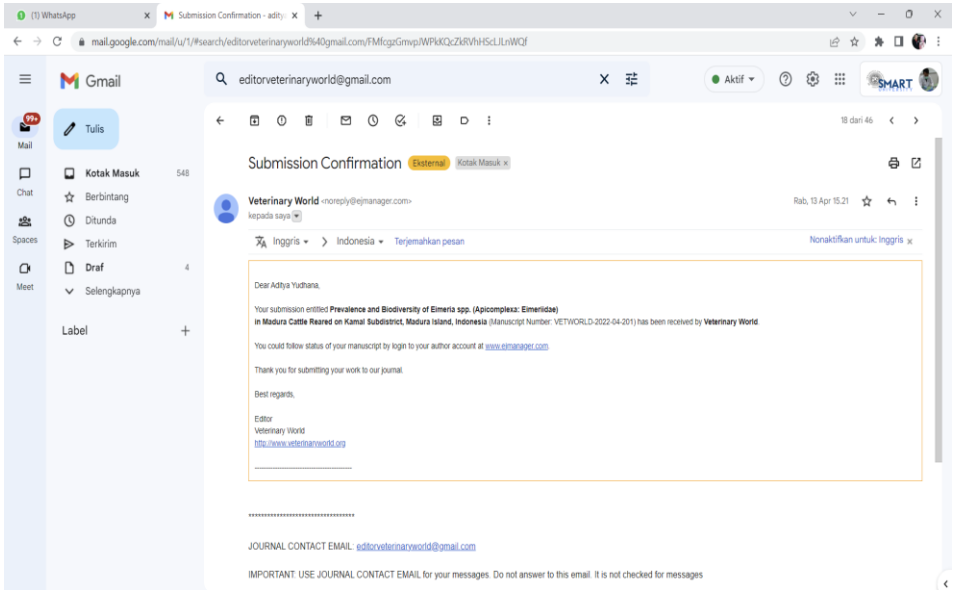
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Dear Aditya Yudhana,
Your submission entitled Prevalence and Biodiversity of Eimeria spp. (Apicomplexa: Eimeriidae) in Madura Cattle Reared on Kamal Subdistrict, Madura Island, Indonesia (Manuscript Number: VETWORLD-2022-04-201) has been received by Veterinary World.
You could follow status of your manuscript by login to your author account at www.eimanager.com.
Thank you for submitting your work to our journal.
Best regards,
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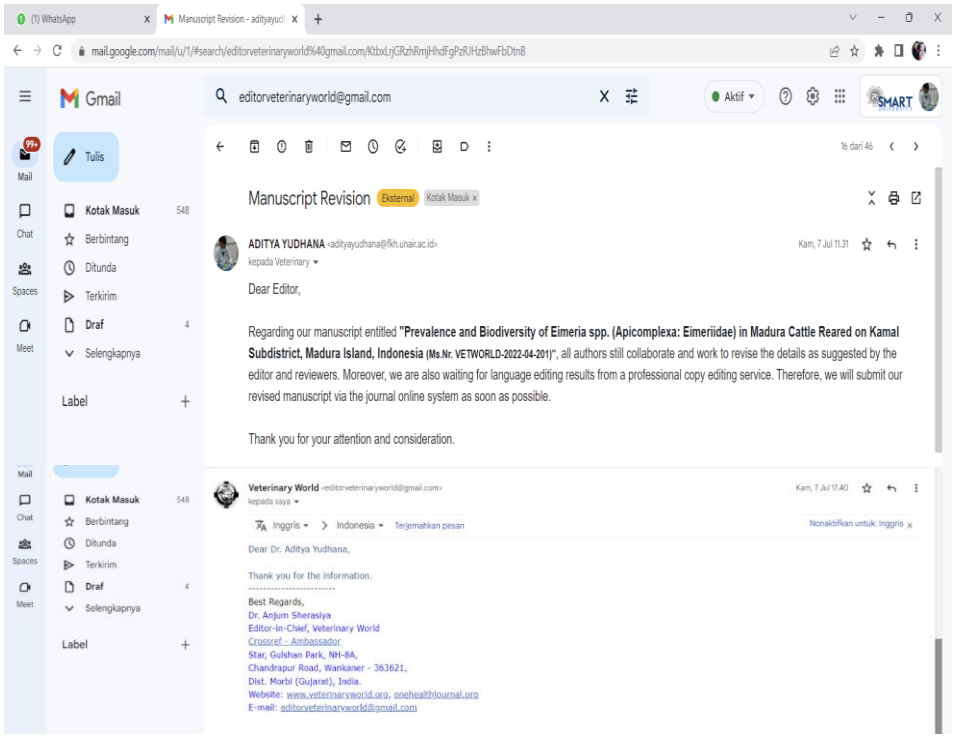
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Dear Editor,
Regarding our manuscript entitled "Prevalence and Biodiversity of Eimeria spp. (Apicomplexa: Eimeriidae) in Madura Cattle Reared on Kamal Subdistrict, Madura Island, Indonesia (Ms.Nr. VETWORLD-2022-04-201)", all authors still collaborate and work to revise the details as suggested by the editor and reviewers. Moreover, we are also waiting for language editing results from a professional copy editing service. Therefore, we will submit our revised manuscript via the journal online system as soon as possible.
Thank you for your attention and consideration.
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Dear Dr. Aditya Yudhana,
Thank you for the information.
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Chandrapur Road, Wankaner - 363621,
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REVIEWERS COMMENTS

EDITORIAL COMMENTS:

- Highlight all corrections/additions in red color font in revised manuscript.
- Please answer all the comments below point-by-point in an accompanying response letter to your revised submission and include your responses at appropriate paragraphs in the revised word file.
- Include all authors name, affiliation, ORCID and email address in revised Word file as per format and style of Veterinary World. Please check latest article from www.veterinaryworld.org for format of this section.
- All reference no. in the text must be in continuous no. as per style of Veterinary World and amend the reference section accordingly if you have not done it.
- Please divide the introduction into 3 paragraphs if you have already not done. Introduction must be divided into 3 paragraphs i.e., 1. introduction 2. significance of the study and 3. aim of the study.
- Include authors\' contributions (refer just below the conclusion section in latest article from www.veterinaryworld.org for format of this section) if you have not added.
- Include Acknowledgements along with source of fund for this study if you have not included.
- All journal names in references must be as per standard journal abbreviation.
- If you will not revise strictly as per suggestion then there will be chance of rejection. So, revise carefully. If you have any query then please email to Editor-in-Chief.

=> Reviewer # 1

In this manuscript, the authors identify the occurrence and verified species diversity of Eimeria spp. from Madura cattle. The proposed study is very important because Eimeria infection causes serious economic problems worldwide. The manuscript is well conducted, has scientific quality and relevance to the field of this journal, but I still have some correction suggestions:
Introduction:
Please include information about economic loss due to coccidiosis in Indonesia. If you feel comfortable, I suggest some references.

Sarwendah Siswi Winasis & Yudha Nurdian. Enormous Economic Impact Compromised by Coccidiosis. https://www.researchgate.net/publication/324833635, April 2018.

W Pawestri, D M Nuraini and M Andityas. The estimation of economic losses due to coccidiosis in broiler chickens in Central Java, Indonesia. Second International Conference on Food and Agriculture 2019, IOP Conf. Series: Earth and Environmental Science 411 (2020) 012030, IOP Publishing, doi:10.1088/1755-1315/411/1/012030.

Material and methods:

Please standardize mL instead ml throughout the manuscript.
Please insert space between 4 and oC.

Discussion:
Please include some discussion about Eimeria species infection in cattle in Indonesia. If you feel comfortable, I suggest this reference:

Ekawasti F, Nurcahyo RW, Firdausy LW, Wardhana AH, Sawitri DH, Prastowo J, Priyowidodo D. Prevalence and risk factors associated with Eimeria species infection in cattle of different geographical regions of Indonesia. Vet World. 2021 Sep;14(9):2339-2345. doi: 10.14202/vetworld.2021.2339-2345. Epub 2021 Sep 6. PMID: 34840452; PMCID: PMC8613789.
Is it possible include in the manuscript, at the discussion end for example, any proposal for public policies to be implemented to control this infection?

Figures
Figures 3A, B, C and D are in low quality resolution. Is it possible to improve them?

=> Reviewer # 2

Line-55: analyzed instead of analized

Sampling methods: Mention the criteria for the sampling.

Also, mention about the sample size calculation.

Line-58, 60: Convert rpm to x g value. It is necessary as rpm is different from company to company.

Mention company and country name for the products mentioned in Materials and Methods.

