FULL PAPER *Parasitology*

Prevalence and Cross-Immunity of *Eimeria* Species on Korean Chicken Farms

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ABSTRACT. Epidemiology of *Eimeria* species in poultry flocks is important to increase the effectiveness of vaccinations and prophylactic strategies on chicken farms. In this study, fecal samples from 356 chicken farms were collected randomly and examined for the prevalence of *Eimeria* species. Through microscopic examination, it was determined that 78.7% of the tested farms were positive in *Eimeria*-infection. Seven *Eimeria* species were detected in all the positive farms by PCR amplification of the internal transcribed spacer 1 (ITS-1) region with species-specific primers. *E. acervulina* and *E. tenella* were the most prevalent, followed by *E. brunetti* and *E. praecox* (87.5, 62.5, 59.3, and 37.5% of the farms, respectively). Each of *E. maxima, E. mitis*, and *E. necatrix* was identified in 31.3% of the farms. Individual positive fecal samples contained multiple *Eimeria* species (mean=3.4). Since *E. maxima* is known to generate antigenic variants, cross-immunity was investigated for four isolates of *E. maxima* from the poultry farms in different regions of Korea. The extent of cross-protection varied from 54.3 to 100% against the heterologous isolates. The results obtained from this large-scale survey will be a useful reference for controlling coccidiosis in the poultry industry.

KEY WORDS: chickens, cross-protection, *Eimeria*, Korea, prevalence.

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Coccidiosis of chickens is one of the most costly diseases affecting the poultry industry worldwide. It is an intestinal parasitic disease caused by intracellular protozoan parasites of the genus *Eimeria*. *Eimeria* infections inflict severe economic loss due to extensive destruction of the enteric epithelium, resulting in reduction of feed conversion, body weight gain and egg production, and increased morbidity and mortality [12, 15, 17]. Seven species of *Eimeria* that infect chickens invade the intestinal epithelium in a region-specific manner, and induce both species-specific immunity and, in some cases of *E. maxima*, strain-specific immunity [17, 20, 21]. Namely, different populations of *E. maxima* including field isolates exhibit substantial immunological diversity [1, 13, 21]. Chickens may be simultaneously infected with multiple species of *Eimeria* in intensively reared poultry farms [16, 18]. This disease in such farms is mainly controlled by the use of chemotherapeutic agents in the feed or water and/or by vaccines using live strains of *Eimeria* [4, 17, 20, 24]. In addition, natural dietary supplements or probiotics have been used to control and prevent *Eimeria* infections [3, 6, 9, 22].

Epidemiological examination and specific diagnosis of *Eimeria* infections would play an important role in the prevention and control of coccidiosis. Although epidemiological studies of *Eimeria* infections on broiler farms have been carried out by morphological and biological features [2, 8, 10], species-specific PCR diagnoses are still lacking for Korean poultry farms. In this study, a species-specific PCR diagnostic tool based on the internal transcribed spacer 1 (ITS-1) sequence was developed to survey the prevalence of *Eimeria* infections in poultry farms. Additionally, cross-protection studies with *E. maxima* isolates to evaluate strain-specific immunity were performed.

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MATERIALS AND METHODS

Collection of fecal samples and counting of oocysts: Fecal samples were collected from 356 randomly selected broiler or layer farms in South Korea between April and November, 2008, and stored at 4°C before counting. Each sample consisted of a pool of ten fresh droppings from multiple chickens. One gram of each fecal sample was homogenized in 5 m*l* of a saturated NaCl solution by a vortex mixer. Oocyst numbers were counted using a McMaster counting chamber. Detection limit of fecal examination was 50 oocysts per gram.

Genomic DNA extraction from oocysts: Before genomic DNA extraction of fecal oocysts, 5 g of fecal sample was added to 25 m*l* of 2.5% potassium dichromate (Samchun Chemicals, Pyongtack, Korea), and incubated with shaking at 28°C for 3–5 days. Sporulation of the oocysts was examined using a microscope at 100× magnification. The samples were washed twice by centrifugation with phosphate buffered saline (PBS, pH 7.4) to remove the potassium dichromate, and resuspended in 5 m*l* of a saturated NaCl solution. Oocysts floated on this solution were collected. Extraction of genomic DNA was performed as formerly described [5]. Briefly, oocysts were washed twice by cen-

trifugation with PBS, resuspended in 0.5 m*l* water and mixed with an equal volume of Ø 0.5 mm glass beads (Bio-Spec Product, Inc., Bartlesville, OK, U.S.A.). The oocysts were ruptured by mini-pestle grinders (Poonglim, Seoul, Korea) for 1 min and lysed in 1 m*l* of lysis buffer [10 mM Tris-HCl, pH 8.0, 25 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 0.5% sodium dodecyl sulfate (SDS), and 0.5 mg/m*l* proteinase K]. The sample was incubated at 56°C for 1 hr with gentle shaking. Genomic DNA was extracted using phenol-chloroform extraction and dissolved in 50 μl TE buffer.

