, West Sulawesi, South Sulawesi, Central Sulawesi, and Yogyakarta (Figure 1), in October 2020. These samples, obtained from fresh cattle feces, were individually preserved in plastic bags and stored at a controlled temperature of 4°C in a refrigerator for subsequent laboratory analyses. Initial examination of the fecal samples in their native state allowed for microscopic detection of oocysts. Positive samples indicating the presence of *Eimeria* oocysts underwent a meticulous purification process.

2.3. Isolation and Purification of *Eimeria* oocyst

The purification of *Eimeria* oocyst utilized the sugar flotation method with a few modifications. In brief, the feces were diluted in 20 ml of distilled water and filtered through a steel mesh. Following centrifugation at 800 ×g for 5 minutes, the sediment was combined with a sugar solution, and distilled water was added

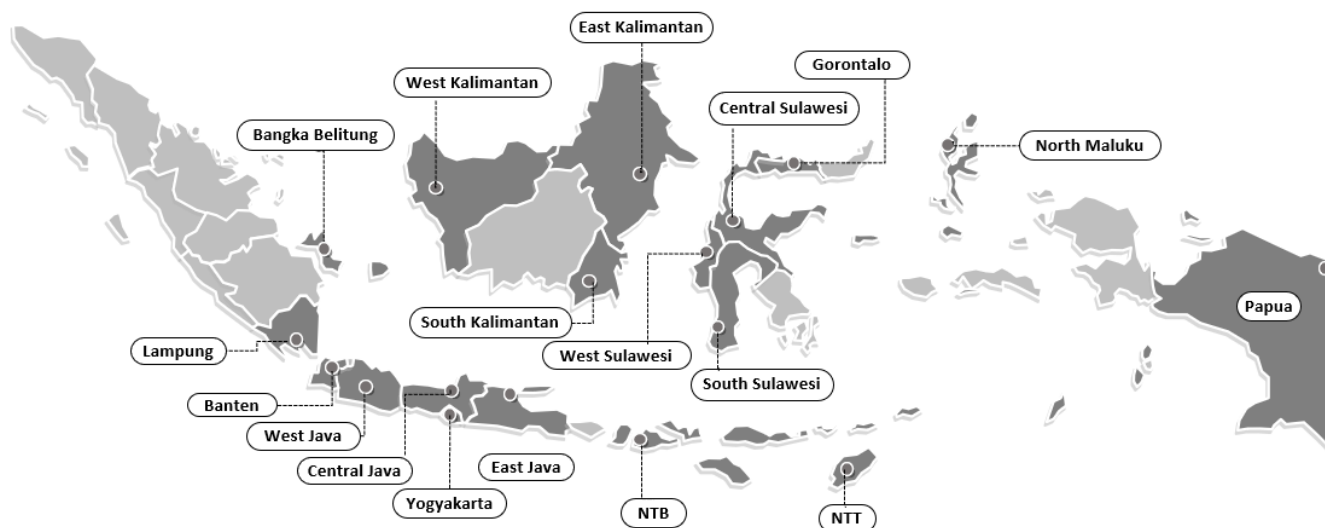


Figure 1. Geographical distribution of sampling areas across 18 provinces in Indonesia

before being plated and centrifuged at 1,200 ×g for 10 minutes. *Eimeria* oocysts, which floated on the surface of the sugar solution, were extracted using a Pasteur pipette and subjected to three washes with distilled water. Finally, the purified oocysts were suspended in 1–2 ml of PBS and stored at 4°C.

2.4. Genomic DNA Extraction

The DNA of *Eimeria* was extracted from oocysts using the Geneaid Genomic DNA Mini Kit, following the manufacturer's protocol with slight modifications as detailed below. The purified samples underwent a freeze-thaw process using liquid nitrogen (N₂) and warm water five times to disrupt the oocysts. Subsequently, the samples were combined with a 3x volume of RBC lysis buffer and centrifuged in a 1.5-ml microcentrifuge tube at 3,000 ×g for 5 minutes at 4°C. The resulting pellet was resuspended in 100 µL of RBC Lysis buffer, and 200 µL of GB Buffer was added, followed by incubation at 60°C for 15 minutes. Next, 200 µL of absolute ethanol was added, and the microtube was vigorously shaken. The mixture was then transferred to the GD column and centrifuged at 15,000 ×g for 5 minutes. The column was washed with W1 Buffer and Wash buffer, with each step involving centrifugation at 15,000 ×g for 5 minutes. The supernatant was collected in a new collection tube, and an elution buffer was added to obtain the eluted DNA, which was subsequently stored at –20°C for further use.

