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RESEARCH ARTICLE

Comparison and analysis of phylogenetic relationship based on internal transcribed spacer 1, ITS-1 fragment of *Eimeria tenella* and *Eimeria acervulina* from broiler chicken in Korea

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Özet

You MJ. Kore'deki broyler tavuklarda *Eimeria tenella* ve *Eimeriaa cervulina*'nın internal transcribed spacer 1, ITS-1fragmentine dayalı filogenetik analizi ve karşılaştırılması.

Amaç: Bu çalışmanın amacı Kore'de kanatlıları infekte eden *Eimeria tenella*, *Eimeria acervulina*'nın tespit edilmesini sağlayacak basit bir PZR gerçekleştirmek ve aynı ve komşu türler ile filogenetik ilişkisini belirlemektir.

Gereç ve Yöntem: İki kanatlı *Eimeria* türünün (*E. tenella* ve *E. acervulina*) ribosomal RNA genlerinin internal transcribed spacer 1 (ITS-1) bölgelerinin sekans analizleri yapıldı ve sahadan elde edilen örneklerle bunlar arasında filogenetik ilişki analiz edildi.

Bulgular: Kore'deki *E. tenella* ve *E. acervulina*'ların 273-bp ve 147-bp uzunluktaki sekansları kısmi nükleotid sekanslarını kullanılarak elde edilen filogramlar ile benzerlik gösterdi. Aynı türler içinde sekanslar *E. tenella* için %98 ve *E. necatrix* için %89 homologdur. Aynı türler içinde homoloji *E. acervulina* için %97, *E. maxima* için %77 ve E. mitis için %76 olarak belirlendi. Her bir Eimarei türünün ITS bölgesi yeterli türler-arası dizi varyasyonuna sahipti ve kullanılan primer Kore'deki herbir isolattan amplifikasyon yapmak için yeterli idi.

Öneri: Bu çalışmada kullanılan moleküler metot benzer *Eimeria* türleri arasında ayrım yapmak için kullanışlıdır ve kanatlı coccidial enfeksiyonlarının epidemiyolojisi ve teşhisi için faydalı metodlar sunmaktadır.

Anahtar kelimeler: *E. tenella*, *E. acervulina*, ITS-1, kanatlı

Abstract

You MJ. Comparison and analysis of phylogenetic relationship based on internal transcribed spacer 1, ITS-1 fragment of *Eimeria tenella* and *Eimeria cervulina* from broiler chicken in Korea.

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Aim: The objective of the present study is the simple PCR assay that permits the detection of the *Eimeria tenella*, *Eimeria acervulina* that infect domestic fowl in Korea and making phylogenetic relation with in the same and neighboring species.

Materials and Methods: The internal transcribed spacer 1 (ITS-1) region of ribosomal RNA genes of two poultry *Eimeria* species; *E. tenella* and *E. acervulina* were sequenced and analyzed the phylogenetic relationship among them from field isolates.

Results: About the 273-bp and 147-bp sequence of ITS-1 of *E. tenella* and *E. acervulina* in Korea were similar reflected in the phylogram constructed using the partial nucleotide sequences. In case of *E. tenella* for homology searching the sequences among the same species was 98% and with *E. necatrix* was 89%. In case of *E. acervulina* sequence similarity within the same species was 97%, whereas the similarity *E. maxima* and E. mitis were 77% and 76%, respectively. ITS-1 region of each *Eimeria* species had sufficient inter-specific sequence variation and the primer is sufficient to amplify each target Korean isolates.

Conclusions: The molecular method examine in the present study appears useful for discriminating among similar *Eimeria* species providing useful methods for diagnosis and epidemiology of avian coccidial infection.

Keywords: *E. tenella*, *E. acervulina*, ITS-1, chicken

Introduction

Eimeria infection causes severe economical loss due to extensive destruction of the enteric epithelium, resulting in reduction of food conversion, body weight gain and egg production, thereby also increase morbidity and mortality (Morris and Gasser 2006, Parket et al 2008) Commonly seven species of *Eimeria* that infect chicken invade the intestinal epithelium usually in region specific manner (Morris and Gasser 2006). Chickens may be simultaneously infected with multiple species of *Eimeria* in intensively reared poultry farms (Morgan et al 2009) . In Korea the most common prevailing *Eimeria* are *E. tenella* and *E. necatrix* (Lee et al 2010). Epidemiological examination and specific diagnosis of *Eimeria* infections would play an important role in the prevention and control of coccidiosis. Most epidemiological studies of *Eimeria* infection on broiler farm have been carried out by morphological and biological features (Allen et al 2005) species specific diagnosis needs for accurate diagnosis and epidemiological studies.

E. tenella, *E. acervulina*, *E. maxima* and *E. necatrix* occur frequently in Korea (Fernandez et al 2003). Species identification has been classically based on observation of oocyst shape, size, color, prepatent period, location of the lesions in the intestine, sporulation time, and characteristics of the endogenous stages in the intestinal mucosa. Hence, coccidiosis can best be diagnosed through immediate necropsy, as attempts to identify characteristic lesions in birds that have been dead for 1 hour or longer are frustrated by the postmortem changes in intestinal mucosa. The entire intestinal tract should be examined microscopically, observing for special diagnostic characteristics, such as clusters of the large schizonts of *E. necatrix*. However, oocyst size and shape are less useful diagnostic characteristics in chickens than once thought, because of the extensive overlapping in size. Lesion scoring (Johnson and Reid 1970) droppings score (Morehouse and Baron 1970) and histopathology methods (H&E or other common histologic stains) require highly trained personnel, and are also limited by further overlap of characteristics among different species. In recent years, more emphasis has been placed on biochemical and physiologic identification, including electrophoresis of metabolic enzymes and PCR (Tsuji et al 1997).