Identification of Eimeria species by PCR: Information on ITS-1 of nuclear ribosomal DNA of *Eimeria* species isolated in Korea was unavailable, therefore, identification of seven *Eimeria* species was carried out with the PCR primers formerly described [5], with minor modifications. The reverse primer of *E. praecox* used was 5'-GCAT-GCGCTAACA**M**CTCCCCTT (GenBank accession No. AF446071). PCR was carried out in 20 μl reactions using PCR PreMix (Bioneer, Daejeon, Korea) containing 1 μl of DNA template, 10 pmol forward primer, and 10 pmol reverse primer. The cycling program was performed under the following conditions: an initial denaturation step for 5 min at 95°C, then 35 cycles at 55 or 65°C for 1 min, 72°C for 1 min, and 95°C for 1 min, followed by a final extension of 72°C for 5 min in a thermal cycler (Bio-Rad, Hercules, CA, U.S.A.). PCR products were verified on 2% agarose gels stained with ethidium bromide.

Cross-protection experiments: Male Cobb 500 chickens were given access to feed and water *ad libitum*, and constant light was provided for duration of the experiment. Prior to *Eimeria* infection, all chickens were reared in brooder pens in an *Eimeria*-free facility and moved into exprimental cages in a separate area where the birds were infected and kept until the end of experiment. *E. maxima* was isolated by flotation on 6–14% sodium hypochlorite and washed 3 times with PBS [6, 25]. Five-day-old chickens were orally infected with 5×10^3 sporulated oocysts of four Korean isolates and transferred to wire-floored grower cages (three birds per cage). Chickens were subsequently given the secondary infection 3 weeks later of 5×10^3 sporulated oocysts of the same or a heterologous isolate. Fecal samples were collected from 6 to 10 days post-infection, and the number of oocysts was assessed using a McMaster counting chamber. Precent cross-protection was calculated as 100 × [1–(oocyst numbers per bird shed by immunized birds following by the secondary infection/oocyst numbers per bird shed by unimmunized birds following by the secondary infection)].

RESULTS

Fecal samples collected from 356 chicken farms were examined for *Eimeria* infection. The overall prevalence of *Eimeria* spp. infection was 78.7% (280 of the 356 farms). Although the number of fecal oocysts per gram varied across the chicken farms, the mean oocyst numbers per gram of the positive samples (n=280) was 20,810 (standard error=3292.7). Multiple species of *Eimeria* were identified among 64 samples randomly selected from coccidia positive samples by PCR with *Eimeria* species-specific primers (Fig. 1A). *E. acervulina* and *E. tenella* were the most prevalent species, identified in 87.5 and 62.5% of the positive samples, respectively. *E. brunetti* and *E. praecox* were present in 59.3 and 37.5% of the positive samples, respectively. Each of *E. maxima, E. mitis* and *E. necatrix* was present in 31.3% of the positive samples. As shown in Fig. 1B, the number of different *Eimeria* species detected in individual fecal samples ranged from one to seven, with a mean of 3.4 species per sample.