Discussion: Follow the journal style for in-text citations. e.g. Lee et al (2018), Ekawasti et al (2019).....

Conclusion: Mention limitations and future scope of the study.

Many sentences are ambiguous and needs to rewrite. Also, follow the journal style strictly.

English needs thorough revision.

Editor\'s comment:
Get professional copyediting from ENAGO or Editage [keep all corrections in track changes (language as well as editorial and reviewers) and paste the certificate in the revised word file] or ask Veterinary World in answer letter for copyediting service (with extra payment) as your manuscript needs extensive copyediting.

RESPONSE LETTER

AUTHOR RESPONSES FOR EDITORIAL COMMENTS:

- All corrections/additions already highlighted in red color font.
- All answer of the comments already written in rebuttal response letter and included in appropriate paragraphs in the revised word file.
- All authors name, affiliation, ORCID and email address in revised Word file as per format and style of Veterinary World has been included.
- All reference no. in the text and section already revised as per style of Veterinary World.
- Introduction section has been divided into 3 paragraphs i.e., 1. introduction 2. significance of the study and 3. aim of the study.
- Author's contribution section has been added in the main text.
- Acknowledgements along with source of fund for this study has been included in the main text.
- All journal names in references already as per standard journal abbreviation.
- All authors were performed revision in the manuscript as suggested by the editor and reviewers.
- English in the manuscript has been improved using professional copyediting Editage (certificate included in the manuscript) as suggested by the editor and reviewers.

AUTHOR RESPONSES FOR REVIEWER 1 COMMENTS:

Introduction:
-Information regarding economic loss due to bovine coccidiosis has been written in this section. However, we did not include references suggestion by the reviewer because of the references discuss about poultry coccidiosis while our study has focused on specific bovine coccidiosis in Madura cattle. Moreover, to our knowledge there is no further references or scientific data which state the economic loss due to bovine coccidiosis in Indonesia.

Material and methods:
-The word "ml" has been standardized to "mL" throughout the manuscript.
-Space between numbers and ^{o}C has been checked throughout the manuscript.

Discussion:
-Section about *Eimeria* spp. infection in cattle in Indonesia, including data update has been added. Thank you for the reference suggestion. Moreover, implementation for public policies is possible but further study regarding the complete prevalence in Madura Island needs to be conducted, and we had been added in conclusion section as the suggestion and limitations of the study.

Figures
-Figures 3A, B, C, and D were already in maximum resolution.

AUTHOR RESPONSES FOR REVIEWER 2 COMMENTS:

-The word “analized” has been changed to “analyzed”.

-Criteria for the sampling and sample size calculation has been added in materials and methods (Line 102-106).

-The “rpm” has been changed to x g value as suggested.

-All products had been included with the company and country name in materials and methods.

-Discussion: Text citations in the manuscript has been checked and rewrite as per journal style.

-Conclusion: The limitations and future scope of the study has been added as suggested.

-Many sentences are rewrite as per journal style and as suggestion by the professional language editing service.

-We already using professional language editing service (Editage) in order to revise strictly regarding the use of English throughout the manuscript. Moreover, the language editing original certificate has been attached in the revised manuscript.

All authors thanks to the editor and also reviewers for the constructive suggestions.

Best Regards.

Occurrence and biodiversity of *Eimeria* spp. (Apicomplexa: Eimeriidae) in Madura cattle reared on Kamal Subdistrict, Madura Island, Indonesia

Poedji Hastutiek[1,2], Nunuk Dyah Retno Lastuti[1], Lucia Tri Suwanti[1,2], Agus Sunarso[1], Dyah Ayu Kusumawati[4], and Aditya Yudhana[1,3]

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Commented [AY1]: The word "Prevalence" has been replaced with "Occurrence" as suggested by the reviewer and language editing service.

Commented [AY2]: Affiliation address, e-mail address, and ORCID ID of all authors has been added.

Abstract

Background and Aim: In Indonesia, Madura cattle are native breeds that are expected to contribute to the improvement of regional meat self-sufficiency. *Eimeria* spp. are protozoans that commonly found in ruminants. This study aimed to identify the occurrence and diversity of *Eimeria* spp. in Madura cattle.

Materials and Methods: In this study, fresh fecal samples were collected from 100 cattle in Kamal Subdistrict, Bangkalan District, Madura Island, Indonesia. Morphological detection was performed using a light microscope, and molecular identification was performed using polymerase chain reaction. DNA amplification was conducted using various species-specific primers for *Eimeria bovis*, *E. zuernii*, *E. auburnensis*, *E. alabamensis*, *E. ellipsoidalis*, and *E. cylindrica*.

Results: The results obtained 21% (21/100) of *Eimeria* spp based on morphological detection. A total of 15 positive samples with 500–25000/mL oocysts were selected for DNA extraction and amplification, resulting in twelve positive samples. Four *Eimeria* spp. were obtained based on molecular identification: *E. bovis*, *E. zuernii*, *E. auburnensis*, and *E. cylindrica*.

Conclusion: Further comprehensive studies are required to understanding the pathogenicity of *Eimeria* spp. in Madura cattle. Therefore, improved and integrated management practices should

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Commented [AY11]: The sentence and percentage of results has been changed as suggested by all authors and the professional language editing service.

be strengthened by local governments to prevent pathogenic diseases and increase national livestock productivity in Indonesia.

Keywords: Biodiversity, *Eimeria* species, Infectious disease, Madura cattle, Madura Island.

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Introduction

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Bovine coccidiosis, caused by *Eimeria* spp. is a parasitic disease that common in cattle, it is caused by *Eimeria* spp. There are approximately 21 *Eimeria* species in cattle, of which *Eimeria bovis* and *E. zuernii* are the most pathogenic species [1]. Coccidiosis in adult animals is often asymptomatic but can be a reservoir for calves [2]. Infection in calves causes diarrhea, dehydration, dysentery, debilitation, and death in severe cases [3] Although most of them are non-pathogenic, *Eimeria* spp. can cause intestinal tissue damage and decrease productivity in meat and milk [4]. In addition, *Eimeria* spp. infection in cattle can increase their vulnerability to other infectious diseases such as pneumonia and, bacterial and viral disease [5, 6]. The economic loss due to coccidiosis in cattle was estimated at USD $ 400 million worldwide. In Mexico, coccidiosis reportedly affects the economics of large and small ruminant, with annual losses up to USD $ 23.7 million [6].

Coccidiosis cases is easily found in managed farms in dirty environments, which are contaminated by *Eimeria* oocysts. Cattle are infected with *Eimeria* spp., through ingestion of sporulated oocysts that contaminate water and feed as the main source of transmission [7]. Factors related to prevalence of *Eimeria* spp. infection in cattle include farm management, age,

and environmental temperature [8]. In Indonesia, the management of farm is mainly based on traditional systems which managed by family units. Madura cattle are one of the main sources of meat in Madura Island, Indonesia. This breed also expected to contribute to the improvement of regional meat self-sufficiency. The manifestation of *Eimeria* spp in the cattle might be affecting the achievement of the program.

To our knowledge, there is no data regarding economic loss due to bovine coccidiosis in Indonesia because the majority of studies only focused on poultry coccidiosis. Only a few studies have reported *Eimeria* infections particularly in cattle in Indonesia. Ananta *et al*. [9] reported 22.4% prevalence of *Eimeria* in cattle in West Java Province and Hamid *et al*. [10] reported 15.5% in Central Java Province. Coccidiosis in Madura cattle was also reported microscopically by Hastutiek *et al*. [11] with a prevalence 75.07%. However, in almost studies, *Eimeria* spp. were only observed morphologically using microscopic examination. The first report of *Eimeria* species based on molecular identification in Indonesia were done by Ekawasti *et al*. [4], who reported that prevalence of each species was 10.4%, 2.8%, 2.1%, 1.4%, 1.1%, and 0.4%, for *E. bovis*, *E. ellipsoidalis*, *E. alabamensis*, *E. zuernii*, *E. auburnensis*, and *E. cylindrical*. Therefore, this study aimed to identify various species of *Eimeria* spp. in Madura cattle using a molecular diagnostic approach.

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Material and Methods

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Ethics approval

All experiments were performed without sacrificing live animals. Thus, ethical approval for animal experimentation was not required. All examinations in the field study were conducted with permission from the Ministry of Agriculture of the Government of Indonesia. Fecal

collection was performed in a noninvasively. No animals were euthanized for the purpose of the field study. No human participants were included in this study.

