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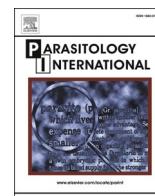
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Copro-parasitological examinations and molecular determination of *Eimeria* species in Madura cattle reared on Madura Island, Indonesia[☆]

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ARTICLE INFO

Keywords:

Eimeria
Indonesia
Madura cattle
PCR

ABSTRACT

Madura cattle, which are native to Indonesia and mainly kept on Madura Island, East Java, are expected to contribute to improving the regional meat self-sufficiency. *Eimeria* spp. are the most pathogenic protozoans among gastrointestinal parasites in livestock but no molecular surveys of *Eimeria* spp. in Madura cattle have been conducted to date. In this study, a total of 183 fecal samples were collected from Madura cattle and 60 (32.8%) were positive for parasites of protozoans and nematodes by the sugar floatation method. Among the samples with parasites, *Eimeria* spp. oocysts were detected in 50 samples (27.3%) with an average OPG value of 1686.1. *Eimeria* spp. were successfully identified to the species level in 26 samples with *Eimeria bovis* being the most prevalent, followed by *E. zuernii* and *E. aubrunensis*. A total of 21 samples showed mixed infection of more than two species of *Eimeria*. *E. bovis* and *E. zuernii* have been recognized as having high virulence and, thus, these parasites are potential sources of severe coccidiosis and the cause of infections in other cattle. Although additional studies are warranted, these results can be helpful for improving the management and productivity of Madura cattle.

1. Introduction

Infections by gastrointestinal parasites including protozoans have been reported in livestock worldwide. Parasitic infections are primarily characterized by gastroenteric symptoms such as watery or bloody diarrhea, which often drastically constrain productivity, through reduced growth or milk yield in cattle [1–3]. Among parasites, *Eimeria* spp. are one of the significant protozoan organisms that cause coccidiosis and result in high morbidity and mortality, especially in calves up to 1 year [4]. The prevalence of gastrointestinal parasites differs by country, region and even management systems on farms.

Although more than 12 members of genus *Eimeria* infect cattle [5], five species cause clinical symptoms such as watery or bloody diarrhea followed by loss of appetite, depression, dehydration, and weight loss, which leads to retarded growth [6]. Two species, *E. bovis* and *E. zuernii*, have the greatest impact due to high virulence and high mortality [6]. As it is sometimes difficult to identify the species of *Eimeria* based on oocyst

morphology in routine fecal examinations, molecular characterization is necessary to accurately make identifications to the species level.

In Indonesia, the population of 16.5 million beef cattle and 0.5 million dairy cattle [7] is concentrated on Java Island (42.6% and 98.9% of beef and dairy cattle, respectively) [8]. Madura cattle are one of the native livestock breeds in Indonesia and are mainly raised on Madura Island, which is located off the northeastern coast of Java Island and contains four districts of East Java Province. The Madura breed is thought to have been developed by inbreeding Zebu (*Bos indicus*) and Banteng (*Bos javanicus*) and is maintained through careful genetic selection [9]. This breed is reported to possess the advantages of high adaptivity to tropical environments, high reproductive performance, high meat quality, and ease of rearing owing to its small to medium size [10]. Recently, the Ministry of Agriculture in Indonesia launched a revitalization program to reduce beef imports and increase domestic production. Based on policies of the government, Madura cattle is listed as one of beef cattle breeds for supplying high-protein food commodities

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

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<https://doi.org/10.1016/j.parint.2021.102478>

Received 7 August 2021; Received in revised form 20 September 2021; Accepted 3 October 2021

Available online 6 October 2021

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and achieving self-sufficiency in Indonesia [11].

In six reports of infection of cattle by *Eimeria* spp. focused on Java Island, the prevalence of parasite infection ranged from 15.33% to 85.07%, and differences could be observed among provinces and districts and even depending on detection methods [12–17]. In only one of the published reports, identification of *Eimeria* spp. to species level was determined by molecular analysis [14]. In the one paper examining the prevalence of protozoan parasites, 71.4% of Madura cattle were reported to be infected and *Eimeria* spp. accounted for 53.4% of the protozoan parasites [16], but identifications were not made to the species level. In the present study, we parasitologically examined the feces of Madura cattle and used molecular methods to identify *Eimeria* species in order to assess the potential risk of severe coccidiosis.

