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Morphological and molecular diagnosis of *Eimeria* sp. that caused fatality in a red-necked wallaby (*Macropus rufogriseus*) in Korea

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ABSTRACT

A red-necked wallaby (*Macropus rufogriseus*) in the Republic of Korea, introduced from Australia, died in 12 d after exhibiting anorexia and diarrhea. Postmortem examination revealed that the wallaby died due to coccidiosis by *Eimeria* sp. Morphologically, *Eimeria* sp. identified closely resembled *E. mykytowyczi*. The 18S rRNA sequence analysis showed that *Eimeria* sp. identified in this study has a 98.5% identity with that in Australian red kangaroo (*M. rufus*). However, owing to insufficient molecular information on marsupial-specific *Eimeria*, exact species could not be determined. Phylogenetically, *Eimeria* sp. identified in this study belonged to clade five of the marsupial group.

1. Introduction

Eimeria is an apicomplexan gastrointestinal parasite that can infect different vertebrate hosts [1]. It may cause coccidiosis in infected hosts, which is characterized by severe diarrhea, sometimes resulting in fatality [2]. Because of the associated morbidity and mortality, coccidiosis is one of the most economically important diseases in livestock and poultry industries [2–4].

Wallaby (Mammalia: Macropodidae), a medium-sized marsupial, is closely related to kangaroo. > 26 species of wallaby are known, including red-necked wallaby, brush wallaby, rock wallaby, nail-tailed wallaby, hare wallaby, short-tailed scrub wallaby, and forest wallaby (https://www.britannica.com/animal/wallaby, accessed on Sep. 12. 2018), and some of them are designated as threatened species (http://www.iucnredlist.org/, accessed on Sep. 12. 2018). Wallabies mainly inhabit Australia, but they have been introduced to geographically distinct countries, including the United Kingdom [5].

Studies have shown *Eimeria* spp. infection in wallaby based on morphological and molecular methods [1,6,7]; however, clinical cases of coccidiosis in wallaby are limited. Herein, we report a fatal case of red-necked wallaby (synonym Bennett's wallaby; *Macropus rufogriseus*) due to coccidiosis caused by *Eimeria* sp. in the Republic of Korea (ROK). Additionally, we analyzed *Eimeria* sp. based on the morphology and molecular characteristics.

2. Case

A three-month-old male red-necked wallaby in the Republic of Korea, introduced from Australia, exhibited anorexia and diarrhea 10 d after introduction. As the wallaby was recently introduced with long-distance transportation, a zoo veterinarian diagnosed that the clinical signs were caused by gastrointestinal dysfunction due to shipping stress.

Treatment was administered as follows: 0.5-liter of 5% dextrose solution (5% dextrose inj., JW Pharmaceutical, Seoul, ROK), 10 ml/kg/h intravenously; enrofloxacin (Baytril, Bayer, Leverkusen, Germany) 10 mg/kg twice a day, intramuscularly. Although the wallaby recovered shortly, he died with severe diarrhea two-days after the treatment. The dead wallaby was submitted to the Animal and Plant Quarantine Agency, ROK, for postmortem examination.

Postmortem examination included necropsy, histopathology, and gastrointestinal parasite inspection. Gross lesions in the dead wallaby revealed that the area around the anus was dirty, with yellowish watery feces, and the tail was covered with feces (Fig. 1A). Necropsy revealed that the lungs were red and incompletely collapsed. The ileum and cecum showed hemorrhages (Fig. 1B), and the mesenteric lymph nodes were two or three folds larger than normal. In the rectum, greenish watery fecal content was observed.