Study sites and sampling methods

Kamal Subdistrict (112.72713 longitude and -7.136996 latitude) is a part of the Bangkalan District on Madura Island. The area spans over 41.40 km^2. Madura cattle (*Sapi Madura*) are a stable, inbred hybrid of Zebu and Banteng (*Bos javanicus*) [12] that originated from the island of Madura. Their body appearance is very similar to that of Bali cattle, which have the same origin as Banteng. The color is reddish-brown with non-specific white patterning on the leg and rump. Adult bulls weigh approximately 250-300 kg. A total of 100 cattle fecal samples were collected from fresh dung (<8 h) and stored in plastic bags containing potassium dichromate. No animals showed specific clinical symptoms when fecal samples were collected. Sample size calculation based on 10% of the total Madura cattle population from each village where located at north, south, east, west, and center part of Kamal Subdistrict.

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Fecal examination

The samples were analyzed using modified sugar flotation methods [13]. Sugar flotation methods were used, with a specific gravity of 1.2 (Gulaku Indonesia, Lampung, Indonesia). Approximately 2-4 grams of feces was diluted with 12 mL of aquadest. The fecal solution was filtered, and the filtrate was transferred to a 15 mL centrifuge tube. The sample was centrifuged at 3000x *g* for 10 minutes. The supernatant was discarded and re-suspended in sugar solution. The suspension was mixed and centrifuged at 3000x *g* for 10 minutes. The supernatant was collected and examined on a glass slide at 100x and 400x magnification under a light microscope. *Eimeria* parasites were identified based on morphological features, such as size, shape, number of

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sporozoites, and other notable characteristics [14]. Qualitative microscopic examination was performed to determine the presence and absence of oocysts. Quantitative examination was performed by counting the number of oocysts per milliliter.

The purification was performed using a positive sample. *Eimeria* oocysts were purified using the sugar flotation method [15]. *Eimeria* oocysts were placed on the surface of the sugar solution using a pipette approximately 1-2 mL. The supernatant was washed three times with distilled water. The pellet was added to 1–2 mL of PBS and stored at 4° C.

Molecular identification

Fifteen morphologically positive samples were subjected to molecular analyses. The selection of molecular samples was based on the number of oocysts, which contain 250-25000 oocysts per milliliter of fecal solution. DNA were extracted using DNAzol (Ohio, USA), according to the manufacturer's recommended procedures. DNA was amplified using the primer pairs *Eimeria* specific (species) primers [15, 16]. Primers were specific for *E. bovis*, *E. zuernii* , *E. auburnensis*, *E. cylindrica*, *E. alabamensis* and *E. ellipsoidalis* (Table 1)**.** In this study, the amplification reaction was performed in a 25µL solution consisting of 12.5 µL of Bioline Mastermix (Bioline, Taiwan), 1 µL of each primer, 8.5 µL destilated water and 2 µL of the DNA template. Amplification involved an initial denaturation phase at 94 °C for 30 s, followed by 35 cycles at 94 °C for 10 s, 52 °C for 20 s, and 72 °C for 20 s, and a final extension at 72 °C for 2 min [15]. Then, 10 µL of PCR products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and visualized in UV transluminator.

Results

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Eimeria spp. were identified in 21 of the 100 (21%) fecal samples by using microscopy. The morphology of *Eimeria* spp., sporulated, and unsporulated oocysts, based on observation under a light microscope, is shown in Figure 1. Of 15 samples amplified by PCR, 12 samples succesfully amplified by PCR. The results of running PCR products showed that four *Eimeria* species were found: *E. bovis*, *E. zuernii*, *E, auburnensis*, and *E. cylindrica* (Figure 3 A-D). Six samples were detected for *E. bovis*, three for *E. ezuernii*, two for *E. aurburnensis* and, one for *E. cylindrica*. *E.bovis* was detected more frequenly in this study. Five samples found in single infection and three samples in mixed infection.

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Discussion

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Many studies have reported the prevalence of *Eimeria* spp. in cattle in different countries. However, lot of studies still used standard microscopy examination to detect oocysts [2, 3, 8, 10]. The number of prevalence was different in each country; the prevalence of *Eimeria* infection reached 75.5% in Colombia, 22.1% in South Korea, 47.09% in Pakistan, and 11.97% in India [3, 8, 16, 17]. Ekawasti *et al*. [4], reported 52.3% prevalence of *Eimeria* on Java Island. Furthermore, bovine coccidiosis has also been reported in Maluku Island as the highest (94.1%) prevalence, followed by Kalimantan (83%), Sumatra (70.3%), Sulawesi (68.9%), Papua (62.3%), and Nusa Tenggara (58.5%) [18]. The variation in prevalence and type of infection can differ depending on the various infection rates, and shedding intensities of individuals. These differences might be due to geographical conditions, sources of feeds, and feeding behavior [19].

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Based on the molecular diagnostic findings, twelve samples were shown positive. *E. bovis* frequently found in this study, followed with *E. zuernii*, *E. aurbunensis*, and *E. cylindrica*. In south Korea, Lee *et al*. [8] reported in which *E. bovis* was identified 79 % and *E. zuernii* 66 % of samples. Ekawasti *et al*. [4] also reported that *E. bovis* (10.4%) is the most prevalent species

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on Java Island, Indonesia. Using PCR as a molecular approach, Lee *et al*. [8] successfully identified three species of *Eimeria*, namely, *E. bovis*, *E. zuernii*, and *E. auburnensis*. Ekawasti *et al*. [4], identified *E. bovis*, *E. ellipsoidalis*, *E. alabamensis*, *E. zuernii*, *E. auburnensis* and *E. cylindrica*. Moreover, in this study, not all of positive samples in the microscopic examination showed positive PCR results. A possible reason for this is the limited number of oocysts in fecal samples. Those findings were supported by the statement of Carvalho *et al*. [20], Mirhashemi *et al*. [21] and Ekawasti *et al*. [4], who explained that a small number of oocysts was not sufficient for species identification using PCR method. The presence of contaminants possibly also inhibited the PCR process during the procedures [4, 15].

Bovine coccidiosis can cause not only growth delays but also decrease of body performance and cattle production. These clinical signs also affect the quality of adult cattle, thus resulting in high morbidity and mortality in calves, which can inhibit the sustainability of livestock production [22]. Theoretically, coccidiosis is a pathogenic disease of young animals, but poor nutritional and environmental management can be potential risk factors for older animals. Adult cattle with chronic infection frequently diagnosed with anorexia, weight loss, emaciation, bloody diarrhea, and blood-stained dung in perineum and tail part [23].

In this study, Madura cattle were infected with either single or mixed *Eimeria* species. Coccidiosis in cattle is typically caused by more than one species of *Eimeria*. A Madura cattle in this study infected with single or mixed *Eimeria*. Bangoura *et al*. [2] reported that 48.6% of cases of diarrhea in calves were caused by a single infection, and 51.4% had mixed infections. Morgoglione *et al*. [24] also reported that 71.2% of cattle were infected with more than one *Eimeria* species. These previous results were different from our study, which showed that a single infection was recorded more frequently compared to mixed infection. In the sampling

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area, the management of cages and sanitation is also known to be improper because feces that were cleared from the cages were dumped right around the cages, it might be potentialy increase the risk of infection and renfection [25, 26]. The majority of cages are also known to be traditional and still not equipped with feces and urine disposal lines.

Management patterns also affect the occurrence of *Eimeria* spp. infection, such as sanitation method, drainage system, population density, cage structure, feeding systems, and drinking sources [27]. Occurrence of infection and intensity of *Eimeria* spp. in cattle were also recorded at lower percentage in cage compared to pasture [28]. Therefore, cattle shed a lot of oocysts through feces in their closed cages every day during the patent period, which can increase the risk of transmission and increase the development cycle of *Eimeria* spp. The clinical signs of bovine coccidiosis frequently appear 2-3 weeks after infection in a contaminated environment condition [29].

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To date, there have been rarely reports of molecular investigations of *Eimeria* spp. especially in Indonesia [4, 30]. Although the number of samples in our study was limited, we revealed that the samples could be identified at the species level for *Eimeria* spp. using the molecular method. Therefore, comprehensive studies are required to further understand the pathogenicity of *Eimeria* spp. infection in Madura cattle and improves productivity through improved and integrated livestock management practices.

Conclusion

The occurence of *Eimeria* spp. infection in Madura cattle in Kamal Subdistrict, Bangkalan District, Madura Island, is 12% detected by PCR using specific species primers. Moreover, this study successfully obtained four species: *E. bovis*, *E. zuernii*, *E. auburnensis* and *E. cylindrica*. The occurrence of *Eimeria* spp. among Madura cattle should be considered

because bovine coccidiosis probably distributed in most parts of Madura Island. Based on these findings, molecular detection of coccidiosis in Madura cattle can be applied not only in one District, but also in several different Districts, which have different condition associated with the risk factors. The biosecurity measures need to be strengthening among traditional farmers, in order to control the transmission of *Eimeria* spp. in Madura cattle.