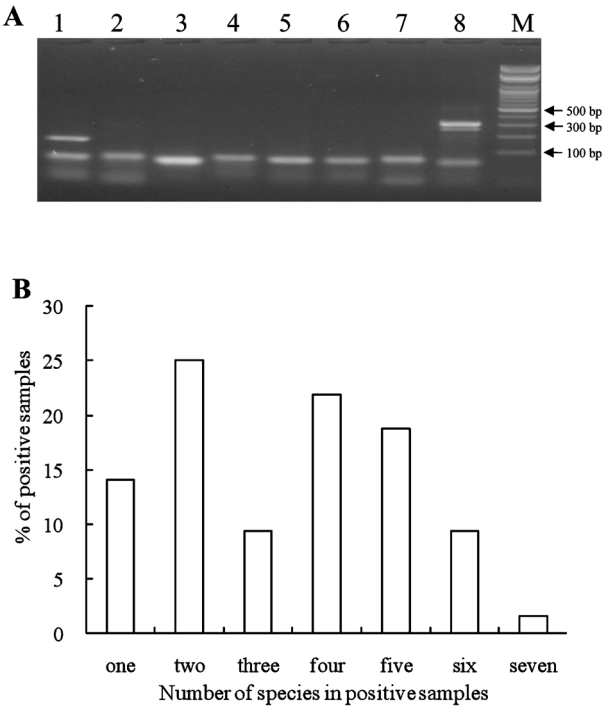

Fig. 1. Identification of multiple *Eimeria* species in the fecal samples. (A) Oocyst genomic DNA was extracted from 64 randomly selected coccidian-positive samples, amplified by PCR with *Eimeria* species-specific primers and analyzed on 2% agarose gels stained with ethidium bromide. Lane 1 was for PCR product amplified by *E. acervulina* specific primers, lane 2 for *E. brunetti*, lanes 3 and 4 for *E. maxima*, lane 5 for *E. mitis*, lane 6 for *E. necatrix*, lane 7 for *E. praecox*, and lane 8 for *E. tenella*. Lane M was 100 bp plus DNA ladder. The example shown is a representative picture of PCR results. (B) Number of *Eimeria* species in fecal samples.

As *E. maxima* is known to be able to generate antigenic variants [1, 13, 21], cross-immunity challenges were carried out among four isolates of *E. maxima* obtained from broiler farms in different regions of Korea. Three isolates (310-2, 310-4, 336-4) were obtained from Jeollabuk-do province, Korea and 291-3 isolate from Gyeongsangbuk-do province, Korea. Each of the four isolates was challenged against