2.5. PCR Assays with Species-specific Primer Sets

DNA amplification was conducted in a two-step PCR process using primer pairs designed to target the ITS-1 fragment, enabling the amplification of both genus-common and species-specific sequences of *Eimeria bovis*. The primer sequences for the genus-common *Eimeria* in the first step and species-specific *Eimeria bovis* in the second step were kindly provided by Kawahara *et al.* (2010) (Table 1). To design genus-common primers capable of amplifying DNA segments

encompassing the entire ITS-1 region across various *Eimeria* species, conserved sequences within the 3' end of the 18S rRNA genes and the 5' end of the 5.8S rRNA genes were targeted.

2.6. Amplification and Sequence Analysis of the ITS-1 Region

In the initial amplification step, a total reaction volume of 25 µL was composed of 1 µL of DNA sample, 12.5 µL of PCR master mix (Promega Corporation, Madison, WI), 1 µL of the forward primer, 1 µL of the reverse primer, and nuclease-free water. The amplification process followed a cycling protocol, starting with an initial cycle at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 55°C for 1 minute, 72°C for 2 minutes, and a final extension at 72°C for 7 minutes.

The second amplification step was carried out under the same conditions as the first step, utilizing products obtained from the initial PCR. Subsequently, 10 µL of the PCR products underwent electrophoresis in a 1.5% agarose gel alongside a 100 bp DNA ladder. Visualization was achieved using a UV transilluminator. Positive samples for *Eimeria bovis* were then further processed for sequencing and subsequent analysis.

2.7. Data Analysis

The sequenced samples were then analyzed for sequence homology using the Basic Local Alignment Search Tool (BLAST), accessible at <https://BLAST.ncbi.nlm.nih.gov/BLAST.cgi>. This homology search aims to determine the percentage of sequence identity between the sample sequences and those in the GenBank NCBI database. The resulting ITS-1 sequences were subsequently aligned using the Multiple Alignment option in the BioEdit application. Phylogenetic analysis of the sample DNA was conducted using the MEGA 11 software, applying the Maximum Likelihood Estimation algorithm. An evaluation was performed with a 1000-bootstrap resampling-based bootstrap test, with the goal of representing the evolutionary history of the analyzed taxa.

Table 1. Details of the genus-common and species-specific primer sets for the detection of *Eimeria bovis* by nested PCR

Target of sequences	Primer sequences (5'-3')		Expected product size (bp)
	Forward	Reverse	
Outer (genus)	gcaaaagtcgtaacacggtttccg	ctgcaattcacaatgcgtatcgc	348-546
Inner (species)	tcataaaacatcacctccaa	ataattgcgataaggagaca	238

3. Results

3.1. Conventional Fecal Examination of *Eimeria* spp.

The nested PCR analysis was initiated subsequent to a qualitative microscopic evaluation of *Eimeria* spp. oocysts in 167 fecal samples collected from beef cattle. The examination was conducted without regard for oocyst size and shape between species. The criteria for positive outcomes were based on the predominantly singular presence of *Eimeria* oocysts. Microscopic observations indicated that 96 out of 167 samples (57.5%) demonstrated positivity for *Eimeria* spp., showcasing a consistent distribution across 18 provinces. The *Eimeria* spp. oocysts identified in the microscopic examination results are illustrated in Figure 2.

3.2. Characterization of ITS-1 of *E. bovis*

In the field isolates, ITS-1 regions were successfully amplified using genus-common primer sets spanning the 3' end of the 18S rRNA gene and the 5' end of the 5.8S rRNA gene. Morphological assessment of oocysts was not conducted in this study; instead, direct processing using nested PCR (nPCR) with two specifically designed primer pairs was employed. The amplification results showed that out of the 96 tested samples, 48 samples (50%) exhibited positive

detection for *E. bovis*, as indicated by a 238 bp DNA fragment (Figure 3). Positive samples were detected in all provinces except West Kalimantan and Yogyakarta (Table 2). Although some samples posed challenges in interpretation, the authors categorized them as negative results based on their confidence.