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Author's Contributions

Commented [AY43]: This part has been added as suggested by the Editor.

PH, LTS, AS, and DAK: Collected fecal samples. PH, NDRL, and DAK: Analyzed the microscopic observation and molecular identification. PH, DAK, and AY: Wrote original draft and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

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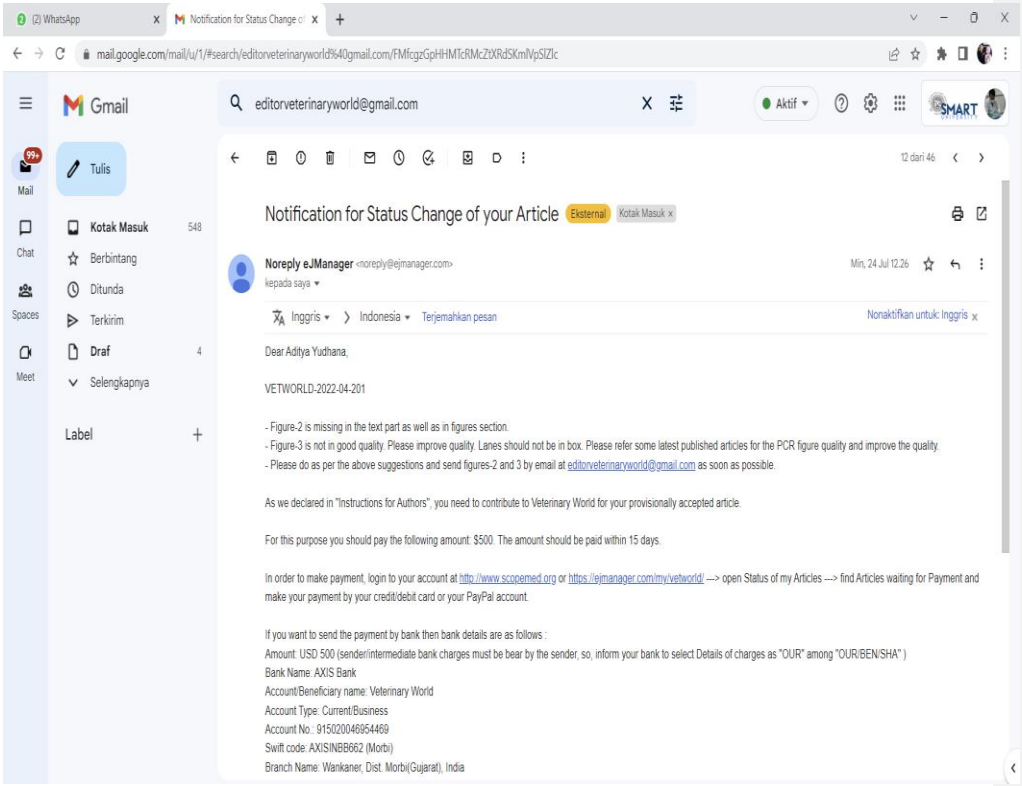
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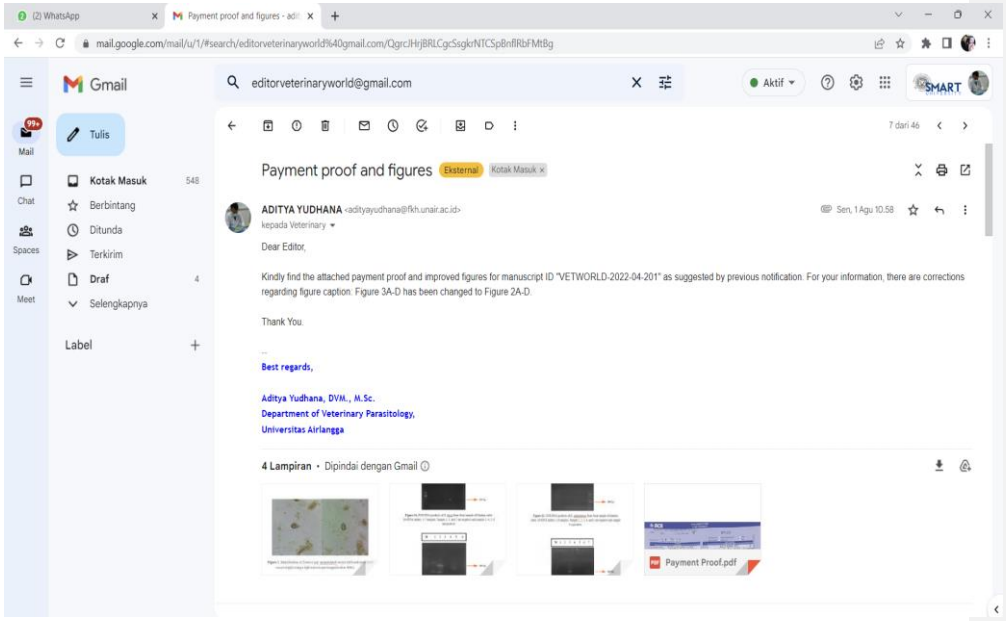
