



Research paper

Molecular characterization of highly pathogenic *Eimeria* species among beef cattle on Java Island, IndonesiaFitrine Ekawasti^{a,b,c}, Wisnu Nurcahyo^{b,*}, April Hari Wardhana^{a,c}, Tomoyuki Shibahara^{c,d}, Masaharu Tokoro^e, Kazumi Sasai^c, Makoto Matsubayashi^{c,*}^a Indonesian Research Center for Veterinary Science, Bogor 16114, Indonesia^b Department of Parasitology, Veterinary Medicine, Gadjah Mada University, Yogyakarta 55281, Indonesia^c Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka 598-8531, Japan^d Division of Pathology and Pathophysiology, National Institute of Animal Health, NARO, Tsukuba, Ibaraki 305-0856, Japan^e Department of Parasitology, Graduate School of Medical Sciences, Kanazawa University, Kanazawa 920-8640, Japan

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ABSTRACT

Gastrointestinal parasites including *Eimeria* spp. are known to affect domestic animal productivity causing watery or lethal bloody diarrhea. However, there are few reports on the detailed distribution of bovine *Eimeria* spp. in cattle, particularly in developing tropical and sub-tropical areas. Using a total of 289 fecal samples collected from beef cattle on Java Island, one of the five main islands of Indonesia, fecal examinations by the Whitlock and sugar flotation methods and molecular surveys were conducted to reveal the prevalence of 6 *Eimeria* spp. As a result of morphological screening using Whitlock methods and sugar flotation, *Eimeria* spp. prevalences of 9.4% and 52.3% were confirmed, respectively. The prevalence was higher in younger cattle [under 1 year (63.9%), 1–2 years (75.0%) and more than in 2 year old cattle (42.3%)]. The prevalences of identified species were as follows: 10.4% for *E. bovis*, 2.8% for *E. ellipsoidalis*, 2.1% for *E. alabamensis*, 1.4% for *E. zuernii*, 1.1% for *E. auburnensis*, and 0.4% for *E. cylindrica*. Moreover, prevalences of 12.8% for *Strongyloides papillosus*, 7.3% for *Trichuris globulosa*, and 0.3% for *Capillaria bovis* were detected. Although the average number of oocysts per gram of feces was < 100 among the positive samples, and cases of heavy infection were limited, the endemicity of these pathogenic *Eimeria* species among farms in Indonesia should be noted.

1. Introduction

Gastrointestinal parasites infecting livestock can sometimes cause gastroenteritis characterized as watery or bloody diarrhea. They often affect the health status in hosts, resulting in decreased meat or milk production [1–3]. Protozoan parasites are thought to be more harmful due to their pathogenicity because they rapidly multiply in the intestinal mucosa, following subsequent cell disruptions by developed zoites. Infection is initiated by ingestion of sporulated oocysts via fecal-oral routes. Infected animals (even without showing symptoms) shed the oocysts in their feces, which are resistant to most disinfectants and environments, and can be potential sources of further infection.

Eimeria spp. are intestinal protozoan parasites that cause coccidiosis, and are considered to be one of the most difficult gastrointestinal

parasites to control. The typical clinical signs of coccidiosis in cattle are watery or bloody diarrhea, followed by loss of appetite, depression, dehydration, and weight loss, leading to retarded growth. To date, > 12 species of bovine *Eimeria* have been reported [4], and *E. bovis*, *E. zuernii*, *E. alabamensis*, *E. auburnensis*, and *E. ellipsoidalis* are thought to be pathogenic due to their causing the clinical signs noted above [5]. Furthermore, two species, *E. bovis* and *E. zuernii*, have a major impact due to their high virulence, particularly in terms of frequent mortality in calves aged under one year of age worldwide [6,7]. Young cattle possess higher sensitivity to infection by *Eimeria* spp., and thus, the prevalence of this disease in calves is higher than in adult cows [6], although the prevalence can be different depending on the country or farm management system [8]. It has been estimated that economic losses due to eimerian infections in cattle is USD 400 million worldwide [8,9].