3.3. Phylogenetic Analysis

The homology measurement of eight samples, each representing a province, was conducted using the BLAST program to confirm that the detected

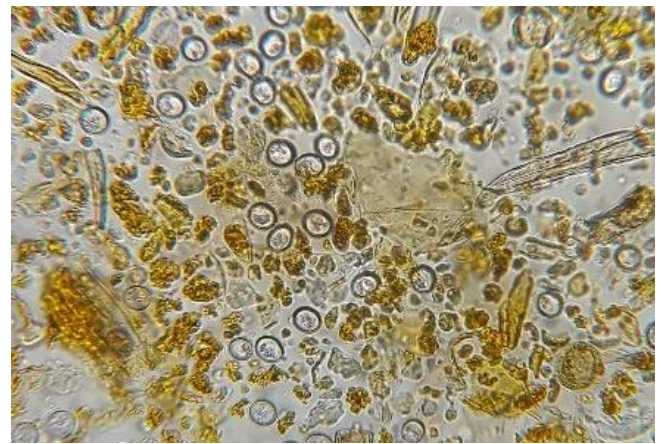


Figure 2. Oocysts of *Eimeria* spp. in the microscopic examination of the samples (magnification 400x)

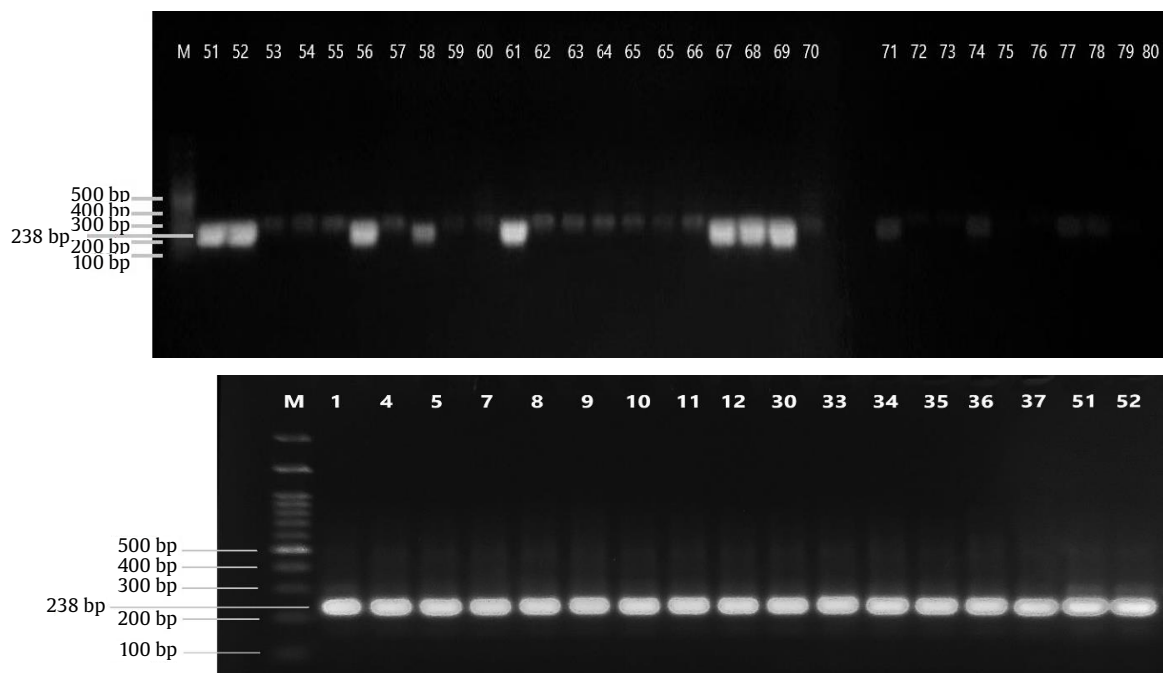


Figure 3. The nPCR product visualization identified within the ITS-1 region of DNA of *E. bovis*. M: molecular markers 100 bp DNA Ladder; Number 1-80: samples number