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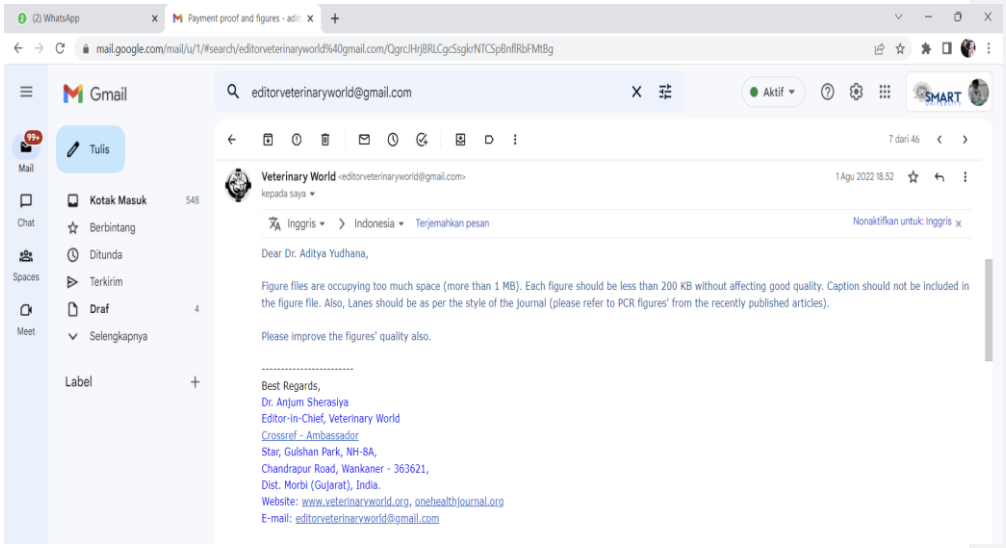
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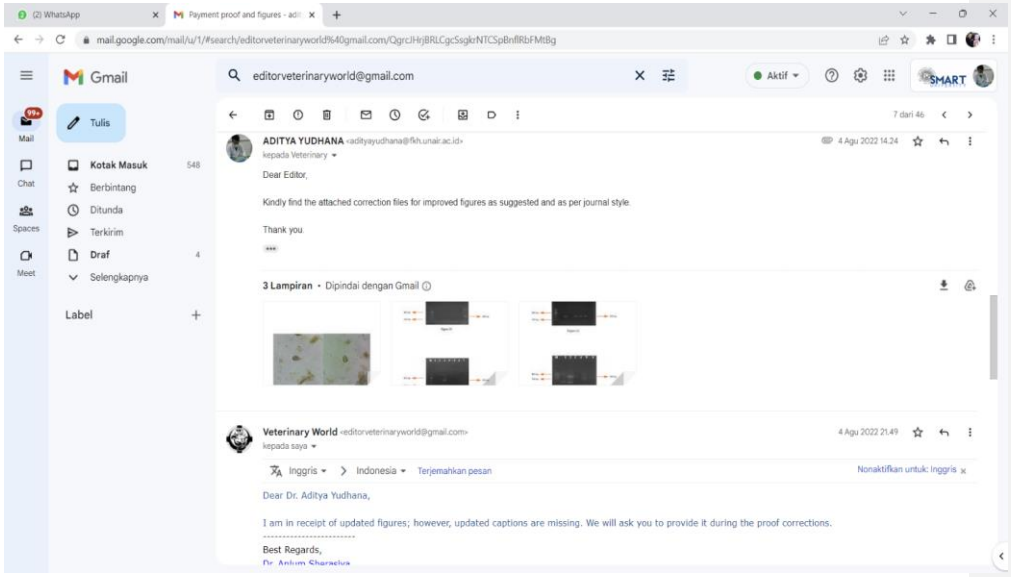
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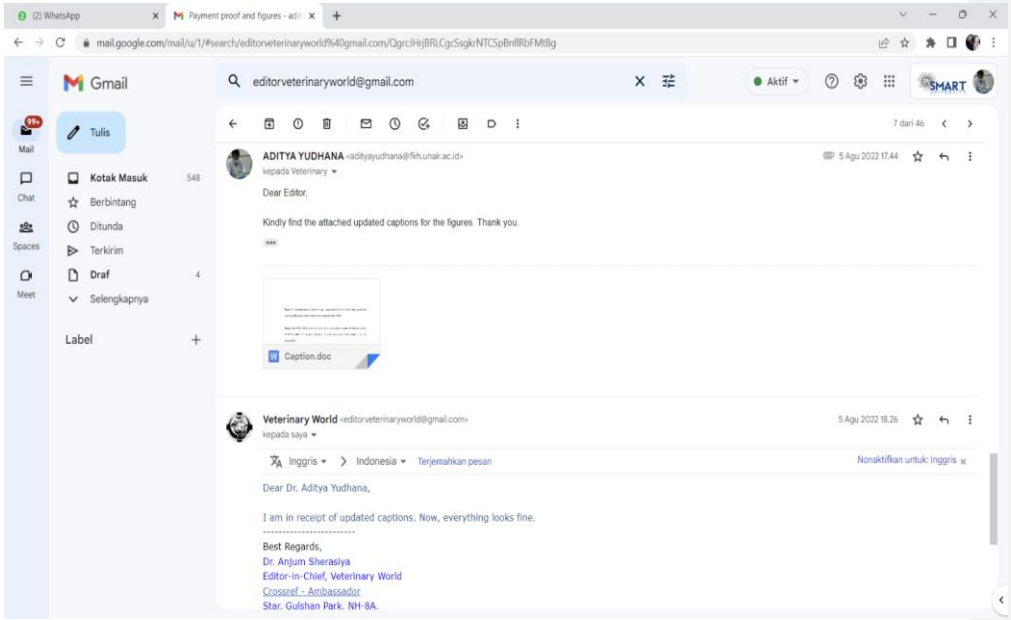
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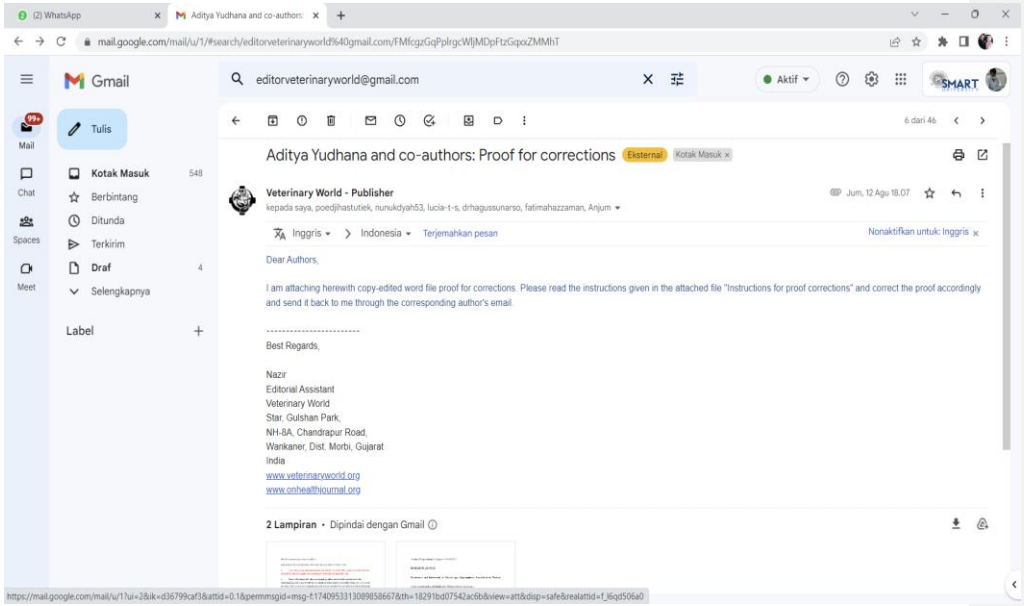
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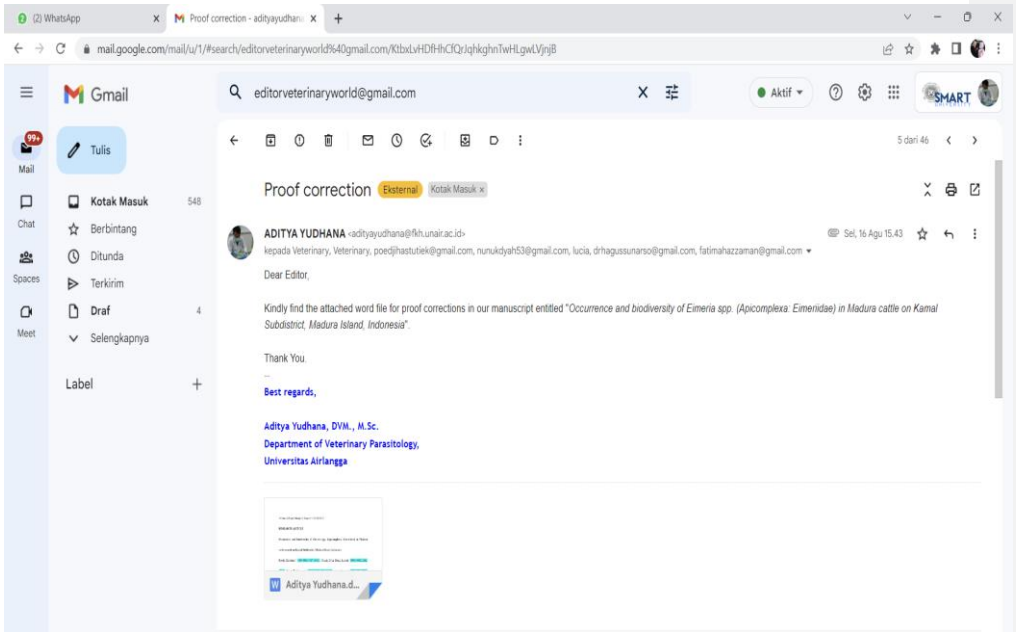
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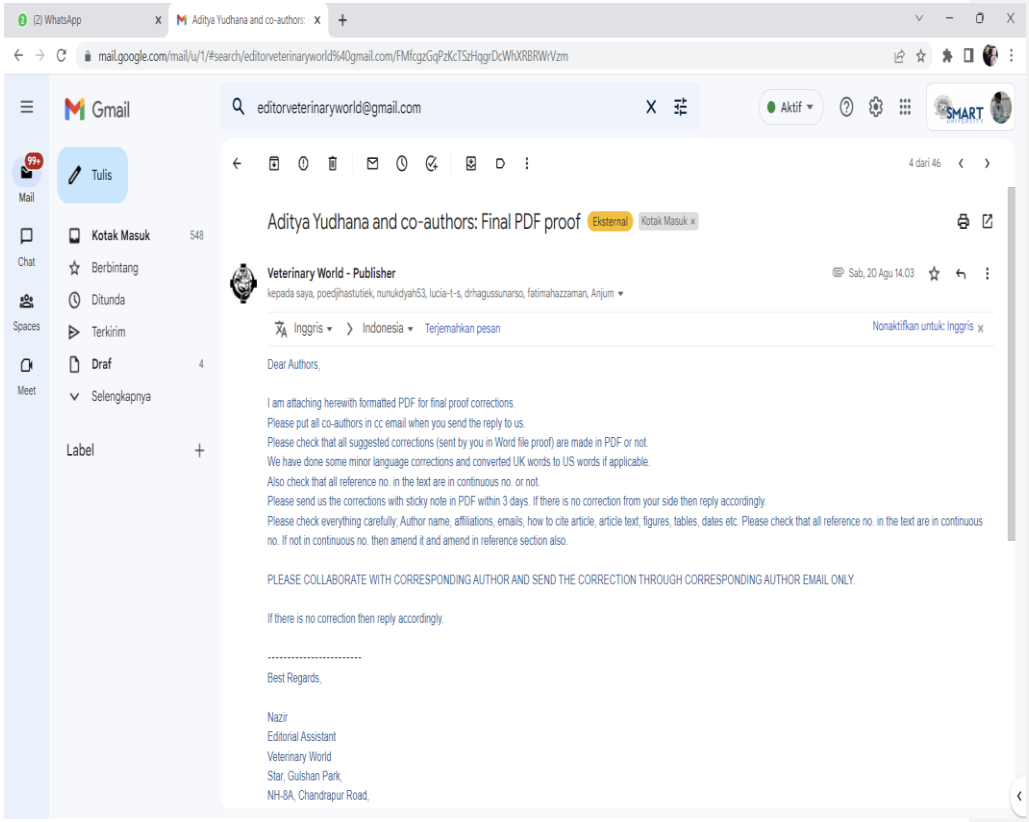