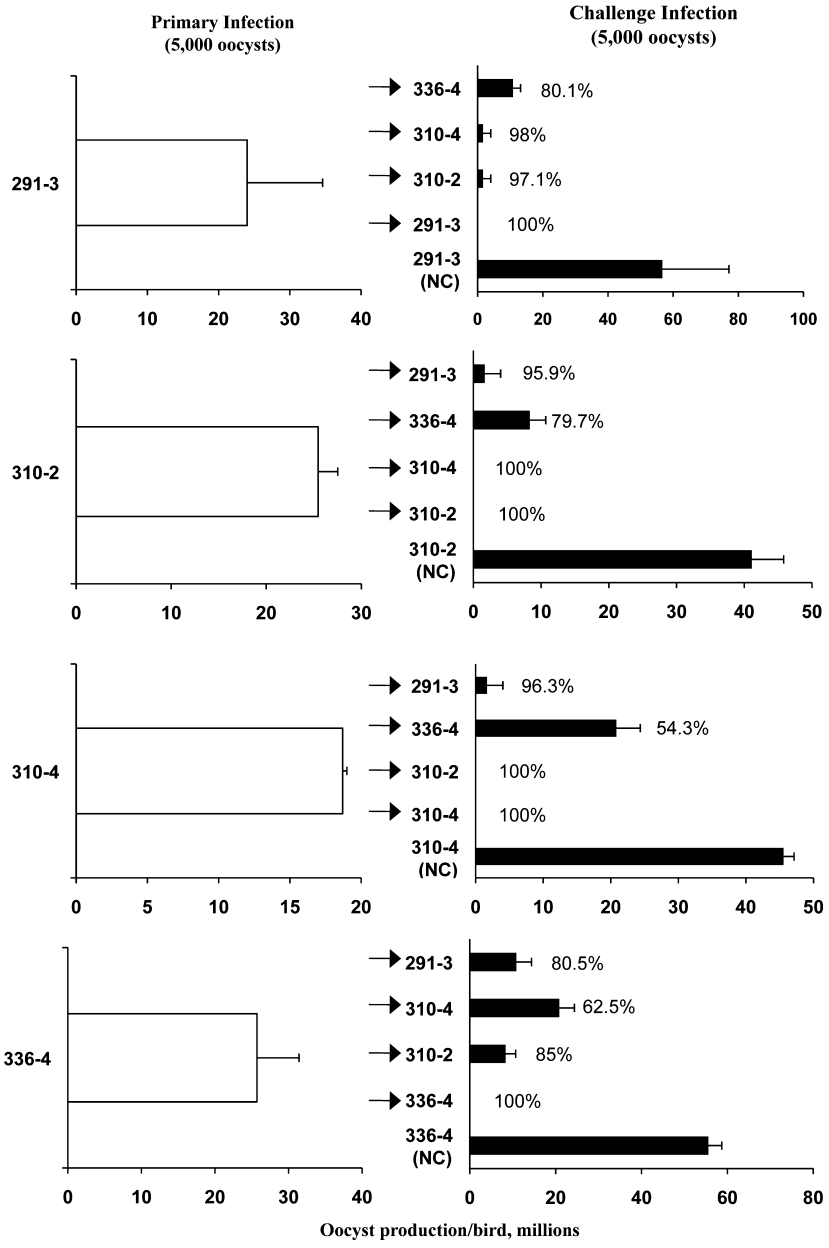

Fig. 2. Cross-protection assays with four isolates of *E. maxima* from the poultry houses throughout Korea. Chickens were orally infected with 5×10^3 sporulated oocysts of four Korean *E. maxima* isolates. Five-day-old chickens were subsequently given a secondary infection 3 weeks later with 5×10^3 sporulated oocysts of the same or a heterologous isolate. Fecal materials were collected from 6 to 10 days post-infection, from which oocysts were counted. Percent cross-protection (%) was calculated as 100 × [1–(oocyst numbers per bird shed by immunized birds following by the secondary infection/oocyst numbers per bird shed by unimmunized birds following by the secondary infection)]. Each bar represents the mean ± SD (n=9). NC means noninfected control at a primary infection followed by a challenge infection.

each other. As illustrated in Fig. 2, isolates 310-2, 310-4, and 291-3 showed high levels of cross-immunity. However, isolate 336-4 did not protect against the other three isolates as effectively, and vice versa. This finding suggests that the 336-4 isolate was antigenically diverse from the other isolates, especially from the 310-4 isolate.

DISCUSSION

Our study provides basic information for the prevention and control of coccidiosis on chicken farms by epidemiology and species-specific identification. PCR methods for specific detection of *Eimeria* species in chicken feces have been used with ITS-1 or ITS-2 of nuclear ribosomal DNA [7, 11, 19, 23]. In this study, all seven *Eimeria* species were detected utilizing a species-specific PCR approach, and antigenic variants among *E. maxima* isolates were identified by cross-protection assays. We identified *E. acervulina, E. tenella*, and *E. brunetti* as the most prevalent species on Korean chicken farms. Several previous reports have revealed differences of the prevalence and composition of *Eimeria* populations in different regions of the world. *E. tenella, E. praecox*, and *E. acervulina* were the predominant species in the local Shandong area in China [23], while *E. acervulina, E. maxima*, and *E. mitis* were the most common species in a region between Sydney and Newcastle, New South Wales in Australia [18]. Here, *E. brunetti* was the third most prevalent species, which was detected in 59.3% of the samples from Korean farms, whereas *E. brunetti* was uncommon in Australia [18] and China [23], being detected in 36 and 8% of the samples, respectively. The dissimilarities of the *E. brunetti* prevalence between these countries might result from differences in management strategies, including use of prophylactic anticoccidial agents and anticoccidial vaccines. Considering that *E. brunetti* is pathogenic and highly prevalent on Korean chicken farms, it is important to have the tools to diagnose and control *E. brunetti* infections.

It is reported that PCR analysis of genomic DNA from the oocysts isolated from fecal samples indicated that most chickens were infected simultaneously with an average of 3.6 different species of *Eimeria* [18]. The present investigation also indicated that chickens could be infected simultaneously with multiple *Eimeria* species (mean=3.4). However, there is no correlation between oocyst number and the number of *Eimeria* species found within an infected animal (data not shown).

E. maxima is known to be highly immunogenic and required a small number of sporocysts to induce protective immunity [4, 20], yet it also appears to have antigenic diversity [1, 13, 14, 21]. This antigenic diversity may be overcome by immunization with the hybrid mixture of *E. maxima*, which showed good protection to variant strains [13]. Cross-protection assays in this study were carried out with four isolates of *E. maxima* from poultry houses in the different parts of Korea. Antigenic differences were identified from these isolates (Fig. 2), supporting the previous finding that geographically separated isolates showed poor cross-protection [14].

In conclusion, this study showed differences in the prevalence of *Eimeria* species found in Korean chicken farms and identified antigenic variants of *E. maxima* species. The results obtained from this large-scale survey will provide a useful reference to design control strategies of coccidiosis in the poultry industry.

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