Table 2. Results of the nPCR test from the examined fecal samples of beef cattle

Province	Number of positive samples for <i>Eimeria</i> spp.	Positive results for <i>E. bovis</i>	
		n	%
Bangka Belitung	6	1	16.7
Banten	21	9	42.9
Gorontalo	5	5	100
West Jawa	3	1	33.3
Central Jawa	2	1	50
East Jawa Timur	3	2	66.7
West Kalimantan	4	0	0
South Kalimantan	1	1	100
East Kalimantan	1	1	100
Lampung	8	3	37.5
North Maluku	6	2	33.3
West Nusa Tenggara	3	1	33.3
East Nusa Tenggara	5	2	40
Papua	8	5	62.5
West Sulawesi	4	3	75
South Sulawesi	12	10	83.3
Central Sulawesi	3	1	33.3
Yogyakarta	1	0	0
TOTAL	96	48	50

Table 3. Homology of samples with reference sequences in the blast program

Number of samples	Sample origin	Reference sequences		Percent identity (%)
		Access code	Country origin	
Sample 24	Gorontalo	AB769575.1	Japan	95
Sample 52	East Jawa	AB769588.1	Japan	96
Sample 56	West Jawa	AB769588.1	Japan	97
Sample 67	Papua	KU351709.1	Turkey	97
Sample 70	Bangka Belitung	AB769588.1	Japan	97
Sample 71	North Maluku	KU351710.1	Turkey	90
Sample 81	West Sulawesi	AB769575.1	Japan	99
Sample 92	Bangka Belitung	AB769583.1	Japan	99

species is *E. bovis*. All these samples exhibited high homology to the ITS-1 sequence of *E. bovis* originating from various countries, with minor differences in the ITS-1 region sequence among isolates of the same species (Table 3). The phylogenetic tree illustrates three groups that explain the species relationships, visually connected to sequence homology. Using ITS-1 from *E. tenella* as an outgroup, three monophyletic clusters of *E. bovis* were observed against the outgroup. All samples in this study formed a distinct cluster separated from the reference group (Figure 4).

4. Discussion

In this study, primers were designed from the ITS-1 gene rRNA site of six *Eimeria* spp. species in cattle, as it is a highly conserved region for studying

their diversity. The ITS-1 region is flexible according to species variations, compared to the overall rRNA gene, showing low intra-specific variation and high inter-specific variation in DNA sequences. This feature facilitates accurate primer design, minimizing the risk of cross-reaction with different species (Kawahara *et al.* 2010).

The specificity of nested Polymerase Chain Reaction (nPCR) was rigorously examined in this investigation. In a preceding trial, the amplification was conducted using conventional Polymerase Chain Reaction (PCR) with the same set of *E. bovis* species primers (results undisclosed). However, the outcomes exhibited less clarity in visualizations, whereas the utilization of nPCR demonstrated more distinct results. This observation suggests that the nPCR technique exhibits superior sensitivity compared to conventional PCR. The iterative