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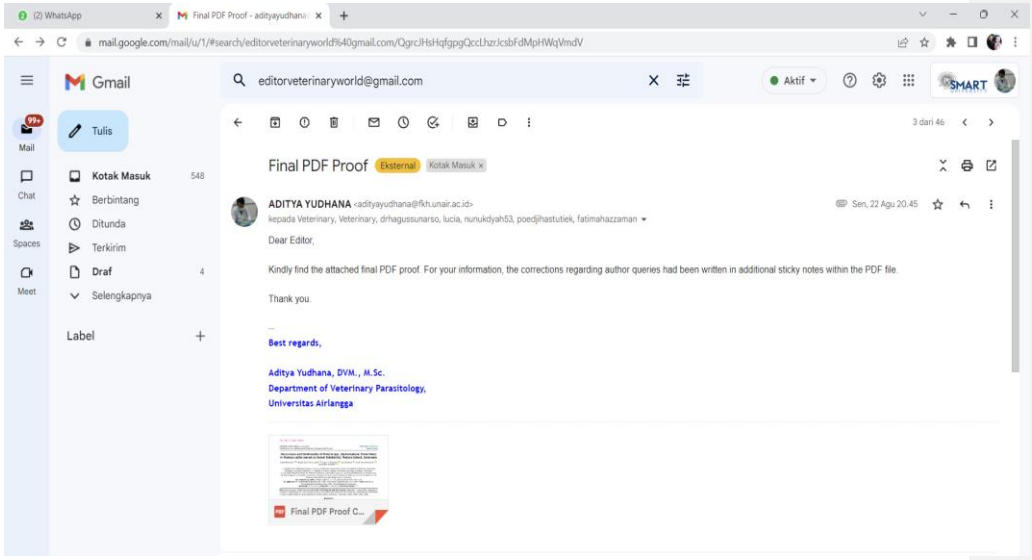
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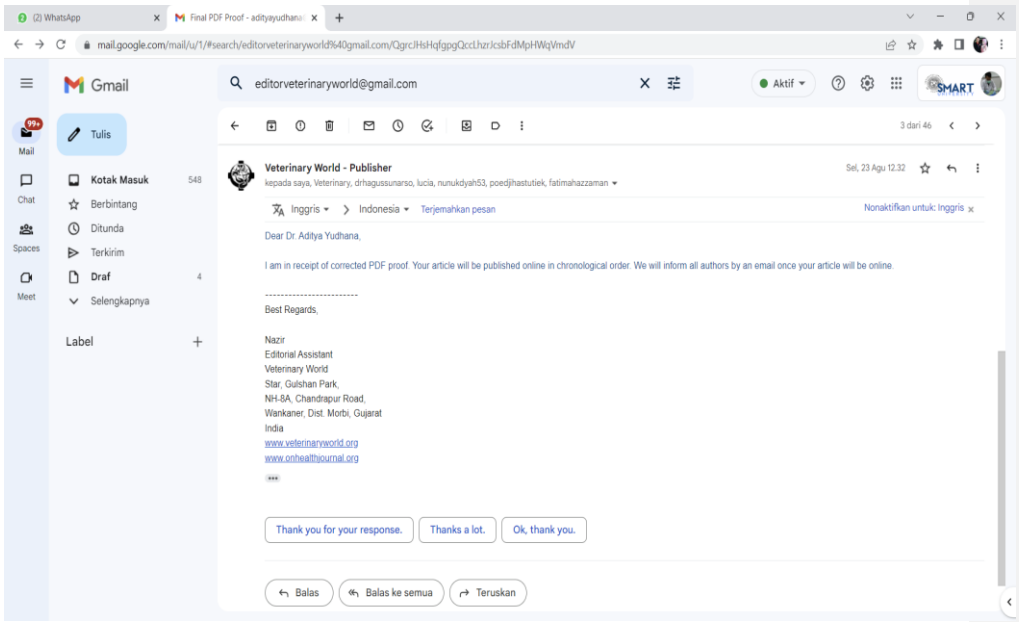
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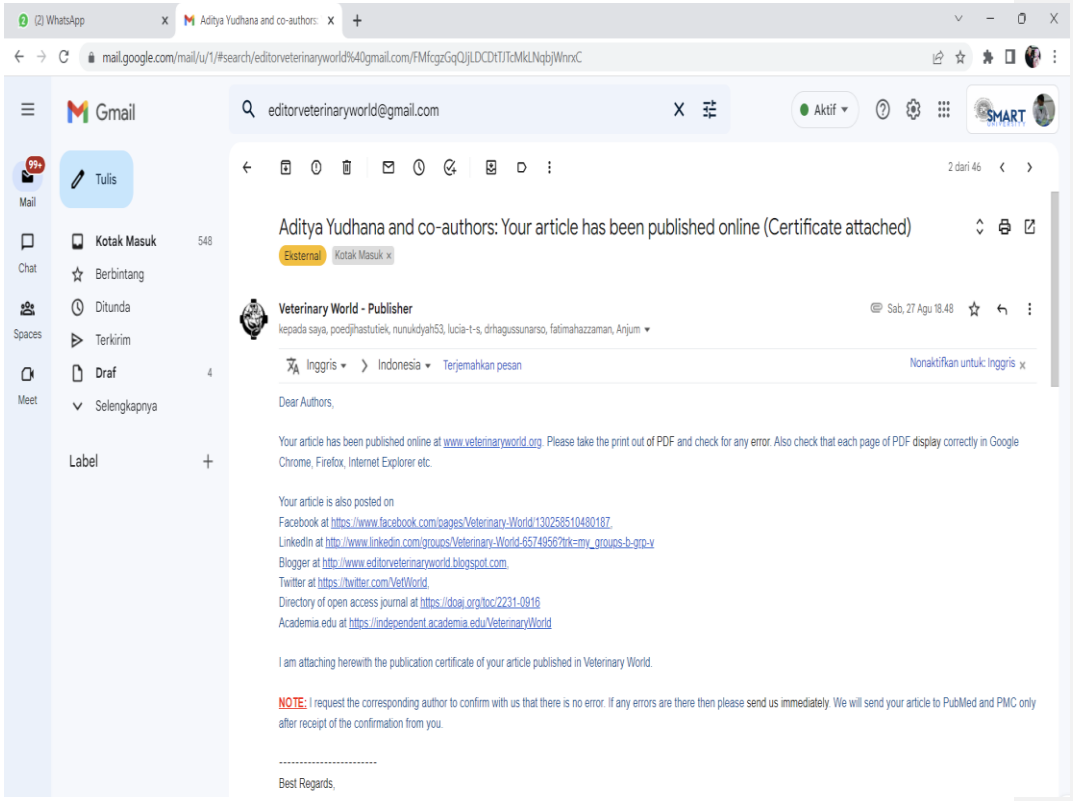
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RESEARCH ARTICLE
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Occurrence and biodiversity of *Eimeria* spp. (Apicomplexa: Eimeriidae) in Madura cattle reared on Kamal Subdistrict, Madura Island, Indonesia