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In Indonesia, the population of cattle was reported to be over 16.5 million beef cattle and over 0.5 million dairy cattle in 2018 [10]. Among the islands, 42.6% and 98.9% of beef and dairy cattle are reared on Java Island [11]. The management of animals is mainly based on small commercial systems, namely by family units in rural areas. The number of cattle per farm is estimated to be < 50 animals, as the management systems actually depend on the farmers. Recently, the ministry of agriculture in Indonesia has begun strategic plans to develop food self-sufficiency programs [12]. It is thought that gastrointestinal parasites infecting cattle may be associated with the achievement of these strategies. However, there are few data on the distribution of gastrointestinal parasites in Indonesia, particularly *Eimeria* spp. In the only two reports, eimerian infections were 22.4% in West Java [13] and 15.5% in Central Java [14], but the species of *Eimeria* were not identified.

Identification of *Eimeria* species has been largely based on the morphology of sporulated oocysts [5], but some species show morphological similarities [15]. Furthermore, only one paper reported the prevalence of three bovine *Eimeria* spp. by PCR [16]. Therefore, molecular characterization is essential to accurately assess the species. Here, we conducted a survey of gastrointestinal parasites targeting *Eimeria* spp. and other gastrointestinal parasites on Java Island, Indonesia, and we report the prevalence of highly pathogenic *Eimeria* spp., *E. bovis* and *E. zuernii*, in Indonesia.

2. Materials and methods

2.1. Study area and samples

Indonesia is the largest archipelago in the world and there are over 13,466 islands. The five largest of these islands are Java, Sumatra, Kalimantan, Papua New Guinea, and Sulawesi. Java is the main island, and has 6 provinces: East Java, Yogyakarta, Central Java, West Java, Jakarta, and Banten. In the present study, a total of 289 fecal samples from beef cattle were collected from 11 districts in Central Java, 2 districts in East Java, and 1 district in Yogyakarta from August to November 2018, and each examined village had more than two farms. The location of these areas is shown in Fig. 1. The average annual temperature is 25 °C. No animals showed clinical symptoms when fecal samples were collected. All samples were taken from the rectums of cattle, preserved in separate plastic bags, and then stored at 4 °C until laboratory examinations.

2.2. Fecal examination

Fecal samples were examined by two flotation methods, using Whitlock universal chambers [17]. and by sugar flotation centrifugation modified based on a previous report [18,19]. Briefly, for detection by the Whitlock method, 3 g of fecal samples were diluted in 17 ml of distilled water, and 40 ml saturated sodium chloride solution was added. Next, 0.5 ml of the sample solution was placed onto a Whitlock universal chamber. After 5 min, we examined and counted the number of *Eimeria* oocysts and nematode eggs in the entire field under light microscopy at a magnification of $\times 200$ or $\times 400$. For the sugar flotation centrifuge method, 1 g of fecal sample was diluted in 9 ml of distilled water and centrifuged at $800 \times g$ for 5 min. After the supernatant was discarded, 10 ml of sugar solution with a specific gravity of 1.2 [e.g., 100 g of sugar (Gulaku Indonesia, Lampung, Indonesia) was added into the 120 ml of distilled water] was added to the sediment, followed by centrifugation at $800 \times g$ for 5 min and was placed onto a glass slide. The entire smear was examined by light microscopy.

2.3. Purification of *Eimeria* oocysts

Using remaining feces (approximately 5–10 g) from positive samples, *Eimeria* oocysts were purified by the sugar flotation method.