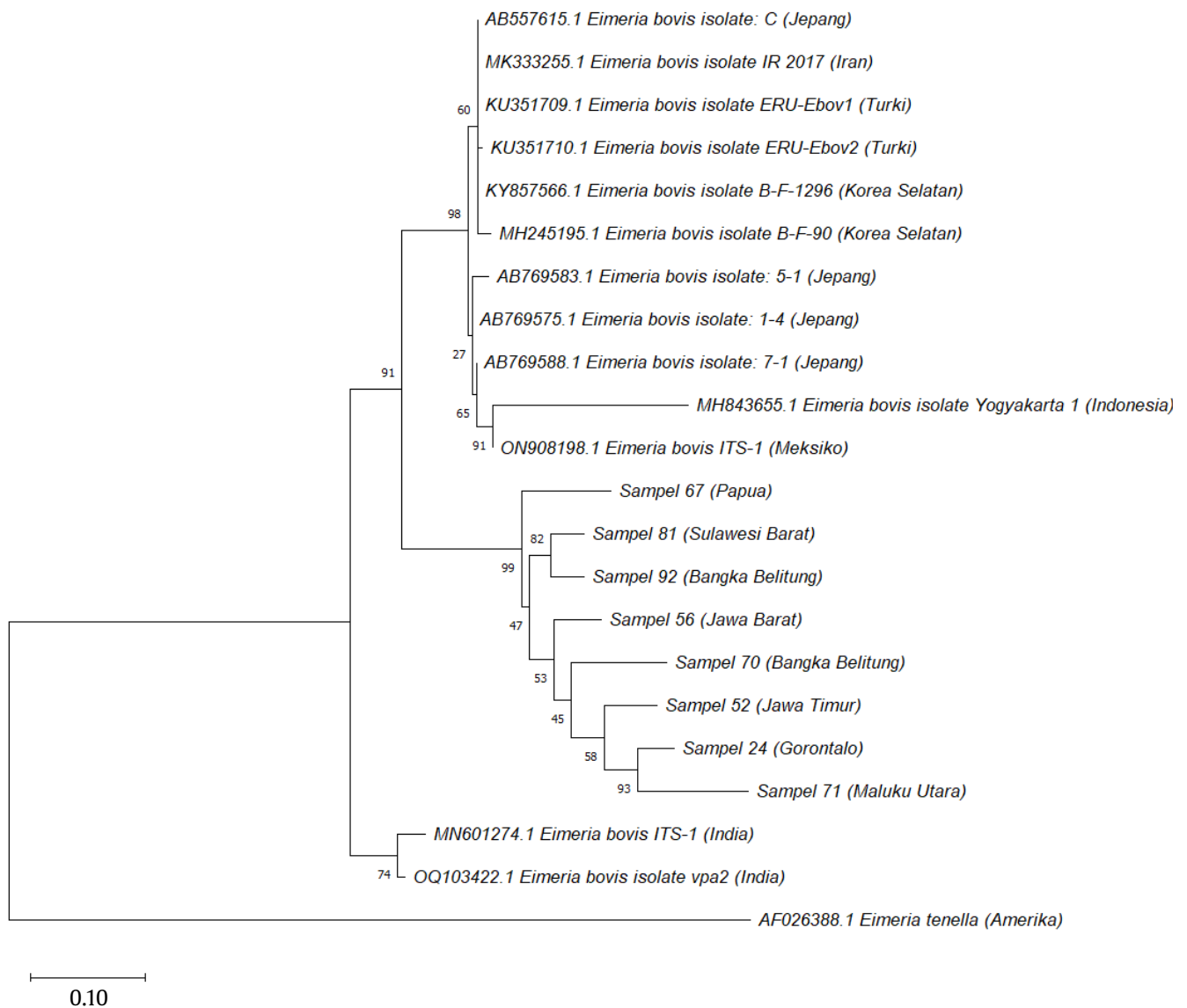


Figure 4. Phylogenetic tree based on ITS-1 sequences for all isolates along with the reference sequences. The tree was rooted in *Eimeria tenella*, with branch lengths representing computed evolutionary distances using the Maximum Likelihood Estimation (MLE) algorithm. Bootstrap support values from MLE are presented on the nodes

process of nested PCR, involving two consecutive amplification steps, enables the reduction of unnecessary contamination products during the amplification process. This technique is adept at mitigating the non-specific amplification of DNA fragments, as the majority of amplicons from the initial reaction contain only the target sequence and its surrounding sequences (Wanger *et al.* 2017).

During the nesting process, the first PCR reaction with genus-specific primers yields the ITS-1 genus *Eimeria* DNA fragment. The product of the first-stage PCR is then utilized as the template for the second-stage PCR reaction, employing specific primers

to obtain the *E. bovis* species DNA fragment. This methodology yields shorter and more precisely specific DNA fragments, significantly enhancing the sensitivity and specificity of the assay (Green and Sambrook 2019; Ekawasti *et al.* 2022).

