Parasitological analyses of *Eimeria* infections in domestic animals and the development of molecular methods for species discrimination

（家畜におけるアイメリア感染の寄生虫学的研究と種鑑別のための分子生物学手法の開発）

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ABBREVIATIONS

ACI	anticoccidial index
AST	anticoccidial sensitivity test
bp(s)	base pair(s)
DPI	days post-inoculation
ITS	internal transcribed spacer
MP	maximum parsimony
NJ	neighbor-joining
OPG	oocysts per gram
PCR	polymerase chain reaction
rRNA	ribosomal RNA
SPF	specific pathogen free
Tm	melting temperature
UD	undefined species type
%OAA	optimum anticoccidial activity

PREFACE

Many medical and veterinary important parasites belonging to the phylum Apicomplexa, including *Plasmodium, Toxoplasma, Cryptosporidium, Eimeria* and *Theileria* species, have a global distribution. These pathogens, which can cause serious diseases, remain a persistent threat to humans and livestock and are responsible for a huge number of human deaths and economic losses every year.

Bovine coccidiosis is the parasitic disease caused by *Eimeria* infection and the economically important disease for cattle production and daily farming through the world (11, 16, 17, 31). Young calves between 3 weeks and 6 months old commonly show clinical symptoms of coccidiosis following the first exposure (60) as a consequence of their naive immune status (10, 57). More than 20 species of *Eimeria* parasites have been described to infect cattle (*Bos taurus*) (25), although just 11 species have been detected in Germany and Japan and 13 species have been reported in the United States (14, 15, 25, 41). *Eimeria bovis* and *E. zuernii* are considered to be highly pathogenic that the species could often cause life-threatening bloody diarrhea (1, 9, 39, 54). Coccidiosis in chickens is also caused by closely related, but genetically distinct *Eimeria* parasites. The disease has globally incurred huge economic damage to the modern intensive poultry industry as a result of the necessary costs of prevention, treatment, sub-optimal performance and mortality (53). Pathognomonic signatures of chicken coccidiosis include a variety of clinical signs including diarrhea ranging from watery to hemorrhagic, body weight reduction and sudden death. The disease can be caused by any of seven *Eimeria* spp. that can infect their definitive host, *Gallus gallus*. Among these species, *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix* and *E. tenella* are most pathogenic and important species in the poultry industry.

To date identification of *Eimeria* species which infect cattle has been exclusively based upon a morphological approach, observing oocyst appearance (Table P-1) (12). Individual features of oocysts of each species were described by Levine and Ivens (25, 26). For a long time, their practical method has been exploited in many studies and field surveys to discriminate bovine *Eimeria* species (15, 41, 50). Similar approaches have been used to diagnose chicken coccidiosis, supplemented by the examination of macroscopic lesions formed at particular regions of the intestine. Stricter identification of chicken *Eimeria* species has relied on parasitological or pathological aspects such as variations in prepatent period (time spent for one generation), variations in sporulation time (the periods for oocyst maturation), clinical signs and histopathological characteristics (23). These tests are mostly carried out in the laboratory after the refinement and inoculation of field strains of chicken *Eimeria*. However, the

requirement for professional expertise and sufficient experience in diagnosis has proven limiting. The discrimination of *Eimeria* to the species level can be difficult, unreliable and subjective in spite of professional identification skills due to overlapping morphological features and clinical signs compounded by intra-species variation (Table P-2) (29). Infection by multiple *Eimeria* species exacerbates this problem.

In response, new user-friendly molecular methods for species-specific detection of bovine and chicken *Eimeria* are required and can complement classical clinical and epidemiological applications. Building on present genomic knowledge of the phylum Apicomplexa, including *Eimeria* parasites, some molecular methods have been developed for the identification of species using conventional polymerase chain reaction (PCR) technique or the PCR-based capillary electrophoresis technique. These methods have been applied the 5S ribosomal RNA (rRNA) gene, the small subunit rRNA gene and the internal transcribed spacer (ITS)-1 or ITS-2 region as a target site (5, 7, 19, 20, 21, 27, 35, 36, 37, 38, 55, 62, 64). Among these target sites, ITS-1 has been proven to be one of the most attractive one, including sequence derived from the gap between the 18S and 5.8S rRNA genes (6, 47, 51, 52). This target is present in multiple copies within each *Eimerian* genome, providing excellent sensitivity, complimented by the occurrence of elevated levels of genetic heterogeneity. The extent of this heterogeneity has been demonstrated for all seven chicken *Eimeria* species, where species-specific DNA sequences have been successfully utilized for diagnostic purposes as conventional PCR and real-time PCR targets (20, 24, 27, 56).

To minimize the economic damage caused by coccidiosis in modern intensive poultry production prophylactic treatment is standard. Major prophylactic strategies against chicken coccidiosis include dietary supplementation with anticoccidial drugs and administration of live anticoccidial vaccines. However, there are now deep concerns about residues of antibiotics and other drugs in poultry products, especially among people in developed countries, stimulating strong demands to ban all drugs from livestock feeds. As a result, food production derived from organic livestock without chemicals or antibiotics feeds is increasing in Japan as well as many European countries. It is estimated that live anticoccidial vaccines have been applied to one hundred million birds per year in Japan (61). Three commercial live anti-coccidiosis vaccines have been registered in Japan. Trivalent TAM™ (Nisseiken Co., Ltd., Tokyo, Japan) contains three precocious attenuated strains representing *E. acervulina*, *E. maxima* and *E. tenella*. Monovalent Neca™ (Nisseiken Co., Ltd.) contains a precocious attenuated strain of *E. necatrix*. Pentavalent Paracox®-5 (MSD Animal Health, Milton Keynes, U.K.), which contains five precocious attenuated strains representing *E. acervulina*, *E. maxima* (2 strains), *E. mitis* and *E. tenella*, has been imported from the

UK. To date, no anticoccidial vaccine which includes *E. brunetti* has been introduced in Japan since the occurrence of the parasite has historically been considered to be negligible. Several surveys on coccidial infection throughout Japan failed to detect *E. brunetti* in broiler flocks in 1970's (43-45). Nonetheless, two Japanese strains of *E. brunetti* were isolated in Hokkaido and Kumamoto in 1990 (40), suggesting the occurrence of this parasite in Japan. Hokkaido and Kumamoto are located in the northern and southern parts of Japan, respectively, indicating a broad distribution of this parasite. However, no further surveys regarding *E. brunetti* in Japan have been conducted since this last report. The prevalence of *E. brunetti* in Japan has been remained poorly understood until now.

The present thesis describes studies focused on analysis of genomic DNA from *Eimeria* species infecting cattle or chicken, supplemented by parasitological characterization of a field strain of chicken *Eimeria* derived from a commercial farm in Japan. Brand-new PCR assays to detect and differentiate bovine and chicken *Eimeria* species based on their genomic DNA sequences have been developed in these studies. Finally, new insights about chicken *Eimeria* distribution and character have been gained to apply PCR assays with epidemiological studies in Japan.

Table P-1. Oocyst morphology of *Eimeria* species of cattle.

Species	Length (μm)	Width (μm)	Oocyst shape
E. alabamensis	13-24	11-16	Ovoid or piriform
E. auburnensis	32-46	20-25	Elongated ovoid
E. bovis	23-34	17-23	Ovoid
E. brasiliensis	34-43	24-30	Ellipsoid
E. bukidnonensis	47-50	33-38	Pear-shaped
E. canadensis	28-37	20-27	Ovoid or ellipsoid
E. cylindrica	16-27	12-15	Elongated ellipsoid or subcylindrical
E. ellipsoidalis	20-26	13-17	Ellipsoid, slightly ovoid or cylindrical
E. illinoisensis	24-29	19-22	Ellipsoid or ovoid
E. pellita	36-41	26-30	Ovoid
E. subspherica	9-14	8-13	Round or subspherical
E. wyomingensis	37-45	26-31	Ovoid or pear-shaped
E. zuernii	15-22	13-18	Subspherical

Based on data from Daugschies and Najdrowski (12).

Table P-2. Oocyst morphology of *Eimeria* species of chicken.

Species	Length (μm)	Width (μm)	Oocyst shape
E. acervulina	18-20	14-16	Ovoid
E. brunetti	21-30	18-24	Ovoid
E. maxima	22-43	17-30	Ovoid
E. mitis	12-19	11-18	Subspherical
E. necatrix	13-23	11-18	Oblong ovoid
E. praecox	20-25	16-20	Ovoidal
E. tenella	20-26	17-23	Ovoid

Based on data from McDougald and Fitz-Coy (32).

CHAPTER I

Genetic analysis and development of species-specific PCR assays based on ITS-1 region of rRNA in bovine *Eimeria* parasites

INTRODUCTION

Bovine coccidiosis is the disease caused by *Eimeria* infection in cattle and the most economically important disease of cattle throughout the world (11, 16, 17, 31). Calves between 3 weeks to 6 months age are particularly susceptible to clinical coccidiosis (60), reflecting naive states to *Eimeria* infections (10, 57). More than 20 species of bovine coccidia have been reported (25). There are 11 species identified in Germany and Japan, and 13 species in the United States (14, 15, 25, 41). Of these species, *Eimeria alabamensis*, *E. auburnensis*, *E. bovis*, *E. ellipsoidalis* and *E. zuernii* are recognized pathogenic (12). *E. bovis* and *E. zuernii* are highly pathogenic since they usually cause bloody stool (1, 9, 39, 54). Infections caused by the above-mentioned 4 species or other ones may exhibit subclinical signs to transient non-hemorrhagic diarrhea (12).

Currently, morphological observation of oocysts is the only practical method to identify species within bovine coccidia (12). The detailed features described by Levine and Ivens (25, 26) for bovine coccidial oocysts have been cited in many reports to determine the species (15, 41, 50). However, the morphological method is not fully reliable since several species have confusing features alongside the presence of intra-species variation (29). Furthermore, morphological observations combined with fecal examination are very labor-intensive and require skillful techniques. Therefore, it is essential to develop a more rapid, reliable and cost effective method.

Inter-species differences in 18S ribosomal RNA (rRNA) gene sequences of bovine coccidia have been known to be small (28), and are not efficient for the identification of species based on PCR assays. Therefore, the development of sensitive and reliable technique to detect and identify correct species is the primary requirement.

Knowledge of apicomplexa at the genomic level has been deepened continuously, and several PCR methods for the molecular identification have been presented (5, 38, 55, 62). In these methods, one of the attractive genomic DNA targets is the internal transcribed spacer 1 (ITS-1) region spanning between two rRNA genes (6, 47, 51, 52). This region is located between the 3' end of 18S rRNA gene and the 5' end of the 5.8S rRNA gene in each transcription unit. Due to heterogeneity of both sequence compositions and lengths among different species, the ITS-1 region is a promising target to design the specific primers. Furthermore, the ITS-1 region belonging to a multiple copy gene family provides large number of targets for PCR assays. In a recent report (27), inter-species-specific diversities were shown within the ITS-1 regions from 7 chicken *Eimeria* species, and the species-specific DNA sequences were used for the diagnostic purpose (20, 24, 27, 56).

To date, a phylogenetic analysis has not been reported in *Eimeria* species of cattle. In

this chapter, the results of the analysis conducted for 21 ITS-1 sequences to define the phylogenetic relationship, and inter- and intra-species variation existing among 18 *Eimeria* field collections are described. The reliability and applicability of PCR assays depending on the specific ITS-1 region for identification of bovine *Eimeria* are also described.

MATERIALS AND METHODS

Parasites. More than 100 samples of cattle feces were collected from commercial farms throughout Japan. They were examined for the presence of oocysts by using a microscope. Species of *Eimeria* (*E. alabamensis*, *E. auburnensis*, *E. bovis*, *E. cylindrica*, *E. ellipsoidalis*, *E. zuernii* and others) were determined morphologically by observing 50 oocysts in each sample according to the standard presented by Levine and Ivens (25, 26). Oocysts were separated from the fecal debris and concentrated by the flotation technique with a saturated sodium chloride solution.

DNA extraction procedures. Harvested oocysts with the number of 5,000 -250,000/sample were sedimented, and then suspended in 0.5 ml tissue lysis buffer (a component of High Pure PCR Template Preparation Kit, Roche Diagnostics, Mannheim, Germany). The suspension was transferred into a round bottom tube with 250 mg of 0.5 mm glass beads. The tube was vortexed for 2 min to disrupt the oocysts. DNA was extracted from 0.3 ml lysate using the commercial kit in accordance with the manufacturer's direction. Finally, DNA was eluted with 0.2 ml elution buffer and stored at −20 ˚C until use.

Amplification and sequencing of the ITS-1 region. In order to design *Eimeria* genus-common primers with the purpose of amplifying DNA segments containing whole ITS-1 region from various *Eimeria* species, conserved sequences at the 3' end of the 18S rRNA genes and at the 5' end of the 5.8S rRNA genes of *E. acervulina*, *E. maxima*, *E. tenella* and *Toxoplasma gondii* (the GenBank with Accession Numbers: AY779492, AY779386, AF026388 and X75429) were used. The genus-common primer sets used were, Forward: 5'-GCA AAA GTC GTA ACA CGG TTT CCG-3', and Reverse: 5'-CTG CAA TTC ACA ATG CGT ATC GC-3'.

A total of 20 µl reaction mixtures containing 10 µl Premix Taq (Ex Taq Version, Takara Bio Inc., Shiga, Japan), 1 µl of the 10 µM primer sets (0.5 µM each), and 1 µl of genomic DNA template were used to amplify the ITS-1 regions. Reaction cycles consisted of an initial denaturing step at 94 ˚C for 30 sec followed by 35 cycles at 94 ˚C for 10 sec, 55 ˚C for 30 sec, 72 ˚C for 30 sec with final extension at 72 ˚C for 2 min using a 9800 Fast Thermal Cycler (Life Technologies Corporation, CA, USA). After determining the size on agarose gel electrophoresis, the PCR products were purified by using a Wizard SV Gel for the PCR Clean-Up System (Promega Corporation, WI, USA) and cloned into the pGEM-Teasy Vector (Promega Corporation) in accordance with the manufacture's instruction. At least 10 transformed clones for each product were used for plasmid extraction. Plasmids were extracted by using a Quick-Gene Plasmid kit S (FUJIFILM Corporation, Tokyo, Japan). At least 3 inserts were chosen and sequenced

from the ITS-1 clones with identical size, while other inserts with different sizes were also sequenced individually. Plasmid DNA sequencing were carried out by using BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corporation) with forward T7 primer using ABI PRISM 310 Genetic Analyzer (Life Technologies Corporation).