2. Materials and methods

2.1. Study areas and examined cattle

The study areas were Kamal and Socah subdistricts of the Bangkalan district of Madura Island, East Java, Indonesia. In August 2019 to November 2020, a total of 183 fecal samples were collected from cattle on 50 farms in the Socah subdistrict and 22 farms in the Kamal subdistrict with the following breakdown of age categories: <6 months, 9 and 13, respectively; 6 months to 2 years, 48 and 18, respectively; and > 2 years, 54 and 41, respectively. One to five feces samples were randomly collected on each farm. Most feces were normal, except for 3 diarrhea and 5 soft ones. The collected samples were stored at 4 °C until analysis.

2.2. Fecal examination

Feces were examined by the sugar centrifugal flotation method [18,19]. Briefly, 1 g of fecal samples was used and centrifuged at 800 ×g for 5 min. After the supernatant was discarded, sugar solution with a specific gravity of 1.2 was added, followed by centrifugation, then the upper layer was placed on a glass slide. The entire smear was examined by light microscopy. The species of detected parasites were identified based on the published data [20].

2.3. Purification of *Eimeria* oocysts

Eimeria oocysts were purified from the remaining feces (approximately 5–10 g) by the sugar flotation method as previously described [14]. Briefly, feces were diluted in distilled water and filtered through a steel mesh or gauze. After centrifugation at 800 ×g for 5 min, the sugar solution was added, and the sample was centrifuged at 1200 ×g for 10 min. The *Eimeria* oocysts floating on the surface were recovered using a Pasteur pipette and washed with distilled water. The purified oocysts were resuspended with 1–2 ml of PBS, the number of the oocysts was counted under a microscope after putting 15–20 µl of the solution on a slide glass, and OPG (oocysts per 1 g feces) values were calculated. Samples were stored at 4 °C until use in molecular identification analysis.

2.4. Molecular identification of *Eimeria* spp.

DNA of *Eimeria* oocysts purified as described above was extracted by DNazol (Molecular Research Center, Cincinnati, Ohio, USA) according to manufacturer recommended protocol but with the addition of five freeze-thaw cycles before extraction. For identification of six *Eimeria* spp., including pathogenic species (*E. bovis*, *E. zuernii*, *E. alabamensis*, *E. aubrunensis*, *E. ellipsoidalis*, and *E. cylindrica*), PCR targeting the internal transcribed spacer 1 (ITS-1) region of the ribosomal RNA gene was carried out as reported previously [21]. PCR products were subjected to electrophoretic separation on agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

3. Results and discussion

Out of a total of 183 fecal samples examined by the sugar flotation method, 60 samples (32.8%) were positive for parasites of protozoans and nematodes. Among 72 farms surveyed, 27 of 50 farms in Socah and 16 of 22 farms in Kamal subdistricts had positive samples, and oocysts of *Eimeria* spp. were detected in 50 fecal samples (27.3%) (Table 1) (Fig. 1). No age-dependent tendency could be identified in the data. The prevalences of *Eimeria* spp. in cattle of Indonesia were reported in six papers to date, ranging 15.33% to 85.07% [12–17], and those of Madura cattle were 66.0% at Socah and 88.2% at Kamal [16]. Compared to previous data, positivity in our study tended to be lower. Although detailed information in some reports could not be confirmed, these differences in prevalences might be attributable to detection methods, sampling seasons, or management strategies of the farms. Especially, the feces were collected only from April to May as ending of rainy season in previous report [16] and our samples were collected in a relatively long period. Because it was implicated that humid climate could play an important role in development to infectious oocysts [22], further studies are needed to resolve the reason for the different prevalences including examined seasons such as rainy or dry ones.

Based on designation of OPG values of 1–499, 500–5000, or more than 5000 as light, moderate, and high infections, respectively [23], the average OPG in the 50 positive samples in the present study of 1686.1 (Table 1) and only five samples with OPG more than 5000 indicates that most of the examined cattle were lightly infected with *Eimeria* spp. although the OPG values could be changed during patent period of the infection. Additionally, because OPG values of the five soft (4 samples infected with *E. bovis*, including 3 samples with mixed infection by *E. zuernii*, and 1 sample with mixed infection by *E. zuernii* and *E. aubrunensis*) and three diarrhea (1 sample infected by *E. bovis*) fecal samples were 500–4000 and 7000–14,500 OPG, respectively, suggesting the possible link between abnormal forms of feces and *Eimeria* infections.

