NOTE
Avian Pathology

Morphological and molecular identification of Eimeria spp. in breeding chicken farms of Japan

Makoto MATSUBAYASHI1–3), Tomoyuki SHIBAHARA1,4), Tomohide MATSUO5), Toshimitsu HATABU6), Junya YAMAGISHI7,8), Kazumi SASAI1,2) and Takashi ISOBE9)*

1)Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka 598-8531, Japan
2)Asian Health Science Research Institute, Osaka Prefecture University, Osaka 598-8531, Japan
3)Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Airlangga University, Surabaya 60115, Indonesia
4)National Institute of Animal Health, NARO, Tsukuba, Ibaraki 305-0856, Japan
5)Laboratory of Parasitology, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima 890-0065, Japan
6)Laboratory of Animal Physiology, Graduate School of Environmental and Life Science, Okayama University, Okayama 700-8530, Japan
7)Research Center for Zoonosis Control, Hokkaido University, Sapporo, Hokkaido 001-0020, Japan
8)Global Station for Zoonosis Control, GI-CoRE, Hokkaido University, Sapporo, Hokkaido 001-0020, Japan
9)Japan Livestock Technology Association, Bunkyo, Tokyo 113-0034, Japan

ABSTRACT. There have been no reports of the prevalence of Eimeria spp. in poultry breeding farms in Japan unlike those of broiler farms. From 2017 to 2018, we examined the prevalence of Eimeria spp. on breeding farms in Japan by oocyst morphology and PCR analyses. A total of 143 samples was collected from 37 breeding farms in 21 prefectures of Japan. We detected oocysts of seven species at 34 of 37 breeding farms by PCR, and we identified E. brunetti at 51.5% of farms found to be positive for Eimeria. The differences in the identification of Eimeria spp. between the morphology and PCR assay methods of oocysts were pronounced for E. maxima and E. necatrix. We confirmed that molecular tools were more suitable for accurately estimating prevalence of Eimeria spp., and these findings suggest that E. brunetti could be widespread in Japan.

KEY WORDS: breeding farm, chicken, Eimeria, Eimeria brunetti, PCR

Members of genus Eimeria cause coccidiosis, which is characterized by watery or bloody diarrhea and the cause of death in livestock due to severe enteritis [2, 4]. Thus, infection by members of this genus has been recognized as a serious threat to the livestock industry worldwide. Although seven species of Eimeria have been reported in chickens, each species possesses different pathological potential [19]. Namely, E. necatrix and E. tenella, and, in some cases, E. brunetti cause lethal bloody diarrhea, while E. maxima, E. acervulina, and E. mitis are rather less pathogenic but affect weight gain, and E. praecox is regarded as being the least pathogenic [9, 14]. To date, anticoccidials such as polyether ionophore antibiotics or live anticoccidial vaccines with attenuated strains have been used to control disease.

In Japan, several surveys on the prevalence of Eimeria spp. have been conducted on broiler farms [15–17]. In these reports from the 1970s, six Eimeria spp., not including E. brunetti, were identified based on morphology of the detected oocysts. Later, Nakamura et al. reported identification of E. brunetti on poultry farms (no detailed description of the farms was given) in 2 different areas of Japan by enzyme electrophoresis [12], and then, Kawahara et al. identified E. brunetti on 21 of 32 poultry farms (10 of 15 breeder farms for broilers, 4 of 4 of breeder farms for layers, 6 of 7 of layer farms, and 1 of 6 broiler farms) by real-time PCR for five Eimeria spp. [7]. Additionally, an isolate of E. brunetti from a breeder farm was found to cause clinical coccidiosis and show lethal pathogenicity by experimental infection [8]. These results suggest that E. brunetti, which had not been detected 4 decades ago, could be distributed in Japan, although further surveys have not been performed. However, live coccidial vaccines against E. brunetti are not available in Japan.

*Correspondence to: Isobe, T. t-isobe@jlta.jp
©2020 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: https://creativecommons.org/licenses/by-nc-nd/4.0/)
Generally, breeder and layer chickens in Japan are kept for a long time (approximately 64–65 and 107–108 weeks, respectively), unlike broiler chickens, which are kept for approximately 7–8 weeks. These chickens are thought to acquire immunity against the infections of *Eimeria* spp. through previous infection with a lower dose of parasites or through commercially available live vaccinations. With this background, investigations of *Eimeria* spp. in poultry have mainly focused on broiler flocks because younger chicks are more susceptible to wild infections than older ones [1, 18]. To our knowledge, there have been no reports of the prevalence of all seven *Eimeria* spp. in breeder flocks in Japan to date. Thus, we cannot sufficiently evaluate the possibility that breeder farms might possess potential risks for occurrence of coccidiosis by the long rearing. Here, we surveyed prevalence of *Eimeria* spp. at breeder farms by two methods in order to assess the current distribution of parasites. One method was the detection of *Eimeria* oocysts from the feces of breeder stocks based on the morphological characteristics. Furthermore, we used a molecular tool by species-specific PCR to exactly identify oocysts of seven species.