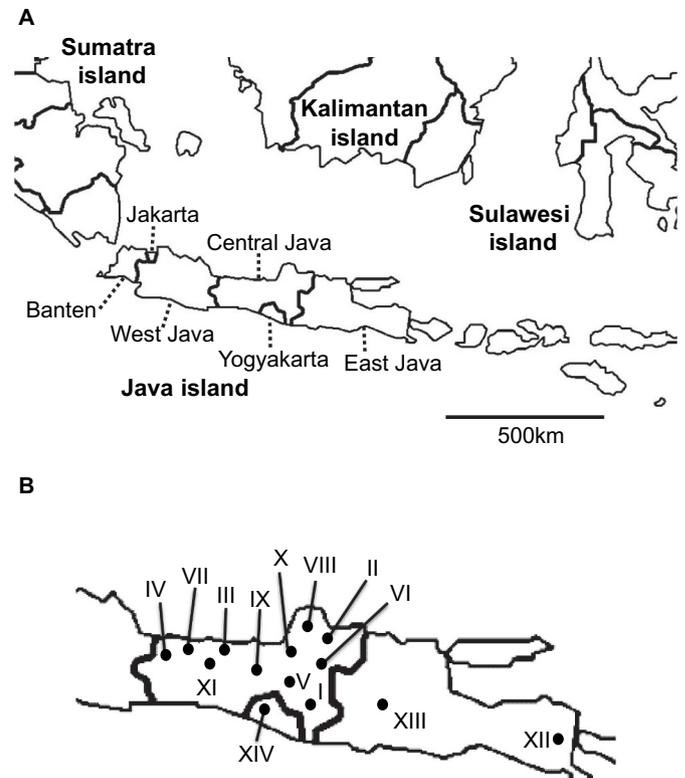


Fig. 1. Maps showing the main islands in Indonesia (Java, Sumatra, Kalimantan, and Sulawesi) (A) and the locations of examined villages on Java Island (B). The numbers in Fig. 1B are also used in Tables 1 and 3.

Briefly, feces were diluted in distilled water and filtrated with a steel mesh. After centrifugation at $800 \times g$ for 5 min, sugar solution was added to the sediments, and distilled water was overlaid and centrifuged at $1200 \times g$ for 10 min. The *Eimeria* oocysts floated on the surface of sugar solution were recovered using a Pasteur pipette, and were washed with distilled water three times. Finally, the purified oocysts were resolved with 1–2 ml of PBS and stored at 4 °C.

2.4. Molecular identification of *Eimeria* spp.

For DNA extraction, 400 μ l of *Eimeria* oocysts purified as described above were used. They were freeze-thawed five times to release genomic DNA [3,20]. After centrifugation at $5400 \times g$ at 3 min, 200 μ l of the supernatant was used based on the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). For identification of six *Eimeria* spp. including pathogenic ones (*E. bovis*, *E. zuernii*, *E. alabamensis*, *E. auburnensis*, *E. ellipsoidalis*, and *E. cylindrica*), PCR targeting of the internal transcribed spacer 1 (ITS-1) region of ribosomal RNA gene was carried out as reported previously [21]. PCR products were subjected to electrophoretic separation on an agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

3. Results

In our fecal examinations of *Eimeria* spp., all 14 districts were found to be positive for *Eimeria* spp., and the sugar flotation method was more sensitive than the Whitlock method [52.3% (151/289) and 9.4% (27/289) of samples, respectively; Table 1]. The number of oocysts calculated using the Whitlock method generated low OPG values (< 100 on average). By the sugar flotation method, the positive ranges were from 30.3% to 81.8% among the villages. The species of *Eimeria* spp. in this study could not be morphologically identified, although some parasites were similar to *E. bovis* or *E. zuernii*. PCR analyses for six *Eimeria* spp.