For the histopathological diagnosis, tissues from the brain, heart,

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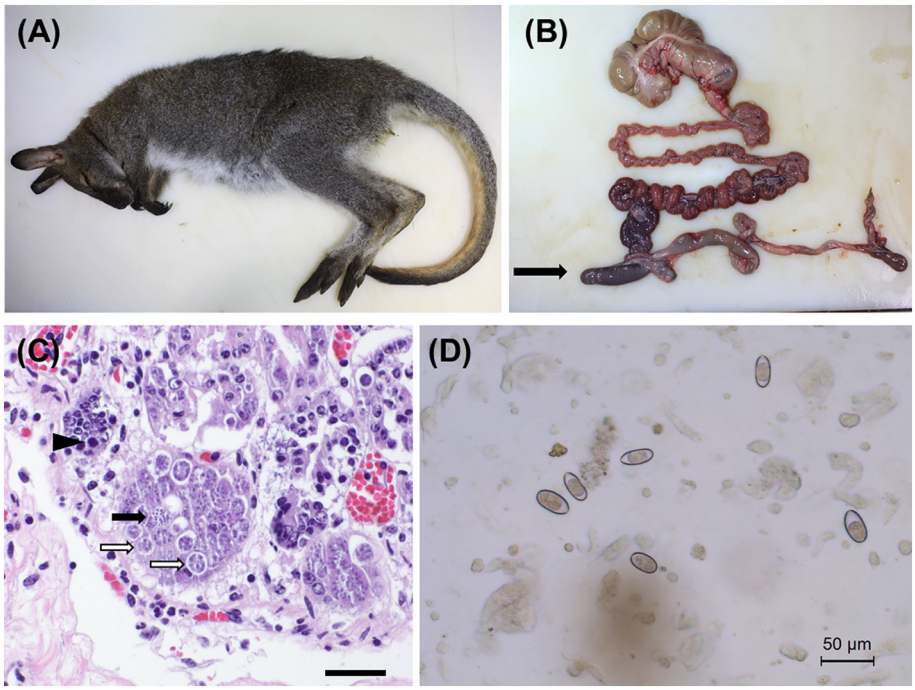

Fig. 1. Postmortem examination of a three-month-old male wallaby infected with *Eimeria* sp. (A) Gross appearance of the dead wallaby. (B) Gastrointestinal organs. Hemorrhage in the ileum and cecum (black arrow). (C) Microscopic examination of the cecum by hematoxylin and eosin staining. Heavy infection and destruction of colonic glands by macrogametocytes (white arrows), large schizonts (black arrow), and developing oocysts (arrowhead). (D) Unsporulated oocysts of *Eimeria* sp. identified in the fecal sample. Bar = 50 μm.

lung, spleen, kidney, liver, and intestines were collected, fixed in 10% buffered neutral formalin, and embedded in paraffin. The paraffin-embedded sections were cut, dewaxed, and stained with hematoxylin and eosin. Histopathologically, the small intestine showed necrotic enteritis, and large schizonts and macrogametocytes were observed in the cecum and colon (Fig. 1C). The villi were stumpy and had disappeared. Granulomatous lymphadenitis was observed in the mesenteric lymph nodes. No lesions were observed in other tissue samples.

To evaluate gastrointestinal parasite infection, fecal samples obtained from the small intestine and cecum were evaluated by the flotation method using sodium nitrate as previously described [8]. The degree of infection was evaluated based on the number of oocysts per gram (OPG) of fecal samples as previously described [2]. From the fecal flotation method, heavy infection (OPG ≥ 10,000) of *Eimeria* oocysts was observed (Fig. 1D). Microscopically, the mean size of the oocysts was 32.2 μm (27.2–34.6; SD, 1.9) × 17.1 μm (14.4–19.0, SD, 1.7). Additionally, the oocysts were ovoid, irregular ellipsoid, colorless, and had an ambiguous micropyle with smooth thin wall. Based on the morphological features, *Eimeria* sp. identified in this study resembled *E. mykytowyczi* [7].

Other gastrointestinal parasites and pathogens causing diarrhea, including *Giardia*, *Cryptosporidium*, *Blastocystis*, and *Enterocytozoon*, were tested by PCR as previously described [9–12]. The genomic DNA was extracted from the fecal sample obtained from the small intestine and cecum using the QIAamp Mini Stool Kit (Qiagen, Hilden, Germany). All the tests were negative except that for *Cryptosporidium* in the cecum sample, but *Cryptosporidium* was negative in the small intestine sample. Based on the clinical signs, identification of *Eimeria* oocysts, and histopathological diagnosis, the cause of death was confirmed as coccidiosis by *Eimeria* sp.