The result of ITS-1 sequences were aligned by using the biological sequence alignment editor (BioEdit version 7.0.9.0 for Windows, Ibis Biosciences, CA, USA) with ClustalW multiple alignment option without manual optimization. The alignment was used to calculate pair-wise identity percentages between species. Phylogenetic analysis was conducted using the software MEGA 4 (59) and inferred under the neighbor-joining (NJ) algorithm and nucleotide substitution model of p-distance with the resampling nodal support of 1,000 bootstrap replicates, under the Maximum Parsimony (MP) analysis using the close-neighbor-interchange algorithm. Species-specific primer sets were designed from the unique sequence of the ITS-1 region of each of the six *Eimeria* species. All ITS-1 sequences resulted from this research have been registered in the GenBank.

PCR assays with species-specific primer sets. All primers were synthesized by Japan Bio Services Co., Ltd. (Saitama, Japan). The reaction mixture and PCR condition used were the same as mentioned above except for the 35 cycles which at 94 ˚C for 10 sec, 55 ˚C for 20 sec, 72 ˚C for 20 sec. After amplification, 10 µl of PCR product was electrophoresed on a 1.5% agarose gels with a 100 bp DNA ladder (New England Biolabs, MA, USA). DNA fragments were visualized with ethidium bromide staining under UV illumination. In a series of PCR assays, six species-specific primer sets were tested for their reactivity by using 6 representative DNA samples. Moreover, artificially reconstituted DNA sample containing DNAs of each of the six species with the quantity equivalent to 10,000 oocysts/ml was used.

RESULTS

Morphometric classification on field collections. Fecal samples were collected from commercial cattle farms and isolated bovine coccidia oocysts. Based on the morphological characteristics of oocysts in these samples, *Eimeria* species present in the field samples was identified.

The samples that predominantly contained single *Eimeria* species were selected. Three representative samples each with a single dominating species without detective levels of other co-infected species were obtained from different farms, resulted in a total of 6 samples for 5 species (one sample each for *E. alabamensis*, *E. bovis*, *E. cylindrica* and *E. ellipsoidalis* and two samples for *E. zuernii* as shown in Table 1-1). Eleven samples (one sample for *E. zuernii* and two each for *E. alabamensis*, *E. auburnensis*, *E. bovis*, *E. cylindrica* and *E. ellipsoidalis*) consisted of a single predominant species with mixed infection with other species. On the other hand, one sample (Specimen ID; Eaub-03) mainly contained species other than the above-mentioned 6 species. In addition, 4 (Specimen ID; Eaub-02, Ecyl-03, Eell-02 and Ezue-01) out of 18 samples contained two or more species other than the 6 target species in this research.

Sequence analysis. In all *Eimeria* field collections, the ITS-1 regions were amplified successfully with the genus-common primer sets spanning at the 3' end of 18S rRNA gene and at the 5' end of 5.8S rRNA gene. The ITS-1 sequences of each *Eimeria* species were verified in correlation to the morphological feature of the oocysts detected in the fecal samples. Those that were not defined to known *Eimeria* species were categorized as the undefined species type (UD). The result of sequence analysis corresponded compatibly with the morphological findings, and the identical ITS-1 sequences were detected commonly across the 3 representative samples for all 6 species. Several types of ITS-1 sequences were mixed in *E. auburnensis*, *E. cylindrica* and *E. ellipsoidalis*, whereas the result of *E. alabamensis*, *E. bovis* and *E. zuernii* was consistent with a uniform sequence without any variation (Table 1-1). Therefore, the ITS-1 sequences of *E. alabamensis*, *E. bovis* and *E. zuernii* were determined at the first step. For the remaining 3 species as *E. auburnensis*, *E. cylindrica* and *E. ellipsoidalis*, the ITS-1 sequences were determined by subtracting those already found in *E. alabamensis*, *E. bovis* and *E. zuernii* from the accounts of mixed ITS-1 sequences.

The ITS-1 regions were composed of 220 bps fragment in *E. alabamensis*, 385 bps in *E. auburnensis*, 380 bps in *E. bovis*, 413-418 bps in *E. cylindrica*, 373-375 bps in *E. ellipsoidalis*, 403-405 bps in *E. zuernii* and 302-440 bps in UDs (Table 1-2). All the sequences from each 3 samples of 6 species had been submitted and registered in the GenBank (accession numbers are shown in Table 1-2). Sequences were aligned and their

similarity percentages were calculated taken into account 22 items including 18 tentative sequences, 3 UDs and an *E. tenella* strain (Table 1-2).

In spite of high homology, there was a minor difference in the sequence of the ITS-1 regions among three samples of the same species as follows; homology ranged from 98.2 to 98.6% in *E. alabamensis*, 99.2 to 99.7% in *E. auburnensis*, 99.5 to 99.7% in *E. bovis*, 91.9 to 93.1% in *E. cylindrica*, 98.9 to 99.7% in *E. ellipsoidalis* and 98.8 to 100% in *E. zuernii*. Inter-species homology between *E. bovis* and *E. zuernii* was the highest (75.2%), followed by *E. auburnensis* and *E. ellipsoidalis*, then *E. cylindrica* and *E. ellipsoidalis* (73.2 and 71%, respectively). The ITS-1 sequences of bovine coccidia contained a lot of repetitions of A and T, and showed AT-rich composition compared with *E. tenella*. AT composition in ITS-1 were 61.4% in *E. alabamensis*, 68.3% in *E. auburnensis*, 67.9% in *E. bovis*, 71.4% in *E. cylindrica*, 69.3% in *E. ellipsoidalis* and 64.8% in *E. zuernii*, 78.2% in UD1, 72.5% in UD2, 68.2% in UD3 and 47.3% in *E. tenella*.

Sequences of the ITS-1 region covering the 18S and 5.8S rRNA genes were used in the phylogenetic analysis. The phylogenetic trees were constructed using the neighbor-joining (NJ) analysis under the nucleotide substitution model of p-distance (Fig. 1-1) and the maximum parsimony (MP) analysis using the close neighbor interchange algorithm (Fig. 1-2). The phylogenetic tree demonstrated three clusters that explain species relation visually connecting with the sequence homology. By using ITS-1 of *E. tenella* as an outgroup, there were three clusters of bovine coccidia. In the major cluster, *E. auburnensis*, *E. cylindrica*, *E. ellipsoidalis*, UD2 and UD3 were grouped. *E. bovis* and *E. zuernii* formed one small cluster with *E. alabamensis* and UD1.

Specificity of PCR assays. The ITS-1 sequences among the *Eimeria* species were arranged in parallel to demonstrate variations and unique regions to design the species-specific primer sets. Species-specific primer sets were established and the expected product size was predicted as summarized in Table 1-3 and illustrated in Fig. 1-3.

The six species-specific primer sets were tested using genomic DNA obtained from six *Eimeria* samples. The predicted sizes of amplified fragments were accurately confirmed correspondingly among species (Fig. 1-4). There were no cross-species amplification and non-specific bands with the exception for *E. auburnensis*. In the case of Eaub-01, there was a PCR product of ITS-1 fragment by the primer sets for *E. bovis* (Fig. 1-4, Lane 3 in the template of Eaub-01) and its fragment size matched to the expected size (238 bps) of ITS-1 fragment of *E. bovis*. Because of the contamination of *E. bovis* in Eaub-01 sample that confirmed by the morphometric assay (Table 1-1), it was difficult to avoid the amplification of ITS-1 fragments of *E. bovis* in *E. auburnensis* using *E. bovis* primer

sets. The homology of the ITS-1 sequence was demonstrated substantially between *E. bovis* and *E. zuernii*, between *E. auburnensis* and *E. ellipsoidalis*, and between *E. cylindrica* and *E. ellipsoidalis*, although no cross-reaction was shown between each species.

PCR assays using species-specific primer sets designed in this chapter were further evaluated using mixed oocyst sample. A genomic DNA derived from a mixed sample containing 10,000 oocysts/ml each of 6 species was prepared. The amount of each DNA in the PCR reaction mixture was equivalent to that from 20 oocysts, assuming no loss during the processing procedures. Even the mixed sample with DNA of 6 species, all species-specific primer sets amplified a single product with the expected sizes (Fig. 1-5). *E. auburnensis* and *E. bovis* primer sets also specifically amplified and produced single bands, respectively.

DISCUSSION

In the present investigation, inter-species-specific DNA sequences located in the ITS-1 region of the rRNA gene of six cattle *Eimeria* were examined to study their diversity. The ITS-1 regions are variable corresponding to the diversity between species, as compared with rRNA genes, showing a pattern of low intra-specific and high inter-specific variations in the DNA sequence. These features make easy to design species-specific primers, which minimizes the possibility of cross-reactions with different species.

For the three samples each of 6 species collected from 3 different farms, ITS-1 sequences almost identical in the length and nucleotide sequences were obtained from each species group. Regarding chicken *Eimeria*, two lineages distinguishable by the ITS-1 sequence within a single species were reported for both *E. mitis* and *E. maxima* (27). Additionally, separated geographical distribution of *E. maxima* organisms at the continental level has been acknowledged for two different ITS-1 types of *E. maxima* (27). As all bovine *Eimeria* oocysts were obtained from farms in Japan, genetic diversities were not seen in the ITS-1 regions within the species. However, ITS-1 homologies between three samples of *E. cylindrica* were slightly low (91.9 to 93.1%), which might reflect their strain-to-strain variation.

All of the ITS-1 sequences contained an AT-rich composition comparing to that of *E. tenella*. This figure is suggestive of a specific characteristic for the most of bovine coccidia species. The three unclassified ITS-1 sequences had also AT-rich composition, which suggests these belong to those classified in 20 already-known or some unknown bovine *Eimeria* species. Higher homologies were recognized in the ITS-1 sequences between UD2 and *E. auburnensis* and between UD3 and *E. cylindrica*. These relationships were also supported by the phylogenetic analysis as the form sharing close two branches with two homologues in each of the 2 pairs. Based on these results, UD2 and UD3 are thought to be genetically close to *E. auburnensis* and *E. cylindrica*, respectively.

Assuming that bovine coccidia are to be categorized with the ITS-1 sequence patterns, one major cluster with many species and 3 others are formed. This clustering suggests several different origins from which many species have been descended. The phylogenetic analysis of chicken *Eimeria* revealed a monophyletic group consisting of highly pathogenic species including *E. necatrix* and *E. tenella* which is separated from other chicken *Eimeria* species (2). Similarly, *E. bovis* and *E. zuernii* are highly pathogenic and formed a monophyletic group in the position away from other members. It is much interesting that these two species may have same origin in spite of much

different morphological feature of the oocysts.

The specificity of the present PCR assay was evaluated by the several ways in this chapter. At first, proper amplification of the ITS-1 fragments with the expected sizes was achieved in six *Eimeria* with corresponding species-specific primer sets (Fig. 1-4). Second, template DNA samples obtained from six *Eimeria* species were assayed with other 5 species primer sets, with no cross amplification of DNA except for *E. auburnensis* template with the *E. bovis* primer sets (Fig. 1-4, lane 3 in the template of Eaub-01). Among three samples (Eaub-01, Eaub-02 and Eaub-03) containing *E. auburnensis* as the predominant species as determined on the basis of the morphological examination, Eaub-02 was thought free of *E. bovis*. Eaub-01 and Eaub-03 were apparently co-infected with *E. bovis.* As DNA amplification was found even in Eaub-02 using the *E. bovis* primer sets (data not shown), the possibility that contamination with *E. bovis* could not be ruled out. The possibility that the genomic DNA of *E. auburnensis* amplified non-specifically with *E. bovis* primer sets may not be true since *E. bovis* primer sets amplified only one single band from a mixed species template (Fig. 1-5, lane 3). In this study, pure *E. auburnensis* template to validate this finding could be obtained. The problem existing between genome DNA of *E. auburnensis* and *E. bovis* primer sets will be solved after obtaining pure materials.

Lastly, the assay was conducted with a mixed DNA sample of multiple species which contained DNAs equivalent to the amount from 20 or less oocysts for each species. With this DNA sample, the PCR assay was also confirmed to be specific and highly sensitive. Thus, the PCR is much sensitive compared to oocyst-counting using McMaster's method of which the detection limit is 100 oocysts in 1 g of feces.

Present results showed the phylogenetic relationship between bovine *Eimeria* and the diversity of species based on the ITS-1 sequences. These findings suggest that the sequence differences in species could be correlated with the morphological characteristics of oocysts. The assays to differentiate these species by PCR targeting the species-specific ITS-1 region were developed. Results demonstrated that the PCR assay for *Eimeria* species in cattle can be used for the detection and identification of the parasites. The PCR appeared superior compared to conventional fecal examination on oocysts in terms of sensitivity and reliability. A further study is recommended to elucidate the phylogenetic relationship and design ITS-1 primer sets for *E. bukidnonensis*, *E. brasiliensis*, *E. canadensis*, *E. illinoisensis*, *E. pellita*, *E. subspherica* and *E. wyomingensis*, so that most of important *Eimeria* species in cattle could be covered.

Table 1-1. *Eimeria* field collections included in this chapter for ITS-1 sequence analysis and evaluations.

Specimen ID	Prefecture	Cattle breed	Results based on oocyst's morphometrics (%[a])	Results based on aligned ITS-1 sequences	Sequence ID
Eala-01	Hokkaido	Holstein	*E. alabamensis* 70%, *E. zuernii* 30%	*E. alabamensis* type	*E. alabamensis* A
Eala-02 [b]	Hokkaido	Holstein	*E. alabamensis* 100%	*E. alabamensis* type	*E. alabamensis* B
Eala-03	Hokkaido	Japanese black	*E. alabamensis* 96%, *E. bovis* 4%	*E. alabamensis* type	*E. alabamensis* C
				Undefined type 1	Undefined type 1
Eaub-01 [b]	Hokkaido	Holstein	*E. auburnensis* 82%, *E. bovis* 18%	*E. auburnensis* type	*E. auburnensis* A
				E. bovis type	NA
Eaub-02	Hokkaido	Holstein	*E. auburnensis* 64%, others 36%	*E. auburnensis* type	*E. auburnensis* B
				Undefined type 2	Undefined type 2
				Undefined type 3	Undefined type 3
Eaub-03	Gunma	Holstein	*E. bovis* 76%, *E. auburnensis* 24%	*E. bovis* type	NA
				E. auburnensis type	*E. auburnensis* C
Ebov-01	Chiba	F1crossbred	*E. bovis* 98%, *E. ellipsoidalis* 2%	*E. bovis* type	*E. bovis* A
Ebov-02	Miyazaki	Japanese black	*E. bovis* 100%	*E. bovis* type	*E. bovis* B
				E. zuernii type	NA
Ebov-03 [b]	Chiba	Japanese black	*E. bovis* 99%, *E. ellipsoidalis* 1%	*E. bovis* type	*E. bovis* C
Ecyl-01 [b]	Tokyo	Holstein	*E. cylindrica* 100%	*E. cylindrica* type	*E. cylindrica* A
				E. bovis type	NA
Ecyl-02	Iwate	Holstein	*E. cylindrica* 92%, *E. zuernii* 8%	*E. cylindrica* type	*E. cylindrica* B
				E. zuernii type	NA
Ecyl-03	Iwate	Holstein	*E. cylindrica* 74%, others 26%	*E. cylindrica* type	*E. cylindrica* C
Eell-01 [b]	Hokkaido	Holstein	*E. ellipsoidalis* 100%	*E. ellipsoidalis* type	*E. ellipsoidalis* A
Eell-02	Iwate	Holstein	*E. ellipsoidalis* 78%, others 22%	*E. ellipsoidalis* type	*E. ellipsoidalis* B
Eell-03	Iwate	Holstein	*E. ellipsoidalis* 60%, *E. bovis* 40%	*E. ellipsoidalis* type	*E. ellipsoidalis* C
				E. bovis type	NA
				E. cylindrica type	NA
Ezue-01	Chiba	Japanese black	*E. zuernii* 96%, others 4%	*E. zuernii* type	*E. zuernii* A
Ezue-02	Iwate	Holstein	*E. zuernii* 100%	*E. zuernii* type	*E. zuernii* B
Ezue-03 [b]	Miyazaki	Japanese black	*E. zuernii* 100%	*E. zuernii* type	*E. zuernii* C

[a] Percentage of mixed species population.