To date, there have been only two reports of molecular investigations of *Eimeria* spp. in cattle in Indonesia and Korea [14,24]. In the present PCR analyses, we identified *Eimeria* spp. in 26 samples with *E. bovis* being the most prevalent, followed by *E. zuernii* and *E. aubrunensis*. A total of 21 samples appeared to be mixed infections with more than two species present (11; *E. bovis*, *E. zuernii* and *E. aubrunensis*, 6; *E. bovis*, *E. zuernii*, *E. aubrunensis*, and *E. cylindrica*, 4; *E. bovis* and *E. zuernii*). All of mixed infections contained *E. bovis* and *E. zuernii*. Generally, *E. bovis* and *E. zuernii* are recognized as being highly virulent. In other surveys, *E. bovis* and *E. zuernii* were reported to be predominant in Indonesia, Brazil or Nigeria based on morphology [15,25,26], and *E. bovis* was detected with high prevalence in Indonesia and Korea by PCR analysis [14,24]. Our results are in agreement with these previous reports. The remaining 23 samples could not be determined to species level. OPG in 17 of 23 samples was less than 10, likely due to there being an insufficient number of the oocysts in feces for successful PCR amplification or contamination by PCR inhibitors.

Of the other parasites, eggs of nematodes were found in 42 of 183 fecal samples, and *Oesophagostomum* spp. were most frequently detected based on being identified in 26 samples (Table 2). These parasites were previously detected in Indonesia and the prevalences were varied among the studies [12–14,17]. These nematode parasites can be sometimes harmful in cattle due to the reduction in productivities such as weight gain [27]. Oocysts of *Eimeria* spp. were found in 32 in 42 samples positive for nematodes. Infections of nematodes and *Eimeria* spp. are transmitted by ingestion of mature eggs and sporulated oocysts, respectively, and infected animals shed newly developed parasites in their feces in forms that are resistant to most disinfectants and environmental conditions. Parasites can survive for long periods and are potential sources of further infection in breeding environments. Thus, presence of these parasites can indicate direct or indirect infection via fecal-oral routes. In our study, the age-dependency of parasite prevalence could not be clarified, and feces with more than 1000 OPG

Table 1
Summary of study areas, ages of cattle and *Eimeria* spp. identification and prevalence.

| Subdistrict (number of samples) | Age category | No. of cattle examined | Number positive ^a (%) | Average OPG ^b (range) | Species identification by PCR analysis ^c | | | | | | Notes |
|---------------------------------|---------------------|------------------------|----------------------------------|----------------------------------|---|-------------------|-------------------|----------------|-------------------|---------------|---|
| | | | | | <i>E. b</i> | <i>E. z</i> | <i>E. aubr</i> | <i>E. alab</i> | <i>E. c</i> | <i>E. e</i> | |
| Socah (111) | <6 months | 9 | 5 (55.6) | 13.2 (1–50) | 5 | 5 | 3 | – | 1 | – | Coinfected with two (<i>n</i> = 2), three (2) and four (1) species Coinfected with three (2) and four (2) species Coinfected with three (6) and four (3) species |
| | 6 months to 2 years | 48 | 11 (22.9) | 9.6 (1–50) | 4 | 4 | 4 | – | 2 | – | |
| | >2 years | 54 | 13 (24.1) | 9.8 (1–50) | 9 | 9 | 9 | – | 3 | – | |
| Kamal (72) | <6 months | 13 | 3 (23.1) | 10,166.7 (5500–14,500) | 1 | – | – | – | – | – | Coinfected with two species (1) Coinfected with two species (1) and three species (1) |
| | 6 months to 2 years | 18 | 12 (66.7) | 3625.3 (1–15,000) | 4 | 1 | 1 | – | – | – | |
| | >2 years | 41 | 6 (14.6) | 1667 (1–4000) | 2 | 2 | 1 | – | – | – | |
| Total | | 183 | 50 (27.3%) | 1686.1 (1–15,000) | 25 (96.2%) | 21 (80.8%) | 18 (69.2%) | 0 (0%) | 6 (23.12%) | 0 (0%) | |

E. e; *Eimeria ellipsoidalis*. Species-level identification was made in 26 of 50 positive samples and percentages indicate the prevalence of each species among the 26 positive samples.

^a Fecal samples were examined by sugar floatation method.

^b OPG: oocysts per gram.