For the identification and molecular characterization of *Eimeria* sp., the PCR was conducted. *Eimeria* genus-specific semi-nested PCR targeting the 18S rRNA was performed using the primers EIF1/EIR3 (primary PCR) and EIF3/EIR3 (secondary PCR) as previously described [1]. The PCR resulted in an amplicon of the expected size, which was subjected to direct sequencing bidirectionally with the primers EIF3/EIR3 (Solgent, Daejeon, ROK). By the sequencing, a 1307-bp sequence was obtained and it showed 98.5% identity with that of *Eimeria* sp. (JF419337) obtained from Australian red kangaroo (*M. rufus*) by the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD). The phylogenetic analysis was conducted based on the 18S rRNA of *Eimeria* using MEGA 7.0 by the maximum-likelihood method (Tamura 3-parameter model with gamma distribution at invariant sites, 500 bootstrap replications) [13]. The phylogenetic analysis revealed that *Eimeria* sp. identified belongs to clade five of the marsupial group (Fig. 2) [1].

3. Discussion

Studies have shown that the effects of intestinal coccidiosis in mammals vary depending on the host-parasite system. The clinical signs are mainly related to malabsorption induced by villous atrophy; anemia, hypoproteinemia, and dehydration caused by exudative enteritis; colitis caused by epithelial erosion and ulceration [14]. Twomey et al. [15] showed lymph node enlargement, watery intestinal contents, soft feces, eosinophilic enteritis with intralesional coccidial organisms in red-necked wallaby infected with *E. prionotemni*. These findings are consistent with our observations.

According to the previous studies, > 50 species of *Eimeria* have been identified in marsupials, including wallaby [1,3,7]. So far, different studies have focused on the morphological characteristics of *Eimeria*, but only a limited number of studies have focused on the molecular approach in marsupial-specific *Eimeria* [1,3,15]. Differentiation of *Eimeria* spp. based on the morphology has limitations, such as broad host specificity of some *Eimeria* spp., subjective analysis, and intraspecies variation [1,16]. Consistently, the oocysts obtained in this study highly resembled those of *E. mykytowyczi* (25.6–36.5 × 15.2–20.0 μm; irregular elongate/ovoid; ambiguous micropyle; no oocyst residuum). However, the oocysts were also similar to those of *E. wilcanniensis* (30.0–38.4 × 18.0–23.0 μm, ellipsoidal, slightly pointed or slightly ovoid; ambiguous micropyle; no oocyst residuum); therefore, based on the morphology, reliable species confirmation could not be achieved [7].

In the current study, molecular techniques were used to identify *Eimeria* sp. The sequence of *Eimeria* sp. identified in this study showed the highest identity with *Eimeria* sp. (JF419337) identified in Australian kangaroos. However, unfortunately, the morphology of *Eimeria* sp. (JF419337) was not analyzed [1]. Moreover, the molecular information