[b] Representative specimen of each species used in the species-specific PCR assay.

Table 1-2. Pair-wise percentage identities calculated using all available ITS-1 sequences, including a sequence from *E. tenella.*

Sequence ID	Accession Number	Length (bps)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. *E. alabamensis* A	AB557607	220	100	98.6	98.2	26.5	28.5	26.7	27.6	27.6	27.6	22.1	21.2	22.9	29.6	28.7	29.7	27.3	27.0	26.8	37.9	20.6	24.8	18.3
2. *E. alabamensis* B	AB557608	220		100	98.6	26.5	26.5	26.7	27.1	27.1	27.1	22.1	22.1	22.2	27.8	27.7	27.9	27.2	26.8	27.0	38.3	20.9	24.3	17.5
3. *E. alabamensis* C	AB557609	220			100	26.5	28.5	26.7	27.3	27.3	25.4	23.3	22.1	23.0	27.0	26.9	27.1	25.9	27.1	27.3	38.6	21.1	24.6	17.9
4. *E. auburnensis* A	AB557610	385				100	99.2	99.7	51.5	53.7	53.5	67.5	68.6	67.4	73.0	72.7	72.7	49.6	49.5	49.8	39.3	73.4	68.5	28.3
5. *E. auburnensis* B	AB557611	385					100	99.5	51.5	52.2	52.0	67.8	68.7	67.7	73.2	73.0	73.0	49.2	49.0	49.3	39.5	73.6	68.7	28.3
6. *E. auburnensis* C	AB557612	385						100	51.5	53.7	53.5	67.8	68.9	67.7	73.2	73.0	73.0	49.9	49.8	50.0	39.5	73.6	68.7	28.3
7. *E. bovis* A	AB557613	380							100	99.5	99.7	51.8	51.9	50.4	51.8	51.6	51.4	75.2	75.2	75.4	34.2	48.6	51.4	26.9
8. *E. bovis* B	AB557614	380								100	99.5	50.6	52.0	50.6	51.5	51.1	51.3	74.7	74.9	75.2	34.7	48.8	51.7	27.3
9. *E. bovis* C	AB557615	380									100	50.6	52.4	50.2	51.4	51.8	51.1	74.9	74.9	74.2	34.5	48.9	51.7	27.1
10. *E. cylindrica* A	AB557616	413										100	91.9	92.9	71.2	71.3	70.7	52.2	52.4	52.2	40.2	62.2	84.0	27.9
11. *E. cylindrica* B	AB557617	414											100	93.1	70.7	70.8	70.2	51.5	51.7	51.5	39.8	62.3	85.9	29.1
12. *E. cylindrica* C	AB557618	418												100	70.4	70.5	70.0	52.0	52.0	52.0	40.4	62.8	84.7	31.6
13. *E. ellipsoidalis* A	AB557619	374													100	99.7	99.2	53.5	53.5	53.3	39.8	65.3	68.5	28.1
14. *E. ellipsoidalis* B	AB557620	375														100	98.9	53.7	53.7	52.3	40.1	65.5	68.3	28.1
15. *E. ellipsoidalis* C	AB557621	373															100	53.3	53.5	53.0	39.5	64.9	68.3	28.3
16. *E. zuernii* A	AB557622	403																100	100	98.8	35.0	48.2	49.9	32.5
17. *E. zuernii* B	AB557623	403																	100	99.3	35.0	48.2	49.9	32.5
18. *E. zuernii* C	AB557624	405																		100	37.0	47.8	49.9	31.8
19. Undefined type 1	AB557625	302																			100	35.6	39.3	22.8
20. Undefined type 2	AB557626	440																				100	62.8	29.9
21. Undefined type 3	AB557627	415																					100	29.2
22. *E. tenella*	AF026388	563																						100

Table 1-3. Genus-common and species-specific primer sets designed for the polymerase chain reaction.

Species	Primer sequences (5' - 3')		Expected product size (bps)
	Forward	Reverse	
Genus-common	GCAAAAGTCGTAACACGGTTTCCG	CTGCAATTCACAATGCGTATCGC	348-546
E. alabamensis	CATTCACACATTGTTCTTTCAG	GCTTCCAAACTAATGTTCTG	184
E. auburnensis	TAAATTGGTGCGATGAGGGA	GCAATGAGAGAAAGATTTAATA	295
E. bovis	TCATAAAACATCACCTCCAA	ATAATTGCGATAAGGGAGACA	238
E. cylindrica	GACATTTAAAAAACCGATTGGT	GGCTGCAATAAGATAGACATA	304
E. ellipsoidalis	CAACGTTTTTCCTTTTCCTATCA	ACTGCGATGAGAGAGAGCG	148
E. zuernii	AACATGTTTCTACCCACTAC	CGATAAGGAGGAGGACAAC	343

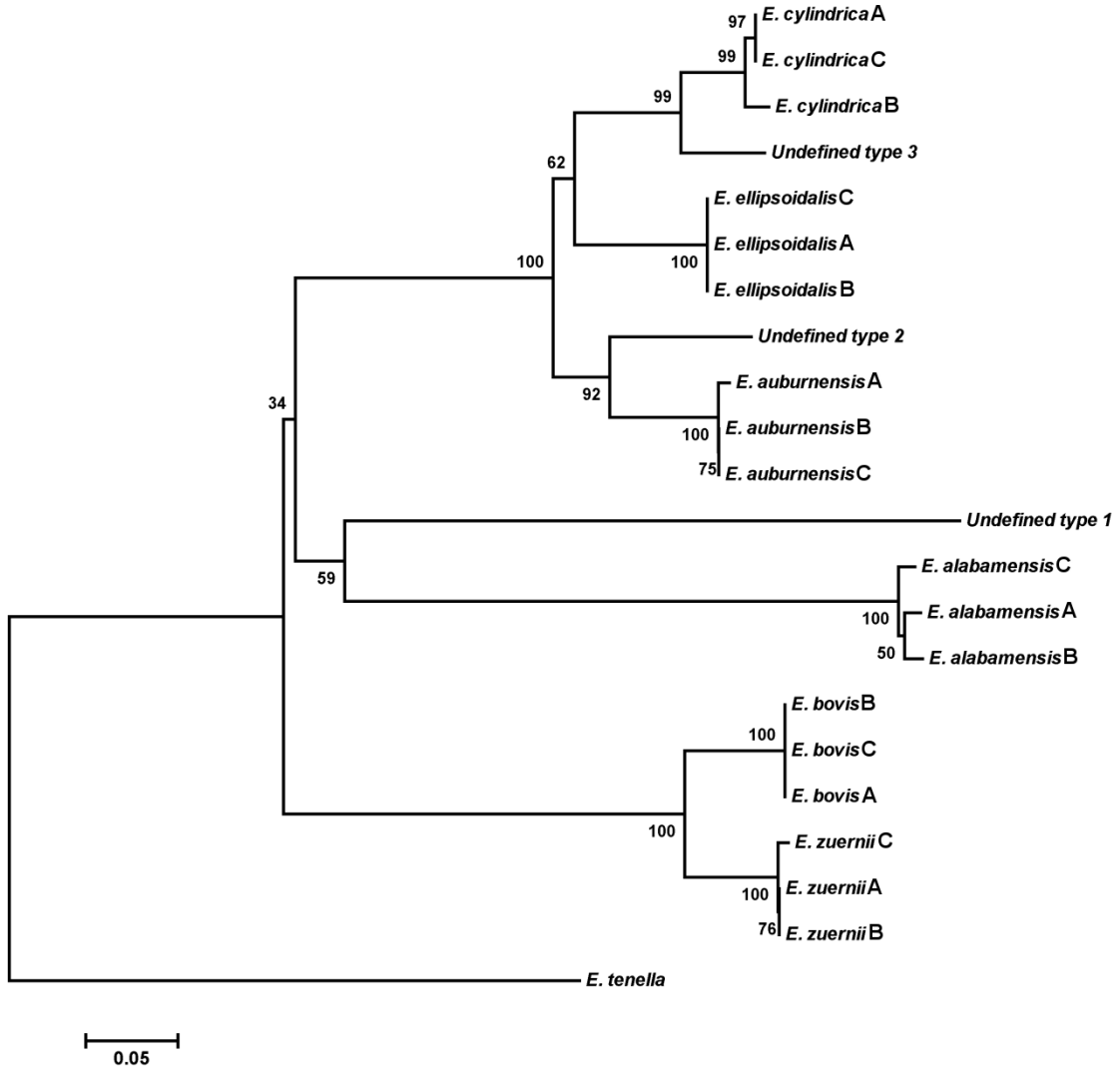


Fig. 1-1. Phylogenetic tree for all available ITS-1 sequences. The tree was rooted on *Eimeria tenella*, branch lengths indicated computed evolutionary distances using the neighbor-joining (NJ) method. Bootstrap support values from NJ are displayed on the nodes.

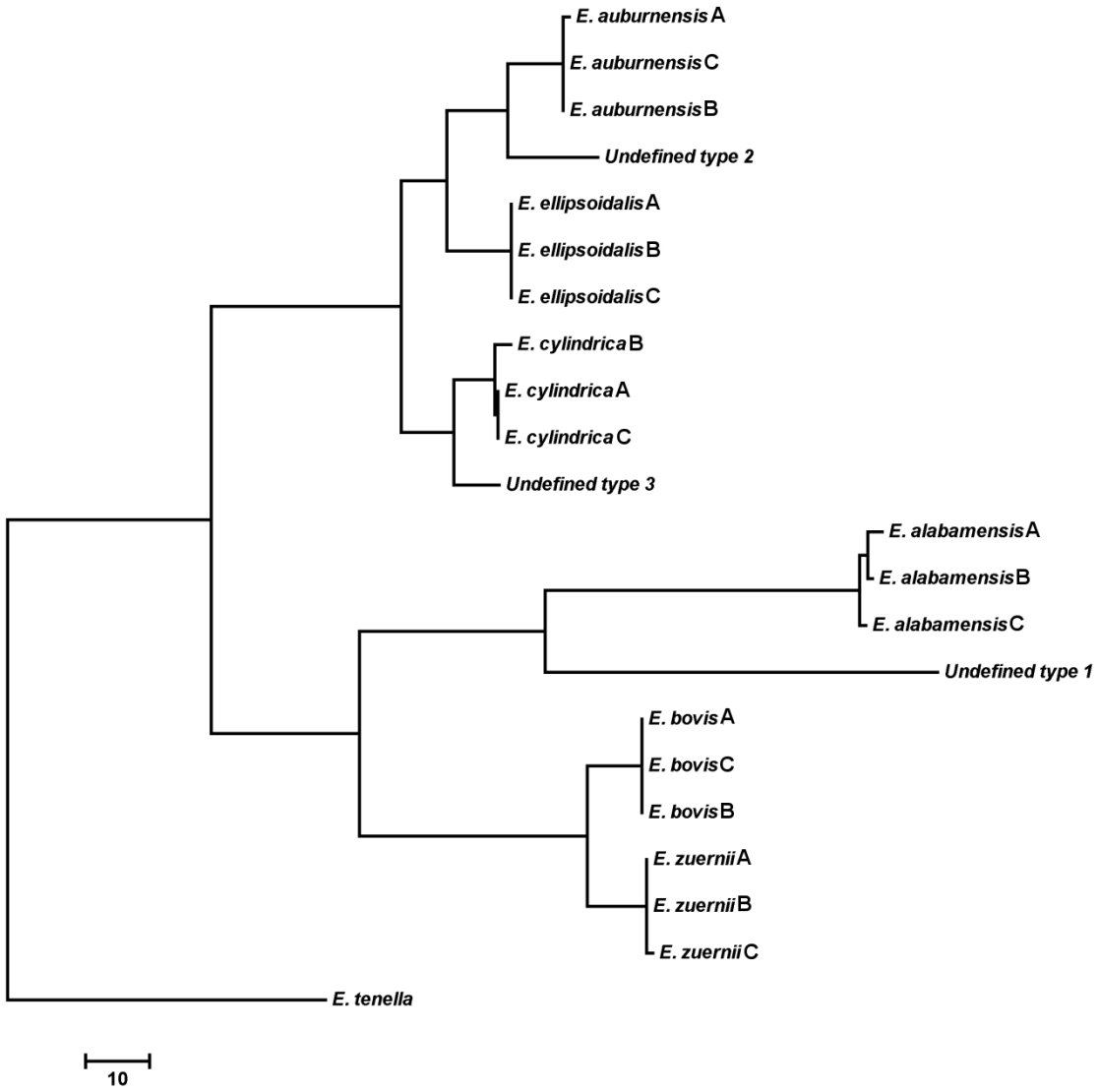


Fig. 1-2. Phylogenetic tree for all available ITS-1 sequences. The tree was rooted on *Eimeria tenella*, branch lengths indicated computed evolutionary distances using the maximum parsimony method.