^c Abbreviations: *E. b*; *Eimeria bovis*, *E. z*; *Eimeria zuernii*, *E. aubr*; *Eimeria aubrunensis*, *E. alab*; *Eimeria alabamensis*, *E. c*; *Eimeria cylindrica*,

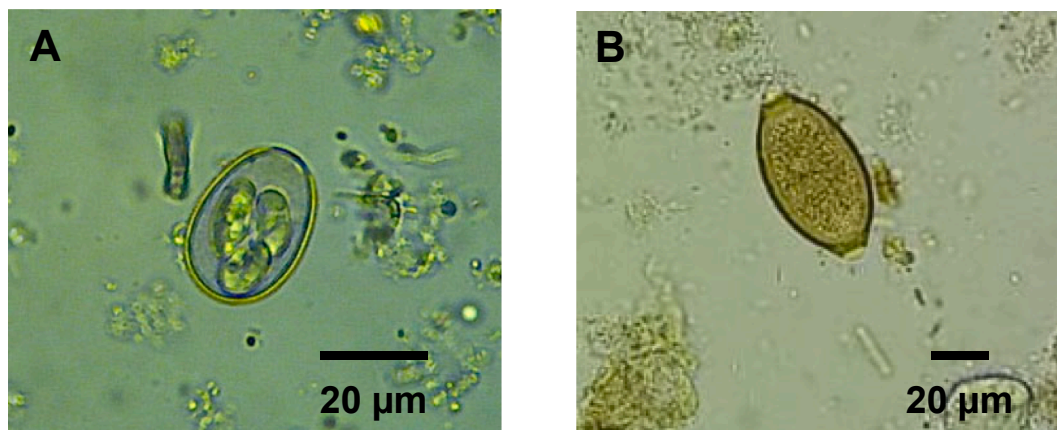


Fig. 1. Photographs of oocyst of *Eimeria* sp. (A) and egg of *Trichuris* sp. (B) from cattle reared in the Socah subdistrict.

Table 2
Summary of study areas, ages and nematodes results.

| Subdistrict (number of farms) | Age category | Number of cattle examined | Number positive (%) | Nematodes identified (percent of total number of cattle examined) | | | | | | |
|-------------------------------|---------------------|---------------------------|---------------------|---|---------------------------|----------------------|---------------------------|-----------------------|---------------------------|------------------------|
| | | | | <i>Oesophagostomum</i> spp. | <i>Strongyloides</i> spp. | <i>Cooperia</i> spp. | <i>Toxocara vitulorum</i> | <i>Trichuris</i> spp. | <i>Mecistocirrus</i> spp. | <i>Capillaria</i> spp. |
| Socah (50) | <6 months | 9 | 3 (33.3) | 2 | 2 | – | – | – | – | – |
| | 6 months to 2 years | 48 | 3 (6.3) | 2 | 2 | – | – | 1 | – | – |
| | >2 years | 54 | 13 (24.1) | 6 | 7 | – | 2 | 1 | 1 | 1 |
| Kamal (22) | <6 months | 13 | 3 (23.1) | 1 | 2 | – | – | – | – | – |
| | 6 months to 2 years | 18 | 7 (38.9) | 5 | 2 | 1 | – | – | – | – |
| | >2 years | 41 | 13 (31.7) | 10 | 1 | 3 | – | – | 1 | – |
| Total | | 183 | 42 (23.0%) | 26 (14.2%) | 16 (8.7%) | 4 (2.2%) | 2 (1.1%) | 2 (1.1%) | 2 (1.1%) | 1 (0.5) |

values of *Eimeria* spp. were sometimes detected in the same farms (data not shown). Differences in age-dependent prevalence might be induced due to management systems of individual farms for breeding and, therefore, longitudinal and quantitative surveys for parasites are

needed to evaluate the occurrence and sources of the coccidiosis and their productivities.

Although the number of samples in our survey was limited, we showed that samples could be identified to species level for *Eimeria* spp.

by the molecular method. Madura cattle are believed to be resistant to various diseases and tick infestations [10]. While resistance to gastrointestinal parasites remains unknown, further studies are required to clarify pathogenicity in local cattle herds and to improve productivity through improved management practices.

Ethics statement

All experiments were carried out without sacrificing live animals. Thus, ethical approval for animal experimentation was not required. All examinations in the field study were conducted with permission of the Ministry of the Environment of the Government of Indonesia. Fecal collection was performed in a non-invasive manner. No animals were sacrificed for the purpose of the field study. No human participants were involved in this study.

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