Poedji Hastutiek[1,2], Nunuk Dyah Retno Lastuti[1], Lucia Tri Suwanti[1,2], Agus Sunarso[1], Dyah Ayu Kurniawati[3], and Aditya Yudhana[1,4]

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Abstract

Background and Aim: In Indonesia, Madura cattle are native breeds that are expected to contribute to the improvement of regional meat self-sufficiency. *Eimeria* spp. are protozoans that are commonly found in ruminants. This study aimed to identify the occurrence and diversity of *Eimeria* spp. in Madura cattle.

Materials and Methods: In this study, fresh fecal samples were collected from 100 cattle in Kamal Subdistrict, Bangkalan District, Madura Island, Indonesia. Morphological detection was performed using a light microscope, and molecular identification was performed using a polymerase chain reaction. DNA amplification was conducted using various species-specific primers for *Eimeria bovis*, *Eimeria zuernii*, *Eimeria auburnensis*, *Eimeria alabamensis*, *Eimeria ellipsoidalis*, and *Eimeria cylindrica*.

Results: The results obtained 21% (21/100) of *Eimeria* spp. based on morphological detection. A total of 15 positive samples with 500–25,000/mL oocysts were selected for DNA extraction and amplification, resulting in 12 positive samples. Four *Eimeria* spp. were obtained based on molecular identification: *E. bovis*, *E. zuernii*, *E. auburnensis*, and *E. cylindrica*.

Conclusion: Four species of *Eimeria* namely *E. bovis*, *E. zuernii*, *E. auburnensis*, and *E. cylindrica* were identified from fecal sample of Madura cattle using PCR method in this study. Further comprehensive studies are required to investigate the pathogenicity of *Eimeria* spp. in Madura cattle. Therefore, improved and integrated management practices should be strengthened by local governments to prevent pathogenic diseases and increase national livestock productivity in Indonesia.

Keywords: biodiversity, *Eimeria* species, infectious disease, Madura cattle, Madura Island.

Introduction

Bovine coccidiosis, caused by *Eimeria* spp., is a parasitic disease that is common in cattle. There are approximately 21 *Eimeria* species in cattle, of which *Eimeria bovis* and *Eimeria zuernii* are the most pathogenic [1]. Coccidiosis in adult animals is often asymptomatic but can be a reservoir for calves [2]. Infection in calves causes diarrhea, dehydration, dysentery, debilitation, and death in severe cases [3]. Although most of them are non-pathogenic, *Eimeria* spp. can cause intestinal tissue damage and decrease productivity in meat and milk [4]. In addition, *Eimeria* spp. infection in cattle can increase their vulnerability to other infectious diseases such as pneumonia and bacterial and viral disease [5, 6]. The economic loss due to coccidiosis in cattle was estimated at USD 400 million worldwide. In Mexico, coccidiosis reportedly affects the economics of large and small ruminants, with annual losses up to USD 23.7 million [6].

Coccidiosis cases are easily found in managed farms in dirty environments, which are contaminated by *Eimeria* oocysts. Cattle are infected with *Eimeria* spp. through ingestion of sporulated oocysts that contaminate water and feed as the main source of transmission [7]. Factors related to the prevalence of *Eimeria* spp. infection in cattle include farm management, age, and environmental temperature [8]. In Indonesia, farm management is mainly based on traditional systems managed by family units. Madura cattle are one of the main meat sources in Madura Island, Indonesia. This breed is also expected to contribute to the improvement of regional meat self-sufficiency. The manifestation of *Eimeria* spp. in the cattle might be affecting the achievement of the program.

To the best of our knowledge, there are no data regarding economic loss due to bovine coccidiosis in Indonesia because most studies only focused on poultry coccidiosis. Only a few studies have reported *Eimeria* infections, particularly in cattle in Indonesia. Ananta *et al*. [9] reported 22.4% prevalence of *Eimeria*

in cattle in West Java Province, and Hamid *et al*. [10] reported 15.5% in Central Java Province. Coccidiosis in Madura cattle was also reported microscopically by Hastutiek *et al*. [11], with a prevalence of 75.07%. However, in almost studies, *Eimeria* spp. were only observed morphologically using the microscopic examination. The first report of *Eimeria* species based on molecular identification in Indonesia was done by Ekawasti *et al*. [4], who reported that the prevalence of each species was 10.4%, 2.8%, 2.1%, 1.4%, 1.1%, and 0.4%, for *E. bovis*, *Eimeria ellipsoidalis*, *Eimeria alabamensis*, *E. zuernii*, *Eimeria auburnensis*, and *Eimeria cylindrical*.

Therefore, this study aimed to identify various species of *Eimeria* spp. in Madura cattle using a molecular diagnostic approach.

Materials and Methods

Ethical approval

No ethical approval was required as samples were collected for diagnostic purposes only. This study not used cattle as the sample but used only fresh fecal samples that had been collected from around the enclosures. However, sample collections in the field were conducted with permission from the Animal Husbandry Department in Bangkalan District, Madura Island.

Study period and location

The study was conducted from January to December 2020. The fecal samples of Madura cattle were collected in the Kamal Subdistrict, Bangkalan District, Madura Island. Morphological and molecular identification of *Eimeria* spp. were conducted in the Laboratory of Veterinary Parasitology, Division of Veterinary Parasitology, Department of Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java Province, Indonesia.

Study sites and sampling methods

Kamal Subdistrict (112.72713 longitude and - 7.136996 latitude) is a part of the Bangkalan District on Madura Island. The area spans over 41.40 km^2. Madura cattle (*Sapi Madura*) are a stable, inbred hybrid of Zebu and Banteng (*Bos javanicus*) [12] that originated from the island of Madura. Their body appearance is very similar to that of Bali cattle, which have the same origin as Banteng. The color is reddish-brown with non-specific white patterning on the leg and rump. Adult bulls weigh approximately 250–300 kg. A total of 100 cattle fecal samples were collected from fresh dung (<8 h) and stored in plastic bags containing potassium dichromate. No animals showed specific clinical symptoms when fecal samples were collected. Sample size calculation based on 10% of the total Madura cattle population from each village were located in north, south, east, west, and center part of Kamal Subdistrict.

Fecal examination

The samples were analyzed using modified sugar flotation methods [13]. Sugar flotation methods were used, with a specific gravity of 1.2 (Gulaku Indonesia, Lampung, Indonesia). Approximately 2–4 g of feces was diluted with 12 mL of Aquadest. The fecal solution was filtered, and the filtrate was transferred to a 15 mL centrifuge tube. The sample was centrifuged at 3000× *g* for 10 min. The supernatant was discarded and resuspended in a sugar solution. The suspension was mixed and centrifuged at 3000× *g* for 10 min. The supernatant was collected and examined on a glass slide at 100× and 400× under a light microscope (Olympus, Guangzhou, China). *Eimeria* parasites were identified based on morphological features, such as size, shape, number of sporozoites, and other notable characteristics [14]. A qualitative microscopic examination was performed to determine the presence and absence of oocysts. A quantitative examination was performed by counting the number of oocysts per milliliter.

The purification was performed using a positive sample. *Eimeria* oocysts were purified using the sugar flotation method [15]. *Eimeria* oocysts were placed on the surface of the sugar solution using a pipette of approximately 1–2 mL. The supernatant was washed three times with distilled water. The pellet was added to 1–2 mL of PBS and stored at 4°C.

Molecular identification

Fifteen morphologically positive samples were subjected to molecular analyses. The selection of molecular samples was based on the number of oocysts containing 250–25,000 oocysts per milliliter of fecal solution. DNA was extracted using DNAzol (Ohio, USA), according to the manufacturer's recommended procedures. DNA was amplified using the primer pairs *Eimeria* specific (species) primers [15, 16]. Primers were specific for *E. bovis*, *E. zuernii*, *E. auburnensis*, *E. cylindrica, E. alabamensis,* and *E. ellipsoidalis*. In this study, the amplification reaction was performed in a 25 μL solution consisting of 12.5 μL of Bioline Mastermix (Bioline, Taiwan), 1 μL of each primer, 8.5 μL distilled water, and 2 μL of the DNA template. Amplification involved an initial denaturation phase at 94°C for 30 s, followed by 35 cycles at 94°C for 10 s, 52°C for 20 s, and 72°C for 20 s, and a final extension at 72°C for 2 min [15]. Then, 10 μL of polymerase chain reaction (PCR) products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide, and visualized in an ultraviolet transilluminator.

Results

Eimeria spp. were identified in 21 of the 100 (21%) fecal samples using microscopy. The morphology of *Eimeria* spp., sporulated, and unsporulated oocysts, based on observation under a light microscope, is shown in Figure-1. Of 15 samples amplified by PCR, 12 samples were successfully amplified. The results of running PCR products showed that four *Eimeria* species were found: *E. bovis*, *E. zuernii*, *E. auburnensis*, and *E. cylindrica* (Figure-2a-d).

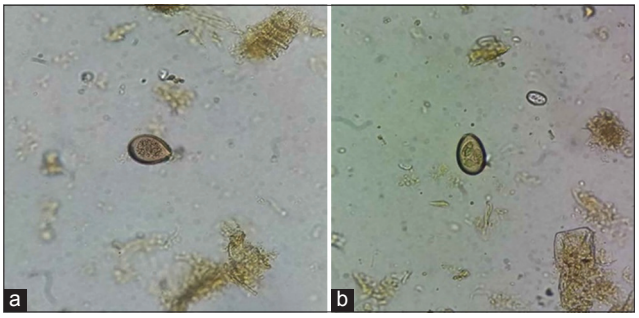

Figure-1: Identification of *Eimeria* spp. unsporulated oocyst (left) and sporulated oocyst (right) using a light microscope (400×).