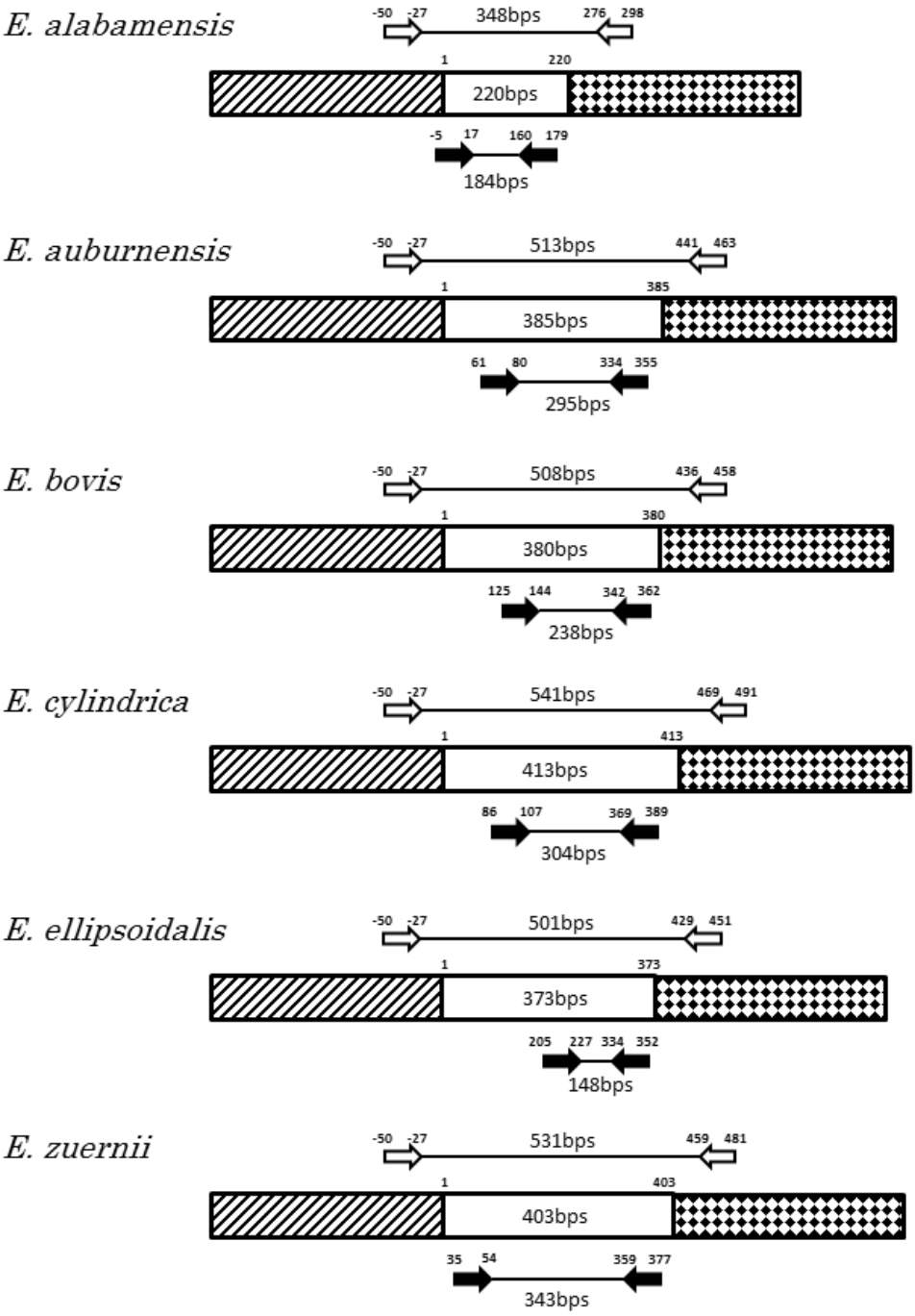
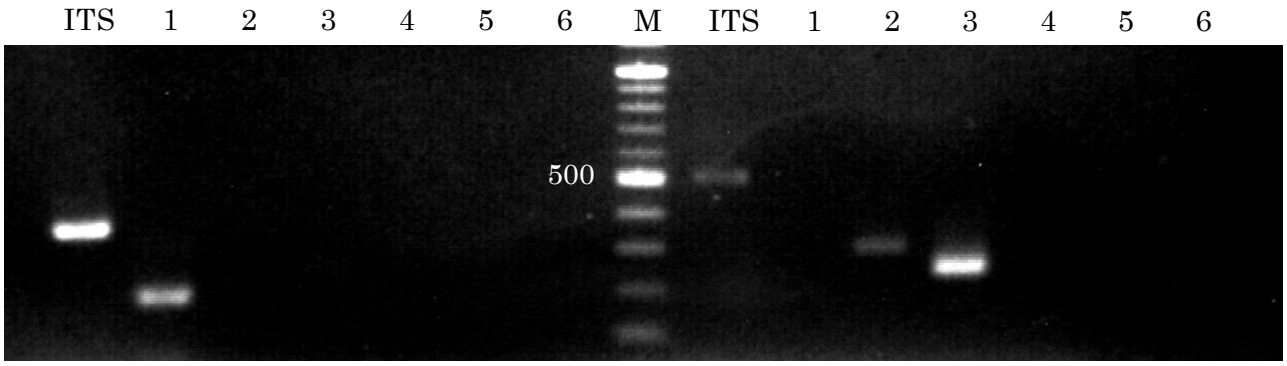
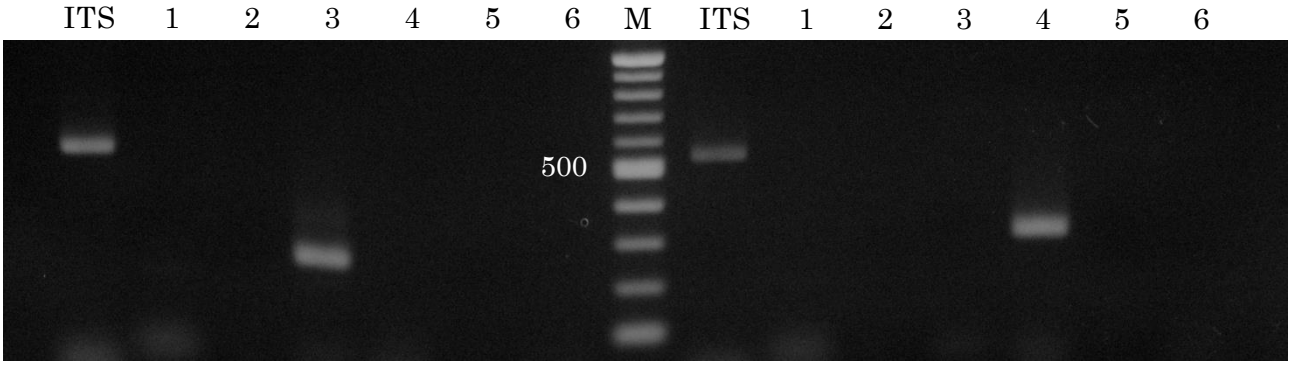


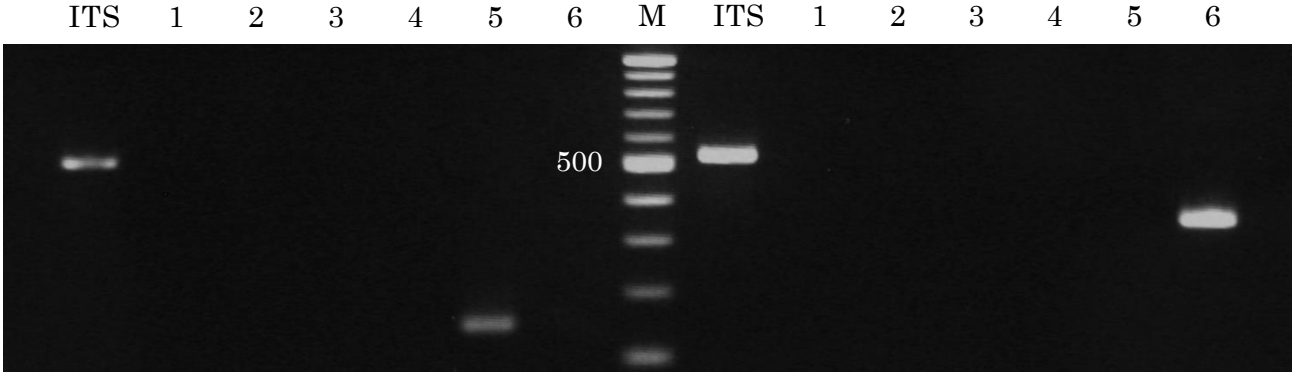
Fig. 1-3. Primer mapping on the shame of each species DNA sequences. Striped box, 18S rRNA gene; Opened box, ITS-1 region; Dotted box, 5.8S rRNA gene; Opened arrow, Genus-common primer set; Solid arrow, species-specific primer set; bps, size of PCR product or ITS-1 region; Small number, the order of nucleic acid residue based on ITS-1 region.



template: Eala-02 template: Eaub-01



template: Ebov-03 template: Ecyl-01



template: Eell-01 template: Ezue-03

Fig. 1-4. Agarose gel electrophoresis of the PCR products obtained from the inter-species specificity assessment of six *Eimeria* species. M: marker ladder (100 base pair). The different primer sets used were as follows: ITS, genus-common; 1, *E. alabamensis*-specific; 2, *E. auburnensis*-specific; 3, *E. bovis*-specific; 4, *E. cylindrica*-specific; 5, *E. ellipsoidalis*-specific; 6, *E. zuernii*-specific. The six species-specific primer sets were tested in their efficiency using genomic DNA template indicated at the bottom.

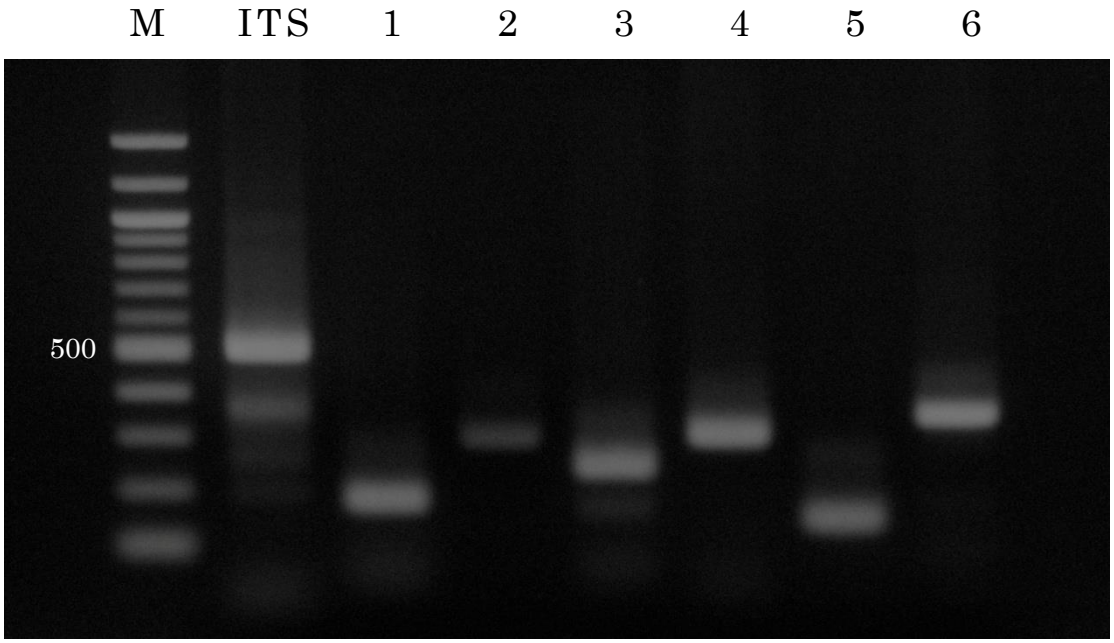


Fig. 1-5. Agarose gel electrophoresis of the PCR products amplified with species-mixed DNA samples by genus-common and species-specific primer sets. M: marker ladder (100 base pair). The different primer sets used are as follows: ITS, genus-common; 1, *E. alabamensis*-specific; 2, *E. auburnensis*-specific; 3, *E. bovis*-specific; 4, *E. cylindrica*-specific; 5, *E. ellipsoidalis*-specific; 6, *E. zuernir*-specific.

SUMMARY

At present, morphological characteristics of oocyst is the only achievable method for the identification of bovine coccidia to the species level. In this chapter, the internal transcribed spacer 1 (ITS-1) region located between large- and small-subunit ribosomal RNA genes of six bovine *Eimeria* species; *E. alabamensis*, *E. auburnensis*, *E. bovis*, *E. cylindrica*, *E. ellipsoidalis* and *E. zuernii*, were sequenced to analyze the phylogenetic relationship among them. In pair-wise alignment, the sequences had highly conserved within species with homologies of over 90%. *E. bovis* and *E. zuernii* were closely related within the same cluster. This cluster and *E. alabamensis* were distant from the major cluster of bovine coccidia that included *E. auburnensis*, *E. cylindrica* and *E. ellipsoidalis*. Since the ITS-1 region of each *Eimeria* species had sufficient inter-specific sequence differences enough to design the primer sets that differentially amplified each of target species, species-specific PCR assays based on the ITS-1 region were also developed to identify the 6 pathogenic species. This PCR assay for the detection and differentiation of *Eimeria* parasite showed higher sensitivity when compared to the conventional morphological examination of oocysts. This is the first attempt for the identification of 6 bovine *Eimeria* parasites at the genomic level and could provide a useful method for diagnosis and epidemiological studies of bovine coccidial infection.

CHAPTER II

Detection of five chicken *Eimeria* species by species-specific real-time polymerase chain reaction assay

INTRODUCTION

Chicken coccidiosis induced by *Eimeria* infections has been found worldwide. Its impact imposes economic losses to poultry industries. The clinical signs are characterized by diarrhea ranging from mucoid and watery to hemorrhagic, reduction in weight or weight gain, high morbidity, and sudden death. Host ranges of *Eimeria* species are highly specific. The eight species infecting chickens are *E. acervulina*, *E. brunetti*, *E. hagani*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella*. Among these species, *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix* and *E. tenella* are highly pathogenic, which cause considerable economic loss to the intensive poultry industry. Detection of oocysts in chicken feces and examination of lesions at particular regions of the gut have been common means to diagnose coccidiosis. Exact identification of *Eimeria* species, mostly carried out in the laboratory, has been conducted traditionally based on morphological and pathological aspects, such as oocyst characteristics, variations in prepatent period (time spent in coccidial generations), sporulation time, clinical signs, intestinal lesions and histopathological characteristics (23). However, these methods of identification require improved skills supported by sufficient experience. Differentiating *Eimeria* species could be difficult due to their similar clinical signs and the developmental characteristics (29). Furthermore, mixed infections make it more difficult to identify the species in the examination of intestinal lesions.