Higher homology was acknowledged within the ITS-1 sequences among all *E. bovis* samples, notwithstanding their geographical diversity. This phenomenon is attributed to the fact that all isolates of *Eimeria* spp. originate from farms in Indonesia and thus can be regarded as the same strain. This inference is supported by phylogenetic analysis, revealing that pathogenic *Eimeria* species from Indonesia cluster

with sequences from *Eimeria* species of diverse geographic origins within the same clade (Figure 4). However, when compared to references, the *E. bovis* sample sequences form a branch with all references except two samples from India. The close relationship, as indicated in the phylogenetic tree, implies a genetic connection between the *E. bovis* samples and the *E. bovis* references. The formed branching suggests that the taxonomic history of the *E. bovis* samples is relatively recent compared to the species originating from India. Based on cluster identity, the well-known pathogenic *E. bovis* displays a distinct identity, forming a monophyletic group in a different position from the outgroup that differs only in species.

Microscopic examination results in this study indicate a widespread distribution of *Eimeria* throughout nearly all regions of Indonesia. These findings support prior research indicating the presence of *Eimeria* in numerous provinces across Indonesia. The highest prevalence was in the Maluku Islands at 94.1%, followed by Kalimantan (83%), Sumatra (70.3%), Sulawesi (68.9%), Papua (62.3%), East Nusa Tenggara (58.5%), Java (53.7%), and Madura Island (12%) (Ekawasti *et al.* 2021; Hastutiek *et al.* 2022). *Eimeria bovis* was the most dominant species detected in several provinces, comprising 10.4% in Java (Ekawasti *et al.* 2019), 81.89% in nine provinces studied by Hamid *et al.* (2019), and in Madura (Hastutiek *et al.* 2022). These data align with the results of this study, where 16 out of 18 provinces confirmed the presence of *E. bovis*. The Special Region of Yogyakarta and West Kalimantan were the two provinces where the presence of *E. bovis* was not detected in this study.

The distribution of coccidiosis cases due to *Eimeria* infection in cattle in Indonesia is influenced by various factors, including climate and weather conditions, farming systems, gender, and age of the cattle (Hastutiek *et al.* 2022). The high prevalence of *Eimeria* in certain regions generally occurs due to the warm and humid tropical climate. Coccidiosis cases are more frequent during the rainy season than the dry season, which is related to the optimal development of the *Eimeria* life cycle (Manya *et al.* 2008; Keeton and Navarre 2018).

Furthermore, the farming system in Indonesia, comprising intensive, semi-intensive, and extensive systems, also plays a role in *Eimeria* dissemination (Hussin, 2016; Ekawasti *et al.* 2019; Lopez-Osorio

et al. 2020). The intensive system is more prone to *Eimeria* spread compared to other systems. Infection of *Eimeria* from one cattle to another occurs due to oocyst contamination in feed originating from cattle feces accumulated around the pen. Most pens used by farmers are still traditional and lack separate disposal areas for feces and urine. This practice leads farmers to dispose of urine and cattle feces around the pen, potentially increasing the risk of *Eimeria* infection and reinfection in a farming area (Marskole *et al.* 2016). *Eimeria* dissemination can also be caused by population density, pen structure, feed, and neglected sources of drinking water (Makau *et al.* 2017).

The primary losses for farmers due to *Eimeria bovis* infection arise from deteriorating cattle health, decreased nutrient absorption capacity, and cases of mortality in calves (Keeton and Navarre 2018). Although some species do not induce clinical symptoms, their infestation within the cattle body can reduce immunity and serve as an entry point for other disease agents.

The identified genetic proximity between *Eimeria bovis* species originating from Indonesia and those from abroad, particularly Japan and India, warrants caution concerning potential outbreaks of coccidiosis in the future. Therefore, continuous efforts must be undertaken to prevent and control this disease and limit the spread of *Eimeria* in cattle. Several control practices can be implemented by improving livestock management, adhering to strict biosecurity practices, and ensuring rapid and accurate early detection of *Eimeria*.

Authors' Contributions

MFN, RWN, DPW, and FE: Research concept and writing the manuscript. MFN, FE, and LWF: Collection and analysis of data. All authors are the main contributors to this work. All authors have read, revised, and approved the final manuscript.

Acknowledgements

The authors would like to thank the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia for financial support with grant number PDUPT NO.1651/UN1/DITLIT/DIT-LIT/PT/2021.

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