Six samples were detected for *E. bovis*, three for *E. zuernii*, two for *Eimeria Aurburnensis,* and one for *E. cylindrica*. *E. bovis* was detected more frequently in this study. Five samples were found with a single infection and three samples with mixed infections.

Discussion

Many studies have reported the prevalence of *Eimeria* spp. in cattle in different countries using a standard microscopy examination to detect oocysts [2, 3, 8, 10]. The prevalence was different in each country; the prevalence of *Eimeria* infection 75.5% in Colombia [3], 22.1% in South Korea [8], 47.09% in Pakistan [16], and 11.97% in India [17]. Ekawasti *et al*. [4] reported 52.3% prevalence of *Eimeria* on Java Island. Furthermore, bovine coccidiosis has also been reported in Maluku Island as the highest (94.1%) prevalence, followed by Kalimantan (83%), Sumatra (70.3%), Sulawesi (68.9%), Papua (62.3%), and Nusa Tenggara (58.5%) [18]. The variation in prevalence and type of infection can differ depending on the various infection rates and shedding intensities of individuals. These differences might be due to geographical conditions, sources of feed, and feeding behavior [19].

Based on the molecular diagnostic findings, 12 samples were shown positive. *E. bovis* was frequently found in this study, followed by *E. zuernii, Eimeria aurburnensis,* and *E. cylindrica*. In South Korea, Lee *et al*. [8] reported that *E. bovis* was identified in 79% and *E. zuernii* in 66% of samples. Ekawasti *et al*. [4] also reported that *E. bovis* (10.4%) is the most prevalent species on Java Island, Indonesia. Using PCR as a molecular approach, Lee *et al*. [8] successfully identified three species of *Eimeria*, namely, *E. bovis*, *E. zuernii,* and *E. auburnensis.* Ekawasti *et al*. [4] identified *E. bovis, E. ellipsoidalis, E. alabamensis, E. zuernii, E. auburnensis,* and *E. cylindrica*. Moreover, in this study, not all the positive samples in the microscopic examination showed positive PCR results. A possible reason for this is the limited number of oocysts in fecal samples. Those findings were supported by the statement of Carvalho *et al*. [20], Mirhashemi *et al*. [21], and Ekawasti *et al*. [4], who explained that a small number of oocysts were not sufficient for species identification

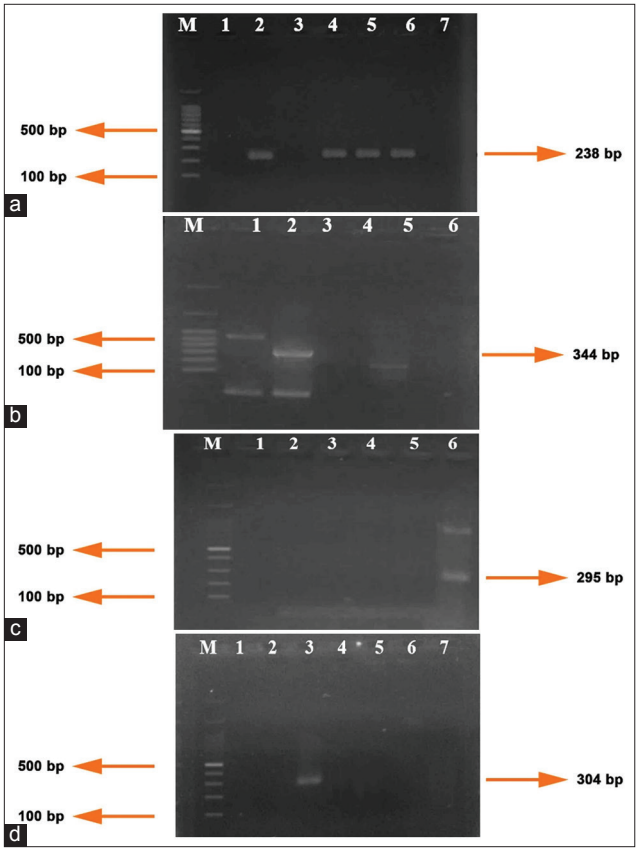

Figure-2: (a) Polymerase chain reaction (PCR) DNA products of *Eimeria bovis* from fecal sample of Madura cattle. M=DNA ladder; 1–7 samples. Samples 1, 3, and 7 are negative and samples 2, 4, 5, and 6 are positive. (b) PCR DNA products of *Eimeria zuernii* from fecal sample of Madura cattle. M=DNA ladder; 1–5 samples. Samples 1, 3, 4, and 5 are negative and sample 2 is positive. (c) PCR DNA products of *Eimeria auburnensis* from fecal sample of Madura cattle. M=DNA ladder; 1–6 samples. Samples 1, 2, 3, 4, and 5 are negative and sample 6 is positive. (d) PCR DNA products of *Eimeria cylindrica* from fecal sample of Madura cattle. M=DNA ladder; 1–7 samples. Samples 1, 2, 4, 5, 6, and 7 are negative and sample 3 is positive.

using the PCR method. The presence of contaminants possibly also inhibited the PCR process during the procedures [4, 15].

Bovine coccidiosis can cause not only growth delays but also a decrease in body performance and cattle production. These clinical signs also affect the quality of adult cattle, thus resulting in high morbidity and mortality in calves, inhibiting the sustainability of livestock production [22]. Theoretically, coccidiosis is a pathogenic disease of young animals, but poor nutritional and environmental management can be potential risk factors for older animals. Adult cattle with chronic infection are frequently diagnosed with anorexia, weight loss, emaciation, bloody diarrhea, and blood-stained dung in perineum and tail part [23].

In this study, Madura cattle were infected with either single or mixed *Eimeria* species. Coccidiosis in cattle is typically caused by more than one species of *Eimeria*. The Madura cattle in this study were infected with single or mixed *Eimeria*. Bangoura *et al*. [2] reported that 48.6% of cases of diarrhea in calves were

caused by a single infection, and 51.4% had mixed infections. Morgoglione *et al*. [24] also reported that 71.2% of cattle were infected with more than 1 *Eimeria* species. These previous results were different from our study, which showed that a single infection was recorded more frequently compared to mixed infections. In the sampling area, the management of cages and sanitation is also known to be improper because feces that were cleared from the cages were dumped right around the cages, which might potentially increase the risk of infection and reinfection [25, 26]. The majority of cages are also known to be traditional and still not equipped with feces and urine disposal lines.

Management patterns also affect the occurrence of *Eimeria* spp. infection, such as sanitation method, drainage system, population density, cage structure, feeding systems, and drinking sources [27]. Occurrences of infection and intensity of *Eimeria* spp. in cattle were also recorded at a lower percentage in a cage compared to pasture [28]. Therefore, cattle shed a lot of oocysts through feces in their closed cages every day during the patent period, which can increase the risk of transmission and increase the development cycle of *Eimeria* spp. The clinical signs of bovine coccidiosis frequently appear 2–3 weeks after infection in a contaminated environment condition [29].

To date, there have been rare reports of molecular investigations of *Eimeria* spp., especially in Indonesia [4, 30]. Although the number of samples in our study was limited, we revealed that the samples could be identified at the species level for *Eimeria* spp. using the molecular method. Therefore, comprehensive studies are required to further investigate the pathogenicity of *Eimeria* spp. infection in Madura cattle and improves productivity through improved and integrated livestock management practices.

Conclusion

The occurrence of *Eimeria* spp. infection in Madura cattle in Kamal Subdistrict, Bangkalan District, Madura Island, is 12% detected by PCR using specific species primers. Moreover, this study successfully obtained four species: *E. bovis, E. zuernii, E. auburnensis,* and *E. cylindrica.* The occurrence of *Eimeria* spp. among Madura cattle should be considered because bovine coccidiosis is probably distributed in most parts of Madura Island. Based on these findings, molecular detection of coccidiosis in Madura cattle can be applied not only in one district but also in several districts, with different conditions associated with the risk factors. The biosecurity measures need to be strengthened among traditional farmers to control the transmission of *Eimeria* spp. in Madura cattle.

Authors' Contributions

PH, LTS, AS, and DAK: Collected fecal samples. PH, NDRL, and DAK: Analyzed the microscopic observation and molecular identification. PH, DAK, and AY: Wrote original draft and revised the manuscript. All authors have read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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