We examined 37 poultry farms (25 broiler breeders, 6 layer breeders, 1 broiler and layer breeder, and 5 breeders unknown for broiler or layer) in 21 prefectures of Japan. The mean number of chickens reared on the farms was 19,578.4 (range, 2,500–73,000), and the mean rearing density was 11.4 chickens/m² (range, 3.8–50.0 chickens/m²). The mean age of the chickens was 213.3 days old (range, 44–470 days old) except for one unknown farm. At 34 of the 37 farms studied, coccidiosis was very rarely or sometimes observed over several years, and 6 farms used an anti-coccidiotat like sulfamonomethoxine or salinomycin for the prevention. As for vaccination use, 17 farms used TAM™ (Nisseiken Co., Ltd., Tokyo, Japan), which contains precocious attenuated strains of *E. acervulina, E. maxima*, and *E. tenella*, and Neca™ (Nisseiken Co., Ltd.), which contains a precocious attenuated strain of *E. necatrix; 9 farms used Neca™ and pentavalent Paracox®-5 (MSD Animal Health, Milton Keynes, UK), which contains precocious attenuated strains of *E. acervulina, E. maxima, E. mitis*, and *E. tenella*; 4 farms used TAM™, Neca™, and Paracox®-5; 2 farms used only Paracox®-5; and 2 farms had unknown vaccine usage. Only 3 farms had no vaccination usage.

From September 2017 to January 2018, 2–4 dropped feces were collected from the ground of 1–4 poultry houses in each farm and stored at 4°C until laboratory examination. All chickens were healthy without any clinical symptoms when fecal samples were collected. Each fecal sample was examined by the sugar flotation method [3] with the modification that the final flotation was conducted by settling for 30 min instead of centrifugation. Oocysts per gram (OPG) were determined using a plankton calculation slide (Matsunami Glass, Osaka, Japan). Briefly, 2 g feces were stirred with 38 ml of 0.5% Tween 80 (Nacalai Tesque, Kyoto, Japan) water for 10 min. One hundred microliter solutions were put on the slide and covered by 36 × 24 mm cover glasses. More than 10 lanes with 0.5 mm wide or entire fields were examined, the number of oocysts were counted, and OPG were calculated.

Species identification of *Eimeria* was conducted under a microscope based on oocyst morphology (size and shape) as previously reported [11]. Fecal examinations were conducted by the Research Institute for Animal Science in Biochemistry and Toxicology (Kanagawa, Japan). Oocysts purified by the sugar flotation method were diluted in 1–2 ml PBS, and DNA was extracted as previously reported [3]. For identification of seven chicken *Eimeria* spp., PCR targeting the internal transcribed spacer-1 (ITS-1) region of ribosomal RNA gene was carried out as reported previously [6]. PCR products were subjected to electrophoretic separation on an agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

As results, we identified oocysts of *Eimeria* spp. in samples from 33 of 37 breeding farms (91.9%) by the sugar flotation method. The maximum OPG at positive farms was <200 at 3 farms, 200 to 1,000 at 14 farms, >1,000 to 10,000 at 7 farms, and >10,000 to 100,000 at 9 farms. The OPG of each *Eimeria* spp. could not be determined. Based on morphology of the isolated oocysts, six *Eimeria* spp. were identified (Table 1). PCR assay of positive samples identified seven *Eimeria* spp., and *E. acervulina* and *E. mitis* were the most prevalent and *E. brunetti* was found in 17 among 33 positive farms. There were mismatches in identification between the two methods and low matched percentages were seen in *E. necatrix* (0%) and *E. maxima* (5.3%). Because these oocysts morphologically have similar characteristics between *E. acervulina* and *E. mitis*, and between *E. maxima* and *E. brunetti*, and among *E. tenella, E. necatrix*, and *E. praecox*, surveys were previously conducted as three type oocysts like smaller, middle, and larger size oocysts [13]. Thus, it is difficult to identify species only based on the morphologies, especially in similar types of