User-friendly molecular methods for species-specific detection of chicken *Eimeria* from the field samples are needed for clinical and epidemiologic application. Nowadays, many methods such as the species-specific conventional polymerase chain reaction (PCR) assay or the PCR-based capillary electrophoresis technique in detecting chicken *Eimeria* species have been described using the 5S rRNA gene, the small subunit rRNA gene, and the internal transcribed spacer (ITS)-1 or ITS-2 region as a target site (7, 19, 20, 21, 27, 35, 36, 37, 51, 52, 55, 62, 64). Real-time PCR offers more advantage by avoiding post-PCR processing steps, which saves time and reduces labor compared with conventional PCR. Real-time PCR assays have been developed for the detection of several infectious agents of avian diseases, such as *Clostridium perfringens* and duck circovirus (18, 63). Application of hybridization probe-based real-time PCR assay was also reported for *E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella* (3, 4, 58). However, there have been no reports describing real-time PCR assay using SYBR Green or other dyes to stain amplified DNA fragments for chicken *Eimeria*. Real-time PCR assay using SYBR Green could be operated at lower running cost than the assay using hybridization probes. Another advantage is that it can be performed for some *Eimeria* species by just

changing the primer set in each reaction mixture. Therefore, the development of SYBR Green-based real-time PCR assay for five *Eimeria* species is described in this chapter.

MATERIALS AND METHODS

Parasites and control samples. The Na strain of *E. acervulina*, Nb strain of *E. brunetti*, Nm strain of *E. maxima*, Nn strain of *E. necatrix* and Nt strain of *E. tenella* isolated from each single oocyst which collected from chicken feces in Japan were used in this study. They have been maintained in the author's laboratory. The correct species identification and purity of all strains were confirmed by PCR using an assay directed toward the ITS-1 (51, 52). Oocyst collection, purification and sporulation followed standard procedures (30). The number of Coccidial oocysts were examined by microscopy observation with the McMaster slide (Fujihira Kogyo, Tokyo, Japan), then suspended in water to be 10^7 oocysts/ml. A 10-fold dilution series of the control samples (10^7 to 10^{-1}) of five species were prepared to determine the detection limit and to analyze the melting curve of real-time PCR products.

DNA extraction procedures. 0.5 ml of the oocyst suspension was centrifuged at 15,000×*g* for 10 min. Supernatant was discarded, and sedimented oocysts were suspended in 0.5 ml of tissue lysis buffer (a component of High Pure PCR Template Preparation Kit, Roche Diagnostics, Mannheim, Germany). The suspension was transferred into a round-bottomed tube with 250 mg of 0.5 mm glass beads, and then it was vortexed for 2 min to disrupt the oocysts. DNA was extracted from 0.3 ml volume of the lysate by using the kit according to the manufacturer's directions. Finally, DNA was eluted with 0.2 ml of elution buffer and diluted 10 times with double-distilled water. Five microliters of the diluted DNA was used per reaction mixture.

Primers. The species-specific primer sets were designed based on species-specific sequence of the ITS-1 region in *E. acervulina*, *E. brunetti*, *E. necatrix* and *E. tenella* using Primer3 software (49). Those for *E. maxima* were modified from previously reported primers (Table 2-1 and Fig. 2-1) (27). These preparations were supplied by Japan Bio Services Co., Ltd. (Saitama, Japan). With the primer sets, the length of amplified fragments was predicted to be 166 base pairs (bps) for *E. acervulina* (the forward and reverse primers share the space of DNA sequence from 295 to 314 bps and from 441 to 460 bps, respectively; accession No. AF026384), 148 bps for *E. brunetti* (forward, from 50 to 67 bps; reverse, from 178 to 197 bps; accession No. AF026383), 148 bps for *E. necatrix* (forward, from 212 to 229 bps; reverse, from 342 to 359 bps; accession No. AF026385), and 147 bps for *E. tenella* (forward, from 2612 to 2631 bps; reverse, from 2739 to 2758 bps; accession No. AF026388).

Real-time PCR. Light Cycler (Roche Diagnostics) was used for the real-time PCR. The preparation of reaction solution consisted of 10 µl of SYBR Premix Ex Taq (Takara Bio, Shiga, Japan), 1 µl of a 10 µM stock of forward primer (final concentration, 0.5 µM), and

1 μl of a 10 μM stock of reverse primer (final concentration, 0.5 μM). The template volume was 5 μl, yielding a final 20-μl volume in the reaction capillary. Cycling reactions were performed under the following conditions: 60 sec at 95 ˚C, followed by 45 cycles of 10 sec at 95 ˚C, 10 sec at 62 ˚C, and 10 sec at 72 ˚C. A melting curve was described by gradual heating from 60 to 95 ˚C at a rate of 0.1 ˚C/sec followed by the incubation at 40 ˚C. To evaluate interspecies specificity, primer sets specific for *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix* and *E. tenella* were tested against a genomic DNA panel of five *Eimeria* species. A genomic DNA panel consisted of five DNA samples which were combined with different four species.

Samples from poultry farms. Pooled fecal samples of several chickens per farm were collected from 32 poultry farms in 2006 and 2007. These samples were submitted for diagnostic investigation to the author's laboratory. Ten grams of the fecal sample was dissolved in 90 ml of phosphate-buffered saline, pH 7.0, and then the sample was vortexed vigorously for 1 min. This dilution has been shown previously to be necessary for efficient material lysis and DNA recovery in chicken gastrointestinal tract samples (63). DNA was extracted from a 0.5 ml volume of the solution according to the DNA extraction procedure mentioned above. A series of real-time PCR assays was done using the five species-specific primer sets for all of the field samples. The melting temperature (Tm) of each field sample was also recorded as a basis of the identification by comparison with the positive control.

The number of oocysts per gram (OPG) in each sample was determined using a standard McMaster technique. The length of 50 random oocysts from each sample was measured by using a calibrated ocular micrometer at ×400 magnification, and categorized into three groups: AMi (small oocysts, <19.0 μm; tentatively *E. acervulina*, *E. mitis*, or both), NPT (medium-sized oocysts, from 19.0 to 24.0 μm; tentatively *E. necatrix*, *E. praecox*, *E. tenella*, or a combination), and BMa (large oocysts, >24.0 μm; tentatively *E. brunetti*, *E. maxima*, or both).

Fisher's exact tests were utilized to evaluate the differences of infection rates between types of farm in each *Eimeria* species.

RESULTS

Analysis for real-time PCR with control samples. Each of the five primer sets was tested by real-time PCR against control samples of all five *Eimeria* species. The amplification of a single product unique to the target species investigated was confirmed. The size of amplification products of the targets was matched with the predicted size of each species (Fig. 2-2). Melting curve analysis of each species showed a single melting peak and a species-specific Tm value (Fig. 2-3). Tm values obtained from PCR products of *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix* and *E. tenella* were 88.8, 83.5, 81.6, 80.2 and 88.3 ˚C, respectively. Two primer sets for *E. brunetti* and *E. tenella* did not react on DNA of other species in mixed DNA samples with any cross-species amplifications (Fig. 2-4). Three primer sets for *E. acervulina*, *E. maxima* and *E. necatrix* amplified a small amount of non-specific PCR products with a mixed DNA sample of other species. However, melting-curves of these three non-specific PCR products did not shape a single acute peak like specific PCR products. Furthermore, non-specific Tm values (round 79 ˚C) of non-specific PCR products of these three species differed enough to distinguish Tm values (80.2 to 88.8 ˚C) of species-specific PCR products. Therefore, if non-specific PCR products are caused in assays, results could not be affected and confused by it.

The linear ranges that show a coefficient of regression >0.99 in the control sample dilution series were from 10^7 to 10^1 oocysts in *E. brunetti* and *E. tenella*, from 10^7 to 10^2 oocysts in *E. acervulina* and *E. maxima*, and from 10^7 to 10^3 oocysts in *E. necatrix* (Table 2-2). The control sample dilution series of each species showed the detection limit of 10^1 oocysts for *E. brunetti*, *E. maxima* and *E. tenella* and 10^2 oocysts for *E. acervulina* and *E. necatrix*.

Identification of *Eimeria* species in feces. Fecal samples from 32 poultry farms, which suffered from chicken coccidiosis, were examined by this assay (Table 2-3). Among the 32 farms, *E. brunetti* was found in 21 farms, *E. maxima* and *E. necatrix* in 16 farms, *E. tenella* in 12 farms, and *E. acervulina* in eight farms. Most prevalent species was *E. brunetti* in breeder and layer chickens, *E. maxima* in broilers (Table 2-4). Furthermore, positive rates of *E. brunetti* have shown significant difference among 4 types of farm by Fisher's exact test, but significant differences have been not shown whether positive rates of any farms were high or low by multiple comparison procedures.

DISCUSSION

This chapter described the application of species-specific real-time PCR using SYBR Green for the diagnosis of five chicken *Eimeria* species. The technique is practically faster than conventional PCR and convenient because the reaction is traced out consecutively by the fluorescent monitoring. Thus, the gel electrophoretic procedure is unnecessary. Furthermore, this assay can handle many samples simultaneously, making this procedure rapid. Real-time PCR assays that depend on hybridization probes have been reported for *E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella* (3, 4, 58). However, there are no reports on real-time PCR assays that use SYBR Green or other dyes to stain amplified DNA fragments for chicken *Eimeria* species. Therefore, SYBR Green-based real-time PCR assay that emits fluorescence at intercalating PCR products without using hybridization probes anymore was performed. SYBR Green-based real-time PCR assay require lower cost than the assay using hybridization probes. Another advantage is that it can be performed for all *Eimeria* species by just changing the primer set in each reaction mixture. In contrast, it is very important to design a specific primer set to avoid non-specific amplification of DNA fragments with other *Eimeria* species. Primer sets designed in this research provide a high level of specificity such that only the target species sequence is detected without any spurious products or primer-dimers because only a single acute peak was gained by melting curve analysis. Although each primer sets for *E. acervulina*, *E. maxima* and *E. necatrix* amplified non-specific PCR products with other species DNA, it is possible to distinguish easily by different Tm values whether the PCR product was specific or non-specific. Tm values produced by the melting curve analysis are specific to each species; therefore, they can be applied as a basis to discriminate individual *Eimeria* species.

Traditional fecal examination usually has a detectable limit of oocysts of >100/ml solution, which has similar sensitivity by the quantitative analysis of the present assay. A fully sporulated oocyst of *Eimeria* species contains eight sporozoites, which has eight the *Eimerian* genomes. In this chapter, about a single copy of *Eimerian* genome per reaction could be detected under the best case conditions. However, because ITS-1, a region of multiple copies of rDNA, was chosen to maximize the detection sensitivity of the assay, it is highly probable that the number of copies of this region differs between species or strains. Therefore, it is impossible to use this region for quantitative analysis for the field samples despite the sufficient correlation of the number of oocysts with the PCR results referring to its linear range.

Coccidia possess the stages of merogony and gametogony in their development in the

host animals. Depending on the species, the parasites at these stages often occur in chicken feces during the severe phase of the disease. Detection of these parasite stages in feces requires a more careful examination to identify which species is present. To shorten the process, direct DNA extraction from a fecal sample can be used because this sample may contain parasites other than oocysts. Findings in this chapter demonstrate that the present PCR assay can be applied to detect *Eimeria* discriminately by species, even in mixed infections.

Among chicken *Eimeria* species, *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix* and *E. tenella* are recognized as highly pathogenic worldwide. On the contrary, the pathogenicity of *E. brunetti* is estimated to be low and the distribution of *E. brunetti* is not well studied. In Japan, previous surveys relying on oocyst detection in commercial chicken flocks failed to detect *E. brunetti* (42, 45) therefor it was supposed that there were no *E. brunetti* in Japan for long time. About 10 years later since these surveys, two strains of *E. brunetti* were isolated from poultry farms in Hokkaido and Kumamoto, located in the northern and southern parts of Japan, respectively, for the first time, reviving the assumption of wide prevalence of the species in Japan (40). In accordance with the report, it was demonstrated in this study that *E. brunetti* was found in 21 farms, 20 of which came from breeder and layer farms. Therefore, the prevalence of *E. brunetti* in Japan is not only quite evident but supposed to be considerably high. Follow up epidemiological studies and analysis of their burden on poultry industry should be highly demanded.

Table 2-1. Species-specific primer sets designed for the real-time PCR.

Species	Primer sequences		Expected product size (bps)
	Forward (5'−3')	Reverse (5'−3')	
E. acervulina	AACCTGACTGTGCAAGCATC	ATCATAGACAGCCGTGCCAG	166
E. brunetti	TTGCGTAAATAGAGCCCT	CATGCAGAAAACTCCAAAAG	148
E. maxima	GTGATTCGTTCGGAAGTTTGC	CTCACCACTCACAATGAGGCAC	145
E. necatrix	GCAGTCGTTCTTGGGTGT	TGCTCACGCCCATACTAC	148
E. tenella	TGGAGGGGATTATGAGAGGA	CAAGCAGCATGTAACGGAGA	147

Table 2-2. Measurable and detectable range determined for the real-time PCR.

Template	No. of oocysts contained in the control dilution series		
	Test range	Linear extension range (coefficient of regression)	Detection limit
E. acervulina	10^7 - 10^{-1}	10^7 - 10^2 (0.9992)	10^2
E. brunetti	10^7 - 10^{-1}	10^7 - 10^1 (0.9947)	10^1
E. maxima	10^7 - 10^{-1}	10^7 - 10^2 (0.9971)	10^1
E. necatrix	10^7 - 10^{-1}	10^7 - 10^3 (0.9971)	10^2
E. tenella	10^7 - 10^{-1}	10^7 - 10^1 (0.9997)	10^1

Table 2-3. Description of the examination of the fecal samples from poultry farms.

Farm	Type of farm[a]	Age (days) at test	Coccidiosis control methods[b]	OPG (Log)	Oocyst categories[c, d]			Detected species by real-time PCR[d, e]				
					AMi	NPT	BMa	EA	EB	EM	EN	ET
1	BB	25	TAM, Neca	2.9	-	+	+	-	-	+	+	+
2	BB	39	TAM, Neca	NT[f]		NT		-	+	+	+	-
3	BB	39	TAM, Neca	NT		NT		+	-	-	+	-
4	BB	48	TAM, Neca	3.0	-	+	-	-	+	+	+	-
5	BB	49	TAM, Neca	3.0	-	+	-	-	+	-	+	-
6	BB	51	TAM, Neca	NT		NT		-	-	+	+	-
7	BB	60	TAM, Neca	4.0	-	+	+	+	+	+	-	+
8	BB	60	TAM, Neca	4.7	-	+	-	-	-	-	+	-
9	BB	70	TAM, Neca	4.0	-	+	-	-	-	-	+	+
10	BB	91	TAM, Neca	NT		NT		-	+	-	+	-
11	BB	111	TAM, Neca	NT		NT		-	+	+	-	+
12	BB	112	TAM, Neca	5.0	-	-	+	-	+	-	-	-
13	BB	175	TAM, Neca	2.0	-	+	+	-	+	+	+	-
14	BB	200	TAM, Neca	4.0	-	-	+	-	+	-	-	-
15	BB	203	TAM, Neca	5.0	-	+	+	-	+	-	-	-
16	BL	40	TAM, Neca	6.6	-	-	+	-	+	-	-	-
17	BL	71	TAM, Neca	NT		NT		+	+	-	+	-
18	BL	80	TAM, Neca	5.6	-	+	-	-	+	-	-	-
19	BL	149	TAM, Neca	6.4	-	+	+	-	+	-	-	-
20	L	71	None	6.1	-	+	+	-	+	+	-	+
21	L	86	Unknown	5.0	-	+	+	-	+	-	+	+
22	L	110	None	5.0	-	-	+	-	+	+	-	-
23	L	140	TAM	4.0	+	+	+	+	+	+	-	-
24	L	140	None	NT		NT		-	-	-	+	-
25	L	157	None	NT		NT		+	+	+	+	+
26	L	210	None	5.5	-	+	+	-	+	-	+	+

27	B	29	Drug	NT		NT		+	-	+	-	+
28	B	37	TAM	5.4	-	+	-	-	-	-	-	+
29	B	42	TAM	3.0	-	+	-	-	-	+	+	-
30	B	45	Unknown	3.7	+	+	+	+	-	+	-	+
31	B	46	Unknown	3.8	+	-	+	+	+	+	-	+
32	B	50	Unknown	NT		NT		-	-	+	-	-

[a]BB, breeders for broilers; BL, breeders for layers; L, layers; B, broilers.