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

Region	Province	District	No. of examined cattle	Fecal examination		PCR analysis ^b							Notes		
				Whitlock method	Sugar floatation method	<i>E. b</i>	<i>E. z</i>	<i>E. aubu</i>	<i>E. alab</i>	<i>E. cylin</i>	<i>E. ellip</i>				
												Positive No. (%)		Average OPG ^a (range)	Positive No. (%)
I	Central Java	Sukoharjo	33	-	10 (30.3%)	-	-	-	-	-	-	-	-	-	-
II		Pati	13	-	9 (69.0%)	1	-	-	-	-	-	-	-	-	-
III		Batang	12	-	9 (75.0%)	2	-	-	-	-	-	-	-	-	-
IV		Brebes	11	-	6 (54.5%)	2	-	-	-	-	-	-	-	-	-
V		Gedawang	11	-	6 (54.5%)	1	-	-	1	-	-	-	-	-	Mixed with two species (1)
VI		Grobogan	10	-	6 (60.0%)	-	-	1	-	-	-	-	1	-	-
VII		Pemalang	10	1 (10.0%)	40.0	1	-	-	-	-	-	-	-	-	-
VIII		Kudus	9	-	5 (55.6%)	-	-	-	-	-	-	-	-	-	-
IX		Getasan	7	-	3 (42.9%)	1	1	-	-	-	-	-	-	-	Mixed with two species (1)
X		Demak	7	-	5 (71.4%)	1	-	-	1	-	-	-	1	-	Mixed with three species (1)
XI		Pekalongan	6	-	3 (50.0%)	-	-	-	-	-	-	-	-	-	-
XII	East Java	Banyuwangi	82	2 (2.4%)	140.0 (40–240)	10	3	2	2	1	2	1	2	2	Mixed infection with four species (1), three species (1), two species (3)
XIII		Kediri	34	1 (2.9%)	40	-	-	-	-	-	-	-	-	4	Mixed with two species (2)
XIV	Yogyakarta	Kulonprogo	44	23 (52.3%)	100.9 (40–480)	11	-	-	-	-	-	-	-	-	-
Total			289	27 (9.4%)	99.3 (40–480)	30 (10.4%)	4 (1.4%)	3 (1.1%)	6 (2.1%)	1 (0.4%)	8 (2.8%)	1	8 (2.8%)	8 (2.8%)	Species were identified in 38 of 151 samples

^a OPG: oocysts per gram.

^b Abbreviations: *E. b*; *Eimeria bovis*, *E. z*; *Eimeria zuernii*, *E. aubu*; *Eimeria auburnensis*, *E. alab*; *Eimeria alabamensis*, *E. cylin*; *Eimeria cylindrical*, *E. ellip*; *Eimeria ellipsoidalis*.

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

Age (y)	No. of examined cattle	Fecal examination		PCR analysis ^b							Notes
		Whitlock method	Sugar floatation method	<i>E. b</i>	<i>E. z</i>	<i>E. aubu</i>	<i>E. alab</i>	<i>E. cylin</i>	<i>E. ellip</i>		
										Positive No. (%)	
< 1	36	15 (41.7%)	23 (63.9%)	8	1	1	-	-	-	-	Mixed with three species (1)
1-2	64	10 (15.6%)	48 (75.0%)	8	-	-	2	-	-	2	Mixed infection with two species (1), three species (1)
> 2	189	2 (1.1%)	80 (42.3%)	14	3	2	4	1	6	6	Mixed infection with two species (6), four species (1)
Total	289	27 (9.4%)	151 (52.3%)	30 (10.4%)	4 (1.4%)	3 (1.1%)	6 (2.1%)	1 (0.4%)	8 (2.8%)	8 (2.8%)	

^a OPG: oocysts per gram.

^b Abbreviations: *E. b*; *Eimeria bovis*, *E. z*; *Eimeria zuernii*, *E. aubu*; *Eimeria auburnensis*, *E. alab*; *Eimeria cylindrica*, *E. ellip*; *Eimeria ellipsoidalis*.

revealed the species in 38 of 151 positive samples, and consequently, *E. bovis* appeared to be the most prevalent. By age, the prevalence of *Eimeria* spp. in cattle aged under 1 year (63.9%) or 1-2 years (75.0%) tended to be higher than in cattle aged > 2 years (42.3%) (Table 2). At all ages, *E. bovis* was identified frequently.

With regard to nematode parasites, cattle from 8 districts were positive. Nevertheless, the prevalence of *Strongyloides*, *Trichuris*, and *Capillaria* were relatively low (Table 3). Similarly to the results for *Eimeria* spp. examination, the sugar flotation method was also highly sensitivity for nematodes, except for *Strongyloides*. A clear trend for age dependence of prevalence was not confirmed (Table 4).

