The Journal of Veterinary Medical Science

NOTE

Avian Pathology

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

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J. Vet. Med. Sci.
82(5): 516–519, 2020
doi: 10.1292/jvms.19-0661

Received: 10 December 2019
Accepted: 13 March 2020
Advanced Epub: 1 April 2020

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.

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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/m^2(range, 3.8–50.0 chickens/m^2). 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-coccidiostat 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 m*l* 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 floatation method were diluted in 1–2 m*l* 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[a)] (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%)

a) Number of farms of each *Eimeria* spp. identified by both morphological characteristic and PCR methods. Percentages identical to both methods are calculated as (number of farms identified by both methods)/ (number of farms identified by morphological characteristics + number of farms identified by PCR–number of farms identified by both methods).

Table 2. Detection of *Eimeria* spp. by PCR and use of live vaccines in 37 examined farms

		Vaccinations[a)]				None	Unknown
		Paracox®	Paracox®+Neca	TAM+Neca	TAM+Paracox®+Neca		
No. of farms for *Eimeria* spp. detected	33	2	9	15	4	1	2
(No. of examined farms)	(37)	(2)	(9)	(17)	(4)	(3)	(2)
E. acervulina	30	2	9	12	4	1	2
E. brunetti	17	2	3	7	3	1	1
E. maxima	16	2	5	4	2	1	2
E. mitis	30	1	9	13	4	1	2
E. necatrix	23	2	7	9	3	1	1
E. praecox	20	1	5	9	2	1	2
E. tenella	12	2	2	3	2	1	2

a) TAM™ vaccine includes strains of *E. acervulina*, *E. maxima*, *E. tenella*, Neca™ vaccine includes strain of *E. necatrix*, and Paracox®-5 includes strains of *E. acervulina*, *E. maxima*, *E. mitis* and *E. tenella*. Underlined numbers indicate the possibility that wild and vaccine strains are included.

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 10^3, 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/or *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).

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