[b]TAM, chicken coccidiosis trivalent live attenuated vaccine containing *E. acervulina*, *E. maxima* and *E. tenella* (Nisseiken Co., Ltd., Tokyo, Japan); Neca, chicken coccidiosis live attenuated vaccine containing *E. necatrix* (Nisseiken Co., Ltd., Tokyo, Japan).

[c]Ami, small oocysts (<19.0 μm; tentatively *E. acervulina* and/or *E. mitis*); NPT, medium-sized oocysts (19.0 to 24.0 μm; tentatively *E. necatrix*, *E. praecox* and/or *E. tenella*); BMa, large oocysts (>24.0 μm; tentatively *E. brunetti* and/or *E. maxima*).

[d]+, detected; -, not detected.

[e]EA, *E. acervulina*; EB, *E. brunetti*; EM, *E. maxima*; EN, *E. necatrix*; ET, *E. tenella*.

[f]NT, not tested.

Table 2-4. Distribution of *Eimeria* species depending on the type of farm.

Type of farm	No. of farms	Number of detected samples by real-time PCR				
		E. acervulina	*E. brunetti*	*E. maxima*	*E. necatrix*	*E. tenella*
Breeders for broilers	15	2	10	7	10	4
Breeders for layers	4	1	4	0	1	0
Layers	7	2	6	4	4	4
Broilers	6	3	1	5	1	4

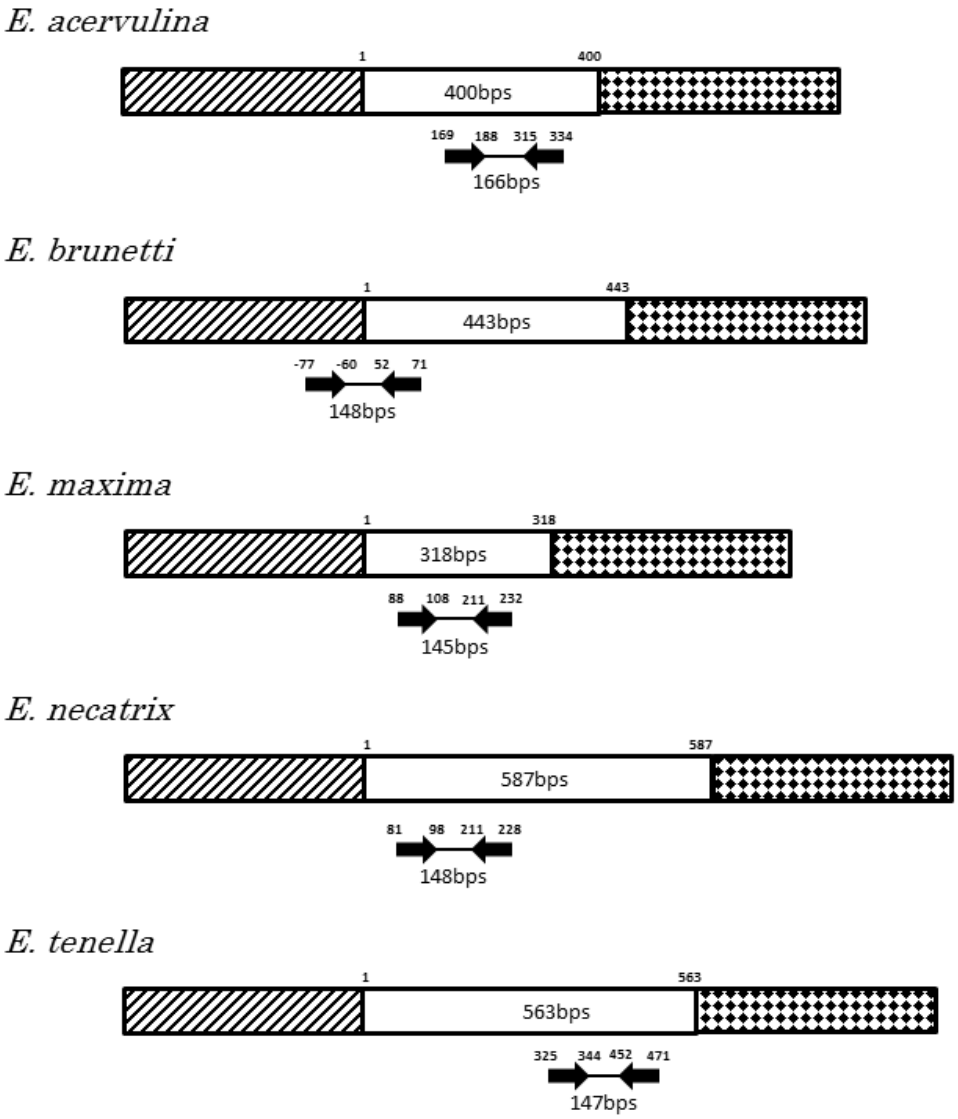


Fig. 2-1. Primer mapping on the shame of each species DNA sequences. Striped box, 18S rRNA gene; Opened box, ITS-1 region; Dotted box, 5.8S rRNA gene; Solid arrow, species-specific primer set; bps, size of ITS-1 region or PCR product; Small number, the order of nucleic acid residue based on ITS-1 region.

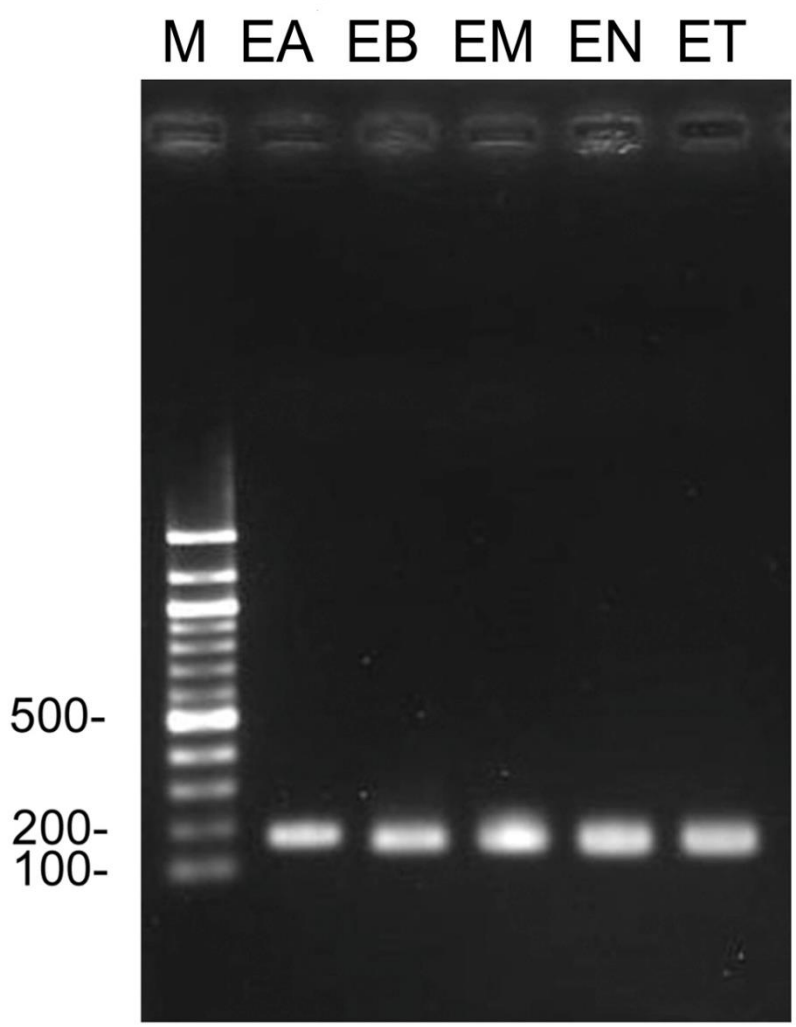


Fig. 2-2. Real-time polymerase chain reaction amplification of ITS-1 region from the control samples. Amplicons were fractionated on 1.5% agarose. Lane M, 100-base pair ladder; EA, *E. acervulina*; EB, *E. brunetti*; EM, *E. maxima*; EN, *E. necatrix*; ET, *E. tenella*.

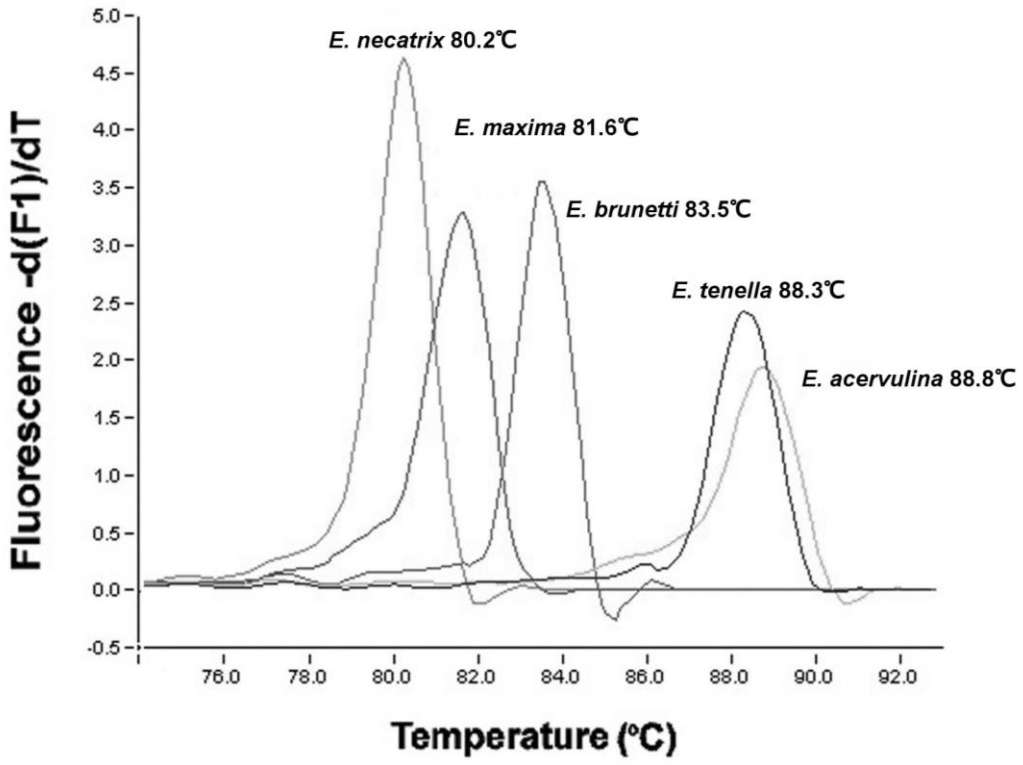


Fig. 2-3. Melting-curves of the control samples by real-time polymerase chain reaction assay.

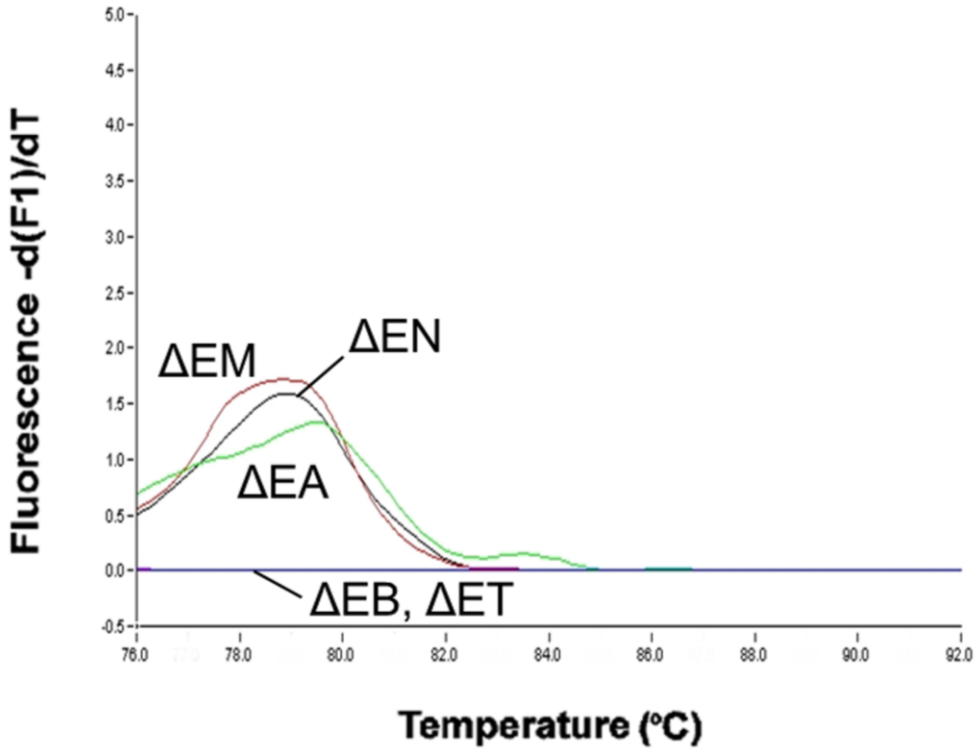


Fig. 2-4. Melting-curves of five DNA samples which were combined with different four species by real-time polymerase chain reaction assay. ΔEA, mixed DNA without *E. acervulina*; ΔEB, mixed DNA without *E. brunetti*; ΔEM, mixed DNA without *E. maxima*; ΔEN, mixed DNA without *E. necatrix*; ΔET, mixed DNA without *E. tenella*.