4. Discussion

In the present study, a survey of gastrointestinal parasites was carried out on Java Island, Indonesia. Two flotation methods were compared (the Whitlock and sugar flotation methods), and the latter method was highly sensitive for the detection of *Eimeria* spp. The Whitlock method using a Whitlock universal chamber with four wells of 0.5 ml was previously modified based on the McMaster method using a McMaster chamber with two wells of 0.15 ml [17,22]. Unlike the sugar flotation method, the Whitlock and McMaster methods are commonly used in Indonesia due to their simplicity and rapidity [14]. They were mainly suitable for counting the number of oocysts or eggs in feces, as well as for detections of light or heavy infections. Although one gram of feces was examined by the sugar flotation method, approximately 25 mg of feces is needed for observation by the Whitlock method. These different volumes of examined feces are indicative of the sensitivity. Therefore, it is necessary to select the best method according to the examinations. Our results for *Eimeria* spp. prevalence (52.3%) were found to be higher than those of previous reports in Indonesia: 22.4% in West Java by the sugar flotation method [13] and 15.33% in Central Java by the McMaster method [14]. Although differences in the prevalence could be due to differences in management at the examined farms or detection methods, we were nonetheless able to report the latest prevalence of *Eimeria* spp. on Java Island.

There have been no reports on the prevalence of *Eimeria* spp. in cattle to date using molecular methods, except for one report on the identification of three *Eimeria* spp. [16]. PCR analyses for six *Eimeria* spp. allowed us to determine the species in 38 samples (including 10 mixed infections) of 151 positive samples and consequently, *E. bovis* was found to be the most prevalent. However, we could not identify the species in the remaining positive samples. The possible reasons might be due to the number of the oocysts in feces or contaminated PCR inhibitors. DNA was extracted from isolates containing 10,000 oocysts/ml in the referenced method [21] and a small number of oocysts (approximately 20 oocysts of each species) were reported to be insufficient for identification of species using PCR [23]. In our fecal examinations, the average number of *Eimeria* spp. oocysts was 99.3. Previously, it was reported that OPG values of 1-499, 500-5000, or > 5000 were evaluated as light, moderate, and high infections, respectively [24]. In addition, examined cattle showed no clinical symptoms, and thus, positive cattle in the present study could be lightly infected with *Eimeria* spp. As a result, *E. bovis* was found to have the highest prevalence among positive cattle. In other reports, it was suggested that *E. bovis* was predominant in Indonesia and Turkey, although they were identified based on their morphologies [14,25]. Their reasons for the predominance have not been clarified, but these results indicate that the highly virulent species could be widespread on Java Island and be a potential source of infections, particularly in the areas of dense breeding.

The age dependence of *Eimeria* spp. prevalence has been reported by others: e.g., 14.8% for < 1 year, 16.9% for 1-2 years and 5.1% for > 2 years in Taiwan [26]; and 2.4% for ≤ 3 weeks, 44% for 4 weeks-3 months, 33.5% for 4-12 months, and 8.4% for > 12 months in Korea [15]. In the present study, infection by *Eimeria* spp. was found in

Table 3
Summary of study areas and results for nematodes.

Region	Province	District	No. of examined cattle	Fecal examination										
				Whitlock method					Sugar floatation method					
				<i>Strongyloides papillosus</i>		<i>Trichostrongylus axei</i>		<i>Trichostrongylus colubriformis</i>		<i>Strongyloides papillosus</i>		<i>Trichostrongylus axei</i>		<i>Trichostrongylus colubriformis</i>
				Positive Nos. (%)	Average EPG ^a (range)	Positive Nos. (%)	Average of EPG	Positive Nos. (%)	Positive Nos. (%)	Positive Nos. (%)	Positive Nos. (%)	Positive Nos. (%)	Positive Nos. (%)	Notes
I	Central Java	Sukoharjo	33	-	-	-	-	-	-	-	-	-	-	
II		Pati	13	7 (53.8%)	202.9 (40–560)	-	-	-	3 (23.1%)	-	-	1 (7.7%)	-	Mixed with two species (1)
III		Batang	12	1 (8.3%)	40	-	-	-	1 (8.3%)	1 (8.3%)	-	-	1 (8.3%)	Mixed with two species (1)
IV		Brebes	11	-	-	-	-	-	-	-	-	-	-	
V		Gedawang	11	2 (18.2%)	140.0 (120–160)	-	-	-	2 (18.2%)	-	-	-	-	
VI		Grobogan	10	-	-	-	-	-	-	-	-	-	-	
VII		Pemalang	10	-	-	-	-	-	-	-	-	-	-	
VIII		Kudus	9	-	-	-	-	-	-	-	-	-	-	
IX		Getasan	7	-	-	-	-	-	-	-	-	-	-	
X		Demak	7	1 (14.3%)	40.0	-	-	-	2 (28.6%)	2 (28.6%)	-	-	-	Mixed with two species (2)
XI		Pekalongan	6	-	-	-	-	-	1 (16.7%)	-	-	-	-	
XII	East Java	Banyuwangi	82	17 (20.7%)	141.2 (40–360)	1 (1.2)	40.0	10 (12.2%)	12 (14.6%)	-	-	-	-	Mixed with two species (1)
XIII		Kediri	34	1 (2.9%)	120.0	-	-	-	2 (5.9%)	-	-	-	-	
XIV	Yogyakarta	Kulonprogo	44	8 (18.2%)	55.0 (40–120)	-	-	-	7 (15.9%)	6 (13.6%)	-	-	-	Mixed with two species (1)
Total			289	37 (12.8%)	128.1 (40–560)	1 (0.4%)	40.0	28 (9.7%)	21 (7.3%)	1 (0.3%)				