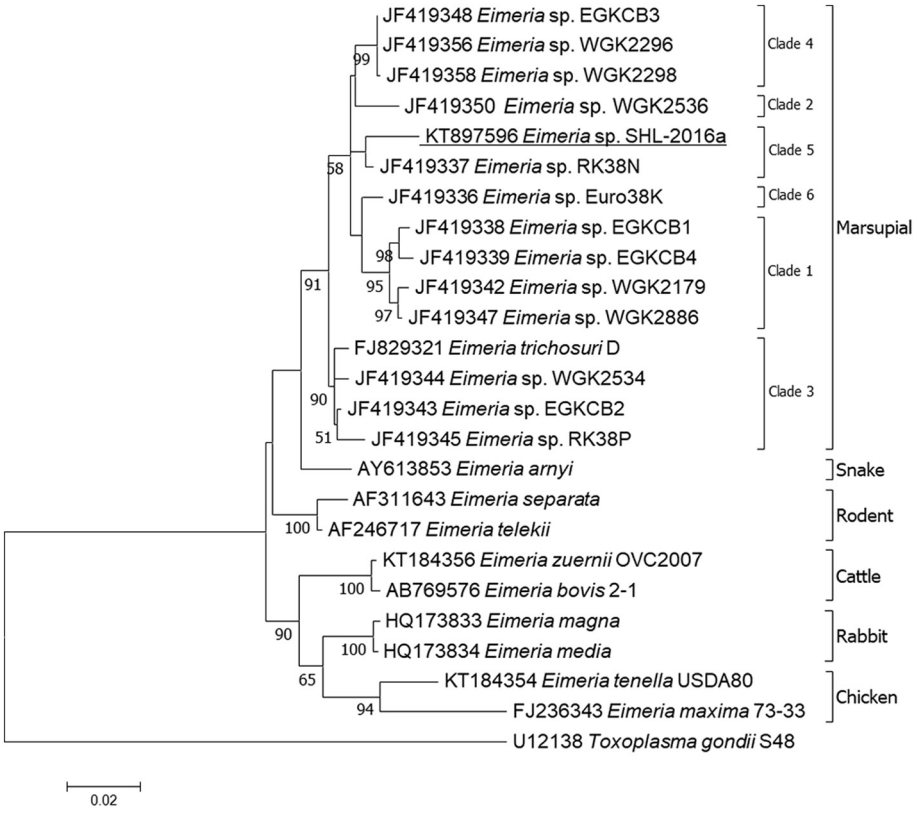

Fig. 2. Phylogenetic analysis of *Eimeria* sp. identified in red-necked wallaby (*Macropus rufogriseus*) using the 18S rRNA. Phylogenetic tree was constructed by the maximum-likelihood method with 500 replications (Tamura 3-parameter model with gamma distribution at invariant sites). *Eimeria* sp. identified in this study is underlined. Bootstrap values < 50 were omitted. Clades are designated as described by Yang et al. [1]. *Toxoplasma gondii* is included as an outgroup. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of *E. mykytowyczi* and *E. wilcanniensis* is not available in the GenBank database. Recently, Yang et al. [1] suggested that *Eimeria* infecting marsupials can be classified into six different clades according to their molecular characteristics based on the 18S rRNA. In the present study, the phylogenetic analysis revealed that *Eimeria* sp. identified belongs to clade five of the marsupial group (Fig. 2) [1]. However, due to insufficient morphological and molecular information on *Eimeria* spp. in marsupials, additional studies have to be conducted to determine the exact species of *Eimeria* sp. identified in this study.

In the ROK, *Eimeria* infection in cattle or chicken has been reported; however, to the best of our knowledge, there is no report of marsupial-specific *Eimeria* [2,4]. Considering the wallaby died 12 d after its introduction, it is reasonable that the wallaby was not newly infected with *Eimeria* sp. in the ROK, but was infected with *Eimeria* sp. in Australia. Although the life cycle of marsupial-specific *Eimeria* has not been well studied, two to three wks of prepatent period of other *Eimeria* species supports this speculation [17]. Twomey et al. [15] also suggested that stress is a predisposing factor for coccidiosis in red-necked wallaby. Therefore, we believe that the wallaby was introduced with asymptomatic *Eimeria* sp. infection from Australia, and immunosuppression caused by shipping stress triggered the clinical signs of coccidiosis.

In conclusion, herein, we report a fatal case of coccidiosis in a red-necked wallaby in the ROK. The fact that the animal was young and immunosuppressed due to long-distance shipping stress might be attributed to the occurrence of coccidiosis, resulting in its death. Considering the case history and life cycle of *Eimeria*, it is believed that *Eimeria* sp. identified in this study was not indigenous and that it originated in Australia. This study broadens our knowledge on coccidiosis and *Eimeria* sp. in marsupials and suggests the importance of preventing coccidial infection before importing animals.

Declaration of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2019.04.008.