Table 1. Prevalence of *Eimeria* spp. among oocysts detected from 33 positive farms and identified by morphological characteristics and PCR

| Species         | Number of oocysts identified by method | Morphology | PCR | Both methods* (percent identical to both) |
|-----------------|---------------------------------------|------------|-----|------------------------------------------|
| *E. acervulina* | 31                                    | 30         | 30  | 96.8%                                    |
| *E. brunetti*   | 14                                    | 17         | 13  | 72.2%                                    |
| *E. maxima*     | 4                                     | 16         | 1   | 5.3%                                     |
| *E. mitis*      | 15                                    | 30         | 14  | 45.2%                                    |
| *E. necatrix*   | 0                                     | 23         | 0   | 0%                                       |
| *E. praecox*    | 21                                    | 20         | 14  | 51.9%                                    |
| *E. tenella*    | 16                                    | 12         | 6   | 27.3%                                    |

*Species* and both methods)/ (number of farms identified by morphological characteristics + number of farms identified by PCR - number of farms identified by both methods).

doi: 10.1292/jvms.19-0661
the oocysts. Actually, in most cases of our study, *E. necatrix* confirmed by PCR was morphologically identified as *E. tenella* or *E. praecox*, and *E. maxima* tended to be misidentified as *E. brunetti* (data not shown). These differences in identification could be due to the coexistence of multiple species in samples as well being attributable to morphological similarities.

We show vaccine usages on the examined farms and detection of *Eimeria* spp. (Table 2). Most of the farms used the vaccine against 4–9 *Eimeria* spp. Among the detected species, *E. acervulina* and *E. mitis* were found with high frequency. Although the reasons remain unknown because the tools to differentiate between vaccine and wild strains are not available, it might be due to oocyst numbers of inoculated vaccine strains, e.g., Paracox*®* and TAM contained *E. mitis* and *E. acervulina* with the highest dose according to the manufacture instructions.

To date, identification of *Eimeria* spp. has been conducted mainly on the basis of observing oocyst morphology as well as infection site(s). However, it is sometimes difficult to distinguish species due to the high degree of similarity among some oocysts with regard to morphological characteristics as described in other reports [5, 10]; thus, molecular techniques have recently been proven to be useful for the clear identification and classification of these parasites, overcoming limitations of traditional approaches [7, 10]. In this study, we had difficulties with the morphological identification of some species such as *E. maxima* or *E. necatrix*. Thus, our experience confirms that it is necessary to use molecular tools to achieve exact identification.

The prevalence of *Eimeria* spp. was differed between countries, diagnostic methods, and situations of the hosts such as ages, treatments to control, and populations. In previous reports of Japan, the prevalence of *Eimeria* spp. were shown to range from 59.2% to 76.6% on broiler farms in 1973–1975 (mostly from 30 to 60 days old or 11 to 80 days old) [15–17], and 72.3% at broiler and 48.0% at layer farms in 2007 (unknown ages) [13]. Although fewer farms were surveyed in the present study, the detection rate (91.9%) was higher than those of previous reports in Japan [13, 15–17]. Because the mean age of the breeder birds was 213.3 days old, high detection rates of *Eimeria* spp. in this study could be due to the long-term rearing. Additionally, OPG for half of the examined farms was less than 10⁴, and no birds showed any clinical symptoms, and thus, they may have been lightly infected.

At least 86.5% of the farms in the present study used live vaccines including *E. acervulina*, *E. maxima*, *E. mitis*, *E. necatrix*, and *E. tenella*. Thus far, we are not able to differentiate strains originating from vaccines from wild strains based on morphological characterization or PCR assay, introducing the possibility that the detected parasites originated from vaccine strains of the species. However, *E. brunetti* and *E. praecox* are strictly wild strains, and more than 50% of eimerian detected farms were found to be positive for *E. brunetti* in the present study. *E. brunetti* was suggested to be the most pathogenic following *E. tenella* and *E. necatrix* in a previous report [8]. Thus, developing live vaccines against *E. brunetti* is desired.

ACKNOWLEDGMENTS. The authors gratefully acknowledge the Research Institute for Animal Science in Biochemistry and Toxicology (Kanagawa, Japan) for conducting the fecal examinations and Mrs. Rika Sekiguchi and Mrs. Noriko Asama for help with the molecular identification assays. This work was supported in part by Grants-in-Aid from the Japan Poultry Breeders Hatcheries Association and the Joint Usage / Research Center (Research Center for Zoonosis Control, Hokkaido University).

REFERENCES

1. Bachaya, H. A., Raza, M. A., Khan, M. N., Iqbal, R., Abbas, R. Z., Murtaza, S. and Badar, N. 2012. Predominance and detection of different *Eimeria* species causing coccidiosis in layer chicken. *J. Anim. Plant Sci.* 22: 597–600.