SUMMARY

To detect five different chicken *Eimeria* species, the SYBR Green-based real-time polymerase chain reaction (PCR) assay for the diagnosis of field-isolated parasites by using their individual species-specific primer sets was applied. The primer sets were originally designed for *Eimeria acervulina*, *E. brunetti*, *E. necatrix* and *E. tenella* based on the sequence of the internal transcribed spacer 1 region of ribosomal DNA, whereas the primer sets for *E. maxima* were derived from sequences reported previously. The detection limit of these assays was defined at 10^2 or 10^1 oocysts depending on species. Melting curves from the real-time PCR assay showed that each species has a single peak and specific melting temperature value. Fecal samples from 32 poultry farms where coccidioses were endemic, were examined using this assay. The data showed that *E. brunetti* was found in 21 farms, *E. maxima* and *E. necatrix* in 16 farms, *E. tenella* in 12 farms, and *E. acervulina* in 8 farms. This survey revealed that *E. brunetti* was highly prevalent in Japan. This technique is not only easy and rapid but also possible to detect *Eimeria* species specifically, and thus, it can be a valuable tool for the diagnosis of chicken coccidiosis.

CHAPTER III

Characterization of *Eimeria brunetti* isolated from a poultry farm in Japan

INTRODUCTION

Chicken coccidiosis induced by *Eimeria* parasites causes huge economic losses to intensive poultry industries worldwide (53). Seven species of *Eimeria* have been reported to infect chickens so far. Among these species, *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix* and *E. tenella* are highly pathogenetic, causing production losses in industry due to clinical or subclinical coccidiosis. Generally, the poultry industry uses anticoccidial drugs or live anticoccidial vaccines as prophylactic therapies to prevent and control the disease. However, there are some concerns about drug residues in poultry products and a strong consumer desire to ban drugs from animal feeds. As a result, the demand for products derived from organic chickens, without feeding of chemicals or antibiotics, tends to increase in Japan as European countries. It is estimated that live anticoccidial vaccines in Japan are applied to one hundred million chickens per year (61). Three live coccidiosis vaccines have been registered and sold in Japan. Trivalent TAM™ (Nisseiken Co., Ltd., Tokyo, Japan) contains precocious attenuated strains of *E. acervulina*, *E. maxima* and *E. tenella*. Monovalent Neca™ (Nisseiken Co., Ltd.) contains a precocious attenuated strain of *E. necatrix.* Pentavalent Paracox®-5 (MSD Animal Health, Milton Keynes, U.K.) which contains precocious attenuated strains of *E. acervulina*, *E. maxima* (2 strains)*, E. mitis* and *E. tenella* has been imported from the UK. However, none of these products contain *E. brunetti* because its occurrence in chicken flocks has been regarded to be less intensive in Japan to date. No oocysts of *E. brunetti* were detected on several national surveys of broiler flocks in 1970's (43-45). In 1990, two strains of *E. brunetti* were first isolated from poultry farms in Hokkaido and Kumamoto. This observation suggested a wider prevalence of *E. brunetti,* as these prefectures were the northernmost and southernmost parts of Japan, respectively (40). No further surveys or cases have been reported since 1991. *E. brunetti* infections have been recently diagnosed in many cases from samples submitted to the author's laboratory from commercial chicken farms (24). This observation leads to a necessity of revision in capability of *E. brunetti* as a pathogen of coccidiosis in Japan in contrast with previous understandings. However, characteristics of the Japanese isolate of *E. brunetti* have not been evaluated yet. For the verification, the characteristics of a Japanese strain of *E. brunetti* on its pathogenicity and sensitivity to drugs are described in this chapter.

MATERIALS AND METHODS

Parasite. The isolate of *E. brunetti*, as the Nb strain, was derived from a single oocyst isolated from feces of breeder chickens in Miyazaki prefecture and has been maintained in the author's laboratory using specific-pathogen-free (SPF) layer chickens. The correct species identification and purity of the strain were confirmed by PCR employing an assay directed towards the ITS-1 (24).

Evaluation of dose-effect. *E. brunetti* was inoculated in a group of ten 35-day-old SPF chickens by oral inoculation with 1×10^2, 1×10^3, 1×10^4 or 1×10^5 sporulated oocysts/bird. The birds were raised in wire-floored cages for 7 days and then necropsied. Pathogenicity was evaluated using growth ratio, mortality and intestinal lesion scores of the chickens. The growth ratio was obtained individually with the following formula: (body weight at termination − body weight at initiation)/ body weight at initiation × 100. Intestinal lesion scores were graded according to Johnson's method (22). A lesion score indicated the degree of pathogenicity of *E. brunetti* with a 0 to +4 scale on 7 DPI as follows: 0, no gross lesions; +1, slight color change of intestinal wall; +2, greyish intestinal wall, thickened intestine in the lower portion or flecks of salmon-colored materials from the intestine; +3, intestinal wall thickened, a blood-tinged catarrhal exudate or soft mucus plugs; +4, extensive coagulation necrosis of the mucosal surface or a dry necrotic membrane.

Evaluation of manifestation over time. The oocysts excreted in feces were counted daily from 4 to 11 days post-inoculation (DPI). To observe the developing stages of the infection with *E. brunetti*, 35-day-old SPF chickens were given 1×10^5 oocysts/bird, and then intestinal tissues were taken daily from two of these chickens from 1 to 8 days. The tissues were paraffin embedded, and sections were stained with hematoxylin and eosin. The density of the population of parasites was ranked at the observation site as 0 (no organisms), +1 (less than 10% of host cells are infected) and +2 (more than 10% of host cells are infected).

Evaluation of drug resistance. To evaluate the drug sensitivity, ten 14-day-old SPF chickens were orally inoculated with or without 1×10^5 sporulated oocysts/bird, kept in wire-floored cages for 7 days and then necropsied. In addition to medicated group with each anticoccidial drug, one unmedicated infected and one unmedicated uninfected (control) groups were tested (Table3-1). As sulfa anticoccidial drugs, diaveridine (19.2 ppm) / sulfaquinoxaline (76.8 ppm) obtained from Sumika Enviro-Science Co., Ltd. (Nishinomiya, Japan) and ormetoprim (75 ppm) / sulfamonomethoxine (225 ppm) obtained from Meiji Seika Pharma Co., Ltd. (Tokyo, Japan) were used in the drinking water on days 0 to 3 after infection. As ionophorous polyether anticoccidial drugs,

salinomycin (50 ppm) obtained from Nichiku Yakuhin Kogyo Corporation (Ayase, Japan) and lasalocid (75 ppm) obtained from Scientific Feed Laboratory Co., Ltd. (Tokyo, Japan) were used in the feed continuously. The sensitivity against anticoccidial drugs is represented by the anticoccidial index (ACI), the optimum anticoccidial activity (%OAA) and the anticoccidial sensitivity test (AST). ACI is calculated as follows: ACI = (relative growth ratio + survival ratio) − (lesion index + oocyst index). The strain was considered sensitive if the ACI was >161, partially resistant if the ACI was 121 to 160 and resistant if the ACI was <120 (33). %OAA is calculated as follows: %OAA = (GSR of drug group – GSR of non-drug group) / (GSR of uninfected control group – GSR of non-drug group) × 100. GSR used in the formula is calculated as follows: GSR = cage weight at termination / cage weight at initiation. A strain was deemed resistant if the %OAA was ≤50%, partially resistant if the %OAA was 51 to 74%, and sensitive if the %OAA was ≥75% (8). AST is calculated as follows: AST = 100 – (mean lesion score of drug group / mean lesion score of none-drug group × 100). AST of 0 to 30%, 31 to 49% and at least 50% indicate resistant, partially resistant and sensitive, respectively (48).

Animal care and use. The experiments presented here were carried out according to the protocols of Animal Care and Use Committee of Nippon Institute for Biological Science (Tokyo, Japan) in accordance with Regulation of Animal Experimentation of Nippon Institute for Biological Science.

Statistics. The mean values for the individual chickens, concerning growth ratio were used for statistical analysis by student's t-test to compare an uninfected control group or a none-drug group to other groups. The Mann-Whitney U test was utilized to evaluate the differences of lesion score of intestine between an uninfected control group or a none-drug group and other groups.

RESULTS

Experimental infection in chicken. Experimentally infected chickens showed a great reduction in their body weight gain which is correlated with the dose of oocysts (Table 3-2). There were 3 mortalities in the group that was infected with 1×10^5 oocysts. Despite showing the great reduction of body weight gains, the intestinal lesions in the infected chickens were rather mild. Typical lesions were found in the rectum, including slight swelling, a color change into whitish-orange and formation of a bellows-like-shape with folded circular rings along the tube. In the other intestinal compartment, the only abnormality observed was a slight paling of the serosal surface. There was no correlation between the output patterns and the number of inoculated oocysts. Oocysts were seen in droppings from 5 to 10 DPI among almost of all groups (Table 3-3). This observation indicates that prepatent and patent periods of the strain were about 5 and 6 days, respectively.

Histopathological study in infected chicken. Histological observation of the developing stages of the parasite is summarized in Table 3-4, showing the infection rate of host cells and stage transition of parasites on each day after inoculation. Mature (arrowheads) and immature (arrows) schizonts were observed in the mucosal cells and subepithelial zone of the disrupted villi of the jejunum at 4 days after infection (Fig. 3-1). Mature female gametocytes (arrowheads) and mature male gametocytes (arrow) were observed in the mucosal cells and sub-epithelial zone of the villi of the rectum at 6 days after infection (Fig. 3-2). The number of female gametocytes was much larger than that of male gametocytes. The onset of parasite development occurred in the upper portion of the small intestine, and subsequently parasites were found in the lower tract. The parasite density in the jejunum of *E. brunetti*-infected SPF chickens was the highest among the locations examined, although the gross lesions were not severe (lesion scores; 1.3 to 1.9 in Table 3-2).

Evaluation of drug resistance. The ACI, %OAA and AST of medicated and unmedicated infected groups are presented in Table 3-5. Of the 4 medicated infected groups, only the relative growth ratio of the salinomycin group was significantly lower than uninfected control group. Significant differences of the relative growth ratio and the lesion index were also shown between none-drug group and other groups. According to ACI and %OAA, the drug sensitivity of *E. brunetti* against salinomycin was shown to be partial resistant. Diaveridine / sulfaquinoxaline, ormetoprim / sulfamonomethoxine and lasalocid were effective so that the *E. brunetti* infection was almost completely blocked. The drug sensitivities of *E. brunetti* against these three drugs were shown to

be sensitive by ACI, %OAA and AST. Salinomycin was also judged to be as sensitive by AST as other three drugs.

DISCUSSION

Severe lesions were often observed in the field chickens infected with *E. brunetti* (13, 32). However, such lesions did not develop in the intestines of SPF chickens under the experimental conditions. In Johnson's paper, it also was described that the severe infections were seldom seen in experimental infections and *E. brunetti* was the most difficult of all species to score (22). This may be due to differences in the susceptibility between commercial field chickens and SPF chickens to *E. brunetti* infection. According to observations in SPF chickens, the organisms first colonized the upper intestine and moved down to the lower portion. Although the accumulation of parasites was higher in the jejunum than in the other compartments, the major parasitized site has been generally considered to be the lower small intestine. Otherwise, the legion may be associated with *Clostridium perfringens* infection. Because necrotic enteritis and coccidiosis often occur concurrently in a field flock (46). Gross lesions resulting from co-infection become much severe than single-infection with the individual pathogen.

Contrary to previous reports (43-45), a recent study revealed that *E. brunetti* was highly prevalent in Japan (24). In this chapter, *E. brunetti* infections with a Japanese isolate cause similar mortalities and suppression of body weight gain to those caused by other tested isolates was shown (13, 32). Thus, it is suggested that the Japanese Nb strain has high pathogenicity similar to strains isolated overseas. Nevertheless, it has been still unknown how *E. brunetti* might affect the productivity of poultry farms in Japan. Therefore, evaluation of the involvement of *E. brunetti* in field coccidiosis cases is highly recommended in detail. The drug sensitivity of *E. brunetti* was also found to be susceptible against diaveridine/sulfaquinoxaline, ormetoprim/sulfamonomethoxine and lasalocid because of the results of ACI, %OAA and AST. These drugs could prevent the infection and the disease by *E. brunetti*. Although the strain was slightly resistant to salinomycin judged by ACI and %OAA, the coccidiostatic drug is generally thought to be slightly ineffective against *E. brunetti* (34).

The reasons for the recent increase in the diagnosis of *E. brunetti* infections in Japan remain unclear. Improvements of the techniques for the detection of *E. brunetti* in field samples based on PCR, rather than morphological diagnosis, could account for this, at least in part. Traditional classification techniques use criteria, such as oocyst size, parasitic site in the intestine, gross lesion figures and variations in prepatent time. It has been well documented that several species overlap in their metric values or patterns of these criteria, leading to the confusion in the species-determination process, especially when mixed infections are present. It is suspicious that the clinical signs attributed to *E. brunetti* infections in breeder pullet flocks have been misdiagnosed as

those of *E. necatrix* or necrotic enteritis by *C. perfringens*. Actually, the single detections of *E. brunetti* by PCR in considerable numbers of samples which field veterinarians had diagnosed as *E. necatrix* infection were often experienced in the author's laboratory (data not shown).

The widely accepted idea on the prevalence of *E. brunetti* was basically derived from information supplied from field surveys in 1970's (43-45). These surveys were performed mainly on broiler flocks. However, *E. brunetti* could not be spread only in broiler flocks consisting of young chickens but also in breeder and layer flocks consisting of old chickens (32). Therefore, the epidemiology of *E. brunetti* should be evaluated using surveys from various areas, farms and flocks in a balanced manner. In fact, a recent survey demonstrated that *E. brunetti* was detected more frequently in samples from bleeder farms than those from broiler farms (24).

From the recent and present findings, the broad distribution and pathogenicity of *E. brunetti* in Japan has been clarified. This result indicates that an appropriate plan to confront the risks associated with outbreaks of this species is required. The fact that *E. brunetti* outbreaks are usually found in older chicken flocks means that using coccidiostatic drugs that are normally applied extensively to broilers would not be suitable for this species. Vaccination would be the best method to mitigate this risk, but appropriate vaccines effective against this species are not available in Japan. Therefore, the development of an effective *E. brunetti* vaccine that is safe for the use in the poultry industry in Japan is urgently required.

Table 3-1. Outline protocol for evaluation of drug resistance.

Drug	Group	Route of drug administration	*Eimeria* infection (1×10^5 oocysts)
Sulfa anticoccidial drug	Diaveridine (19.2 ppm) Sulfaquinoxaline (76.8 ppm)	In water	Yes
	Ormetoprim (75 ppm) Sulfamonomethoxine (225 ppm)	In water	Yes
Ionophorous polyether anticoccidial drug	Salinomycin (50 ppm)	In feed	Yes
	Lasalocid (75 ppm)	In feed	Yes
None	Infected control	NA	Yes
	Uninfected control	NA	None

Table 3-2. The pathogenicity of the Japanese Nb strain of *E. brunetti*.