References

[1] R. Yang, S. Fenwick, A. Potter, A. Elliot, M. Power, I. Beveridge, U. Ryan, Molecular characterization of *Eimeria* species in macropods, Exp. Parasitol. 132 (2012) 216–221.
[2] S.H. Lee, H.Y. Kim, H. Lee, J.W. Kim, Y.R.L. Lee, M.J. Chae, S.I. Oh, J.H. Kim, M.H. Rhee, O.D. Kwon, Y.K. Goo, T.H. Kim, P.J.L. Geraldino, D. Kwak, *Eimeria* species in cattle with diarrhoea in the Republic of Korea regarding age, season and nature of diarrhoea, Vet. Rec. (2018), https://doi.org/10.1136/vr.104600.
[3] J.M. Austen, J.A. Friend, R. Yang, U.M. Ryan, Further characterisation of two *Eimeria* species (*Eimeria quokka* and *Eimeria setonicis*) in quokkas (*Setonix brachyurus*), Exp. Parasitol. 138 (2014) 48–54.
[4] M.J. You, Detection of four important *Eimeria* species by multiplex PCR in a single assay, Parasitol. Int. 63 (2014) 527–532.
[5] P. Havlin, A. Caravaggi, W.I. Montgomery, The distribution and trophic ecology of an introduced, insular population of red-necked wallabies (*Notamacropus rufogriseus*), Can. J. Zool. 96 (2017) 357–365.
[6] M.L. Power, C. Richter, S. Emery, J. Hufschmid, M.R. Gillings, *Eimeria trichosuri*: phylogenetic position of a marsupial coccidium, based on 18S rDNA sequences, Exp. Parasitol. 122 (2009) 165–168.
[7] I.K. Barker, M.G. O'Callaghan, I. Beveridge, Host-parasite associations of *Eimeria* spp. (Apicomplexa: Eimeriidae) in kangaroos and wallabies of the genus *Macropus* (Marsupialia: Macropodidae), Int. J. Parasitol. 19 (1989) 241–263.
[8] W.J. Foreyt, Diagnostic parasitology, in: W.J. Foreyt (Ed.), Veterinary Parasitology Reference Manual, 5th ed., Blackwell Publishing, Ames, Iowa, 2013, pp. 3–10.
[9] H.I. Cheun, T.K. Choi, G.T. Chung, S.H. Cho, Y.H. Lee, I. Kimata, T.S. Kim, Genotypic characterization of *Cryptosporidium* oocysts isolated from healthy people in three different counties of Korea, J. Vet. Med. Sci. 69 (2007) 1099–1101.
[10] H. Lee, S.H. Lee, M.G. Seo, H.Y. Kim, J.W. Kim, Y.R. Lee, J.H. Kim, O.D. Kwon,

D. Kwak, Occurrence and genetic diversity of *Blastocystis* in Korean cattle, Vet. Parasitol. 258 (2018) 70–73.
[11] J.H. Lee, Prevalence and molecular characteristics of *Enterocytozoon bieneusi* in cattle in Korea, Parasitol. Res. 101 (2007) 391–396.
[12] S.H. Lee, D. VanBik, H.Y. Kim, A. Cho, J.W. Kim, J.W. Byun, J.K. Oem, S.I. Oh, D. Kwak, Prevalence and molecular characterisation of *Giardia duodenalis* in calves with diarrhoea, Vet. Rec. 178 (2016) 633.
[13] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (2016) 1870–1874.
[14] F.A. Uzal, B.L. Plattner, J.M. Hostetter, Alimentary system, in: M.G. Maxie (Ed.), Jubb, Kennedy & Palmer's Pathology of Domestic Animals, 6th ed., Saunders Ltd., Edingurgh, 2015, pp. 1–257.
[15] D.F. Twomey, N.J. Vine, M.E. Wessels, R. Wood, W.G. Rosie, *Eimeria prionotemni*-associated coccidiosis in a Bennett's wallaby (*Macropus rufogriseus*), Vet. Rec. 167 (2010) 621.
[16] F. Kawahara, G. Zhang, C.N. Mingala, Y. Tamura, M. Koiwa, M. Onuma, T. Nunoya, Genetic analysis and development of species-specific PCR assays based on ITS-1 region of rRNA in bovine *Eimeria* parasites, Vet. Parasitol. 174 (2010) 49–57.
[17] M.A. Taylor, R.L. Coop, R.L. Wall, Laboratory diagnosis of parasitism, in: M.A. Taylor, R.L. Coop, R.L. Wall (Eds.), Veterinary Parasitology, 4th ed., Wiley-Blackwell, Singapore, 2017, pp. 259–312.