2. Daugschies, A. and Najdrowski, M. 2005. Eimeriosis in cattle: current understanding. *J. Vet. Med. B Infect. Dis. Vet. Public Health* 52: 417–427. [Medline] [CrossRef]

3. Ekawasti, F., Nurcahyo, W., Wardhana, A. H., Shibahara, T., Tokoro, M., Sasaki, K. and Matsubayashi, M. 2019. Molecular characterization of highly pathogenic *Eimeria* species among beef cattle on Java Island, Indonesia. *Parasitol. Int.* 72: 101927. [Medline] [CrossRef]

4. Fatoba, A. J. and Adeleke, M. A. 2018. Diagnosis and control of chicken coccidiosis: a recent update. *J. Parasit. Dis.* 42: 483–493. [Medline] [CrossRef]

5. Haug, A., Gjevre, A. G., Thebo, P., Mattsson, J. G. and Kaldhusdal, M. 2008. Coccidial infections in commercial broilers: epidemiological aspects
and comparison of Eimeria species identification by morphometric and polymerase chain reaction techniques. Avian Pathol. 37: 161–170. [Medline] [CrossRef]
6. Haug, A., Thebo, P. and Mattsson, J. G. 2007. A simplified protocol for molecular identification of Eimeria species in field samples. Vet. Parasitol. 146: 35–45. [Medline] [CrossRef]
7. Kawahara, F., Taira, K., Nagai, S., Onaga, H., Onuma, M. and Nunoya, T. 2008. Detection of five avian Eimeria species by species-specific real-time polymerase chain reaction assay. Avian Dis. 52: 652–656. [Medline] [CrossRef]
8. Kawahara, F., Zhang, G., Suzuki, T., Iwata, A., Naganuma, K. and Nunoya, T. 2014. Characterization of Eimeria brunetti isolated from a poultry farm in Japan. J. Vet. Med. Sci. 76: 25–29. [Medline] [CrossRef]
9. Lillehoj, H. S. and Lillehoj, E. P. 2000. Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. Avian Dis. 44: 408–425. [Medline] [CrossRef]
10. Long, P. L. and Joyner, L. P. 1984. Problems in the identification of species of Eimeria. J. Protozool. 31: 535–541. [Medline] [CrossRef]
11. McDougald, L. R. 2003. Coccidiosis. pp. 974–991. In: Diseases of Poultry, 11th ed. (Saif, Y. M. ed.), Iowa State University Press, Amsterdam.
12. Nakamura, T., Kawaguchi, H. and Imose, J. 1990. Identification of Eimeria brunetti using glucose phosphate isomerase and lactate dehydrogenase. Nippon Juigaku Zasshi 52: 859–860. [Medline] [CrossRef]
13. Nakamura, Y., Kanehira, K., Isobe, T. and Kamio, T. 2010. A nation-wide survey of coccidial infections on chicken farms in Japan (January-March, 2007). Bull. Natl. Inst. Anim. Health 117: 1–10 (in Japanese with English abstract).
14. Nematzadeh, A., Moghadam, G. and Pourabadi, R. F. 2009. Prevalence of Eimeria species among broiler chicks in Tabriz (Northwest of Iran). Mun. Ent. Zool. 4: 53–58.
15. Oikawa, H., Kawaguchi, H., Nakamoto, K. and Tsunoda, K. 1974. Field surveys on coccidial infection in broilers in Japan—results obtained in spring and summer in 1973. Nippon Juigaku Zasshi 36: 321–328. [Medline] [CrossRef]
16. Oikawa, H., Kawaguchi, H., Nakamoto, K. and Tsunoda, K. 1975. Field surveys on coccidial infection in broilers in Japan—results obtained in autumn and winter and summarized in 1973. Nippon Juigaku Zasshi 37: 271–279. [Medline] [CrossRef]
17. Oikawa, H., Kawaguchi, H., Nakamoto, K. and Tsunoda, K. 1977. Field surveys on coccidial infection in broilers in Japan in 1974 and 1975. Nippon Juigaku Zasshi 39: 127–134. [Medline] [CrossRef]
18. Rose, M. E. 1967. The influence of age of host on infection with Eimeria tenella. J. Parasitol. 53: 924–929. [Medline] [CrossRef]
19. Shirley, M. W. 1986. New methods for the identification of species and strains of Eimeria. pp. 13–35. In: Research in Avian Coccidiosis (McDougalld, L. R., Joyner, L. P. and Long, P. L. eds.), University of Georgia, Athens.