Number of oocysts inoculated per bird	Mortality rate	Mean growth ratio[a] (relative growth ratio)	Mean lesion score	
			Jejunum, ileum	Rectum
1×10^2	0/10	38.7 ± 8.3 (0.89)	1.7[b]	0.8[b]
1×10^3	0/10	22.2 ± 8.7[b] (0.51)	1.3[b]	0.6
1×10^4	0/10	9.3 ± 8.2[b] (0.21)	1.3[b]	1.1[b]
1×10^5	3/10	-4.2 ± 4.9[b] (-0.1)	1.9[b]	1.4[b]
None	0/10	43.7 ± 7.4 (1.0)	0	0

[a](body weight at termination – body weight at initiation) / body weight at initiation ×100.

[b]Values with superscripts indicate significant difference ($P < 0.05$) from uninfected control group.

Table 3-3. Oocyst output of the Japanese Nb strain of *E. brunetti*.

Number of oocysts inoculated per bird	Days after inoculation							
	4	5	6	7	8	9	10	11
1×10^2	-	4.7[a]	7.4	7.4	7.2	6.1	5.3	-
1×10^3	-	-	7.6	7.6	7.2	5.1	-	-
1×10^4	-	4.2	7.7	8.1	7.2	6.1	5.8	-
1×10^5	-	4.1	7.2	7.7	7.3	5.3	-	-
None	-	-	-	-	-	-	-	-

[a]Oocyst output (oocysts/g feces) is shown by the base-10 logarithm. Estimated detection limit is 100 oocysts.

Table 3-4. Developing stages of the Japanese Nb strain of *E. brunetti* present in intestinal tissues.

Day after inoculation	Mean scores of organism density[a] (Types of emerging organisms[b])				
	Duodenum	Jejunum	Ileum	Cecum	Rectum
1	0	0.5 (F)	0	0	0
2	0	1 (F)	0	0	0
3	0.5 (F)	1.5 (F)	0	0	0
4	0.5 (F > S)	2 (S)	2 (S)	1.5 (S)	1 (S)
5	2 (S)	2 (S > G)	2 (S > G)	2 (S > G)	2 (S > G)
6	0.5 (G)	1 (G > S)	1 (G > S)	1.5 (G)	1.5 (G)
7	0.5 (G)	1 (G)	1.5 (G)	1.5 (G)	1 (G)
8	0	0	0.5 (G)	1 (G)	0.5 (G)

Two birds were sampled daily after inoculation with 1×10^5 oocysts per bird.

[a]0, no organisms; 1, less than 10% of host cells are infected; 2, more than 10% of host cells are infected.

[b]F, first generation schizonts; S, other generation schizonts or immature gametocytes; G, mature gametocytes.

Table 3-5. Sensitivity of the Japanese Nb strain of *E. brunetti* against anticoccidial drugs[a].

Group	Relative growth ratio	Survival ratio	Lesion index[b]	Oocyst index[c]	Anticoccidial index[d] (ACI)	% Optimum anticoccidial activity[e] (%OAA)	Anticoccidial sensitivity test[f] (AST)
Diaveridine Sulfaquinoxaline	96.51[g]	100	0[g]	0	197[S]	95.8[S]	100[S]
Ormetoprim Sulfamonomethoxine	94.10[g]	100	0[g]	0	194[S]	94.1[S]	100[S]
Salinomycin	49.18[gh]	100	1[g]	5	143[PR]	54.3[PR]	93.8[S]
Lasalocid	96.88[g]	100	0[g]	0	197[S]	97.3[S]	100[S]
Infected control	-4.3[h]	100	16[h]	40	40	NA	NA
Uninfected control	100[g]	100	0[g]	0	200	NA	NA

[a]Each group contains ten birds which were inoculated with 1×10^5 oocysts per bird.
[b]The total amount of lesion score of ten birds.
[c]The index based on the ratio (%) of OPG to the none-drug group. 0~1%, 0; 1~25%, 5; 26~50%, 10; 51~75%, 20 and 76~100%, 40.
[d]ACI = (relative growth ratio + survival ratio) – (lesion index + oocyst index).
[e]%OAA = (GSR of drug group – GSR of non-drug group) / (GSR of uninfected control group – GSR of non-drug group) × 100. GSR; cage weight at termination / cage weight at initiation.
[f]AST = 100 – (mean lesion score of drug group / mean lesion score of none-drug group × 100).
[g]Significant difference ($P < 0.05$) from none-drug group.
[h]Significant difference ($P < 0.05$) from uninfected control group.
[S]Sensitive, [PR]Partially resistant.

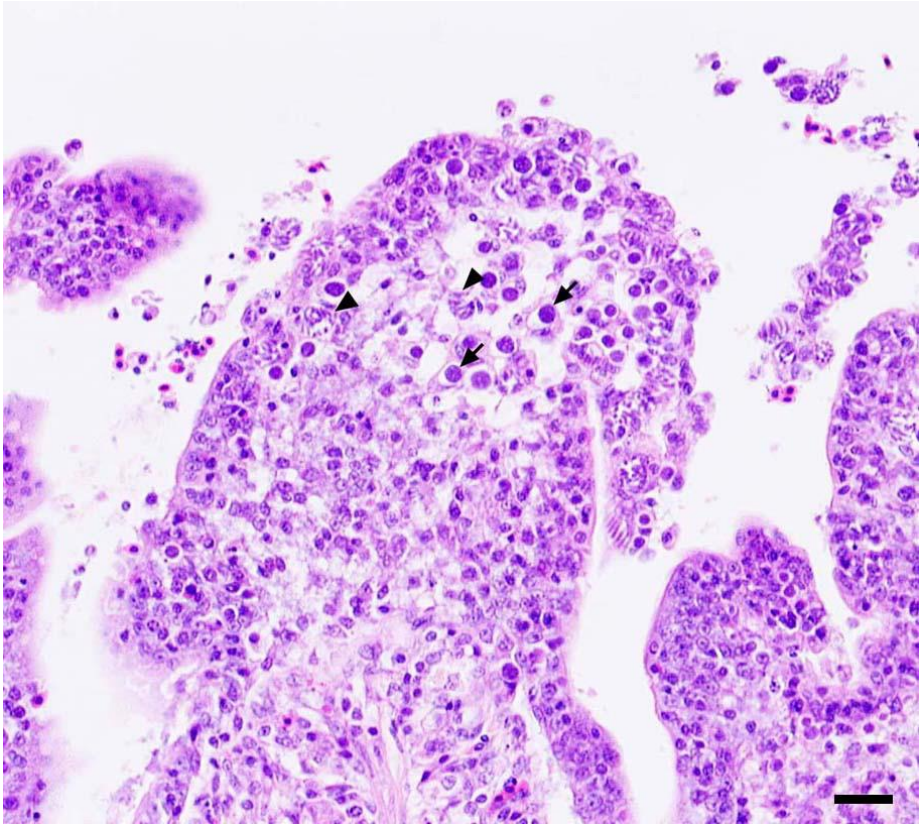


Fig. 3-1. Microscopic findings of the jejunum of 4 DPI. Mature (arrowheads) and immature (arrows) schizonts are observed in the mucosal cells and subepithelial zone of the disrupted villi. HE stain. Bar = 20 μm.

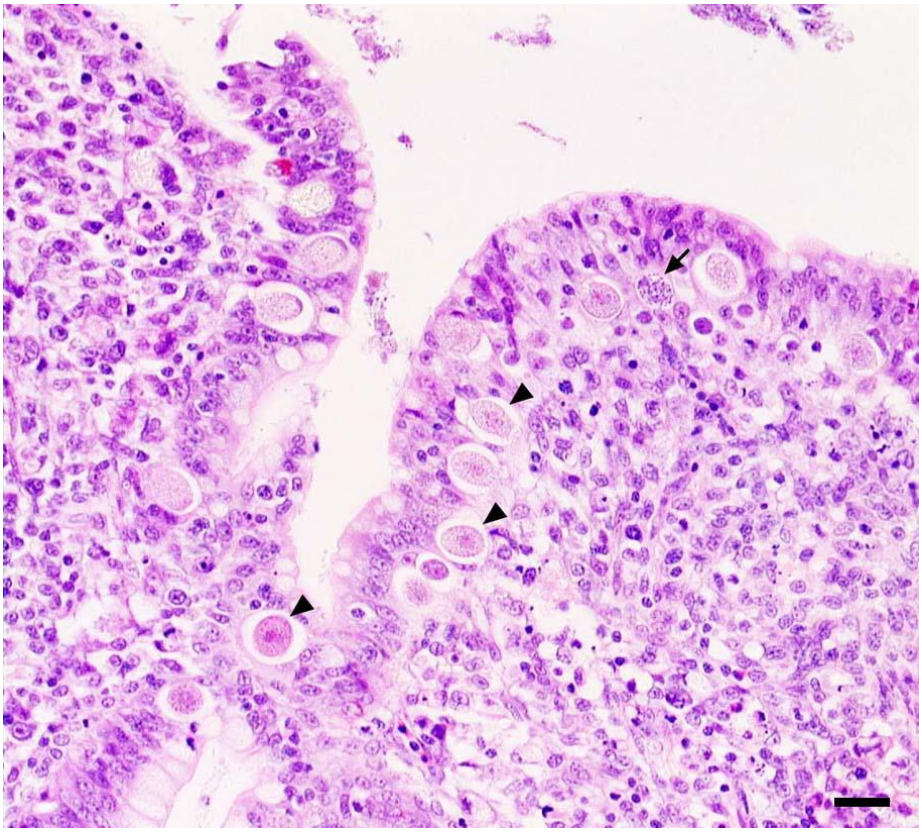


Fig. 3-2. Microscopic findings of the rectum of 6 DPI. Mature female gametocytes (arrowheads) and mature male gametocytes (arrow) are observed in the mucosal cells and subepithelial zone of the villi. The number of female gametocytes is much larger than that of male gametocytes. HE stain. Bar = 20 μm.

SUMMARY

None of anticoccidial vaccines (Trivalent TAM™, monovalent Neca™ and imported pentavalent Paracox®-5) commercially available in Japan contain *Eimeria brunetti*, which has not been regarded as a cause of coccidiosis, because of its low prevalence. However, the author's study has recently revealed the evidence of a high nationwide prevalence of this species. In this chapter, the characteristics of *E. brunetti* which have never been clearly defined in Japan are described. Mortality rates and other disease characteristics caused by the strain (Nb strain) were similar to those reported previously in other studies. Despite great reduction of body weight gains among groups infected with over 1×10^3 oocysts, the intestinal lesions in the infected chickens were rather mild compared to those reported in previous studies. Sulfa drugs and lasalocid were effective so that the *E. brunetti* infection was almost completely blocked. Consequently, it is suggested that *E. brunetti* has a certain range of diversity in pathogenicity and those in Japanese Nb strain is strong enough to cause clinical coccidiosis.

CONCLUSION

The identification of *Eimeria* species which infect domestic animals has been exclusively based upon a morphological approach of observing oocyst appearances in feces; size, shape and color. However, the discrimination of *Eimeria* to the species level can be difficult, unreliable and subjective in spite of professional identification skills due to overlapping morphological features compounded by intra-species variation. Samples derived from domestic animals infected by multiple *Eimeria* species must exacerbate this problem much more. In response, new user-friendly molecular methods for species-specific detection of bovine and chicken *Eimeria* are required and can complement classical clinical and epidemiological applications. The thesis also describes new insights about *Eimeria* species of cattle and chicken, which are obtained during the process to develop the molecular methods.

Chapter I of this thesis describes nucleotide sequences of the ITS-1 region from the ribosomal RNA locus of six bovine *Eimeria* species; *E. alabamensis*, *E. auburnensis*, *E. bovis*, *E. cylindrica*, *E. ellipsoidalis* and *E. zuernii*. The results of analysis conducted for 21 ITS-1 sequences to define inter- and intra-species variation among 18 *Eimeria* field collections and analysis of the phylogenetic relationship of each sequence and *Eimeria* species are also described. These sequences have been used to undertake the most advanced phylogenetic analysis and develop the first diagnostics PCR assays for *Eimeria* species that infect cattle. The nucleotide sequences of the ITS-1 of highly pathogenic two species (*E. bovis* and *E. zuernii*) formed a monophyletic group away from other bovine *Eimeria* species in the phylogenetic analysis. The ITS-1 regions were found to show sufficient inter-species variations for the development of reliable PCR diagnostics to identify species of bovine *Eimeria* in this study. It is the first attempt for the identification and differentiation of bovine *Eimeria* parasites at the genomic level. The PCR assays developed in this study could detect and identify six species of bovine *Eimeria* in species-specific manner. It is expected that the assay is more objective and reliable than the classical method depending on the morphological features of parasites.

Chapter II deals with the development of a SYBR Green-based real-time PCR assay for the quantitative diagnosis of field-isolated parasites using individual species-specific primer sets to detect five different *Eimeria* species of chicken; *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix* and *E. tenella*. Real-time PCR offers the advantage of avoiding post-PCR processing steps, saving enormous time and laboratory labor to accomplish diagnostic examinations compared to conventional PCR. Application of hybridization probe-based real-time PCR for *E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella* was already reported, whereas SYBR Green-based real-time PCR assay for any chicken

Eimeria species had not been reported. This study represented a first attempt to complete SYBR Green-based real-time PCR for chicken *Eimeria* species. The sensitivity was slightly superior to an authentic fecal examination which shows a detectable level over 100 oocysts per 1g feces. *Eimeria brunetti* was found in 21 farms examined by the PCR assay, 20 of which came from breeder and layer farms on this survey throughout Japan. It is the first study to produce clear evidence that *E. brunetti* was highly prevalent in Japan.

Chapter III describes the characterization of a Japanese field strain of *E. brunetti*, defining its pathogenicity and sensitivity to drugs for the first time. Many diagnostic samples submitted recently to the author's laboratory from commercial chicken farms have been found to contain *E. brunetti*, despite the presence of *E. brunetti* in Japan long being regarded as scarce. This report suggests that *E. brunetti* may be common throughout Japan and prompts the reassessment of *Eimeria* species occurrence across the country. Clear reasons for the recent increase in the cases of *E. brunetti* infections in Japan remain unclear. Technical improvement for the detection of *Eimeria* based on PCR could account at least in part for the increase in the number of cases detected. Therefore, morphological and molecular differentiation methods should be combined with each other to gain objective results in epidemiological surveys and studies. It is desirable that a PCR-based technique will provide new epidemiological data to reveal potential problems such as the example of *E. brunetti* prevalence in Japan and thus it helps to formulate strategies for controlling the parasites as quickly as possible.

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SUMMARY

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