^a EPG: eggs per gram.

Table 4
Summary of ages and results for nematode.

Age (y)	No. of examined cattle	Fecal examinations						Notes	
		Whitlock method				Sugar floatation method			
		<i>Strongyloides papillosus</i>		<i>Trichuris globulosa</i>		<i>Strongyloides papillosus</i>	<i>Trichuris globulosa</i>		<i>Capillaria bovis</i>
Positive no. (%)	Average of EPG ^a (range)	Positive no. (%)	Average EPG ^a (range)	Positive no. (%)	Positive no. (%)	Positive no. (%)			
< 1	36	9 (25.0%)	80.0 (40–160)	1 (2.7%)	40.0	4 (1.1%)	5 (1.3%)	–	
1–2	64	6 (9.4%)	80.0 (40–160)	–	–	6 (9.4%)	5 (7.9%)	–	
> 2	189	22 (11.6%)	160.9 (40–560)	–	–	18 (9.5%)	11 (5.8%)	1 (0.5%)	
Total	289	37 (12.8%)	128.1 (40–560)	1 (0.4%)	40.0	28 (9.7%)	21 (7.3%)	1 (0.3%)	

^a EPG: eggs per gram.

younger cattle aged < 1 year (63.9%) and 1–2 years (75.0%) more frequently than in adult cattle aged > 2 years (28.6%). This trend was concordant with previous results. Generally, adult cattle can produce the acquired immunity to be resistant to *Eimeria* infection as a result of their past exposure, but calves are thought to be more susceptible due to a lack of immunity, resulting in clinical symptoms [5].

Regarding nematodes, *S. papillosus*, *T. globulosa*, and *C. bovis* were detected, but the prevalence was low and the age dependence of infection could not be evaluated. These low detection levels were in line with previous reports: 0.5% for *S. papillosus*, 0.5% for *T. globulosa*, and 1.1% for *C. bovis* in West Java, Indonesia [13]. Among these parasites, *S. papillosus* can cause diarrhea and malnutrition, particularly in young animals, more than other species infecting cattle. Thus, control of nematodes, as well as *Eimeria* spp., is necessary. Unlike detection of *Eimeria*, *S. papillosus* was found by the Whitlock method more frequently than by the sugar floatation method. Although the reasons for this remain uncertain, the detection of parasite eggs might be better suited to sodium chloride solution than sucrose solution, as the eggs are easily disrupted.

The farms in the examined areas are generally constructed in small areas, and management, including removal of feces and cleaning of pens, is conducted by the family units. Usually, two to four cattle are reared in a stall together and the cattle are easily moved between the farms [27]. Therefore, the environment in which cattle are raised may increase the risk for transmitting oocysts and infections. Our results suggest that *Eimeria* spp. is more widespread in Indonesia and *E. bovis* was predominant among the detected species. The revealed endemicity of these pathogenic *Eimeria* species among farms in Indonesia should thus be noted.

Declaration of competing interests

The authors declare that they have no conflicts of interest. All authors contributed equally in writing the manuscript.

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