Recently, Ribosomal internal transcribed spacer-1(ITS-1) has been used in coccidiosis diagnosis (Dunn and Keymer 1986, Schnitzler et al 1999, Fernandez et al 2003, Bhaskaran et al 2010) Eukaryotic cells contain ITS, which refers to a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. Read from 5' to 3', this polycistronic rRNA precursor transcript contains the 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S, rRNA, ITS2, 26S rRNA and finally the 3'ETS.

The ITS region is widely used in molecular phylogeny and taxonomy because it is easy to amplify from small quantities of DNA, and has a high degree of variation between closely related species (Bornstein et al 2008, Samarasinghe et al 2008).The present study describes the simple PCR assay that permits the detection of the *E. tenella* , *E. acervulina* that infect domestic fowl in Korea and making phylogenetic relation with in the same and neighboring species.

Materials and Methods

Parasites, animals and experimental infections

Farm droppings randomly from 200 commercial broiler chickens were collected from Jeonbuk-Namwon area of Korea. Oocysts were differentiated in the 2% potassium dichromate solution, stored at 4°C. Re-infection was done in commercial chicken broilers' (Ibaika) at the age of day 14. Oocyst was check by Mc Master Method, diluted at 1.0 X 104 and inoculated in 14 days chick and checking after 10 days.

Oocyst separation and clinical infection

To observe the oocyst in fecal sample saturated saline floating method was carried out. Sample was incubated for at 27°C for 7 days in saturated saline solution and differentiated 2% dichromate solution (Allen and Fetterer 2002). Differentiated oocysts removed and $1.0x10^4$ oocysts were diluted for reinfection. Bleeding caused for autopsy at 5-7 days to evaluate intestinal hemorrhage.

Rate of infection and fecal discharge

Differentiated oocysts diluted to $1.0x10^4$ were infected 4 groups each of which containing 5 birds. Fecal discharge collected from 5 to 10 days. 3-8 days after infection duodenum and cecum intestinal area were recovered and feces were washed with PBS. Recovered in each cell scraper scraping organs were collected. Each sample collected after grinding through a glass bead.

Identification by PCR (ITS1)

Samples containing oocyst were processed for DNA extraction as described previously with little modification (Zhao et al 2001, Fernandez et al 2003, Haug et al 2007). In our experiments oocyst wall broken by vortex in the presence of glass beads and recovered after one minute. Genomic DNA was extracted by adding same volume of mixture comprising phenol/chloroform/isoamyl alcohol (25:24:1) (Fluka, Japan). After adding an equal volume of isopropyl alcohol, genomic DNA was precipitated from the aqueous phase (Merck, Korea). The precipitated DNA was washed twice with 70% ethanol. Some of the intestinal samples did not have oocysts.

The epithelial lining from such intestines were scraped and genomic DNA extracted using as described before. The DNA extracted from sample was used to amplify the ITS-1 region (Haug et al 2007). Genus-specific primers were used for the amplification of ITS-1 region from all pathogenic *Eimeria* spp. of chicken. PCR was performed as mentioned before (Anita et al 2007).

Sequencing and comparing the results

The PCR products of *E. tenella* and *E. acervulina* DNA was recovered by using GFXTM PCR DNA and Gel Band Purification kit (GE Healthcare). The recovered amplified products of the PCR were cloned into T & A Cloning Kit / RC013 T & A Cloning Vector (RBC) and transformation was done in Competent Cells HITTM-DH5a High 108 (RBC). Competent cells for plasmid amplification in benign recovered through Plasmid DNA Purification Kit (Intron). Plasmids were recovered for sequencing, the DNA sequence shown in the NCBI nucleotide was analyzed by BLAST search nucleotide database using a nucleotide (http://www.ncbi.nlm.nih.gov/blast). Compared the results of multiple sequence alignment by Florence Corpet (http://bioinfo.genotoul.fr/multalin/multal.html).

Phylogenetic analysis of ITS-1 sequence

The ITS-1 sequences obtained from Korea isolates were matched with Australian, European, American and Chinese isolates available in the GenBank database. The sequence alignment was performed with ClustalW program, while phylogenetic and molecular evolutionary analyses were conducted with MEGA, version 4 (Tamura et al 2007). A maximum parsimony tree was created using the ITS-1 sequences from Korean isolates and other published sequences. The pairwise percentage identity was calculated using GeneDoc multiple sequence alignment editor, version 2.6.002.

Results

Oocyst and identification by pathology

Fecal sample was collected 5-7 days after bleeding from infected chickens and the oocyst size was average 34.02x24.45 μm (Figure 1. A). In postmortem (Figure 1. B) two enlarged cecum (Figure 1. C, D) was noticeable. Bleeding or morphological changes were observed in the head of cecum.

Identification of Eimeria spp. using PCR

Species-specific PCR was used to identify *Eimeria* spp. All intestinal samples, which were sent for postmortem analysis, were positive. A representative gel picture showing the PCR amplification products from one of the field isolates is provided in Figure 2 ITS-1 region from few of these samples was sequenced to ascertain the sequence homology between the isolates. 279-bp PCR amplicons represents of *E. tenella*; GenBank accession no. AF446074; lane 2, and 147-bp PCR amplicons corresponding to of *E. acervulina*; GenBank accession no. AF446055. Sample $1x10^4$ to re-infection with oocyst the EPG results shows that, other than a period of 6-8 days oocyst discharge less than 200,000. Between 6-8 days oocyst discharge were 960 000, 860 000, 560 000, respectively. Most oocysts were discharged into the 6-8 days between 6 and 7 showed up emissions (Figure 3). Appear bloody period and were similar. The ratio of the density of *E. acervulina* in the appendix is about 3.5 times than *E. tenella*. In the duode-

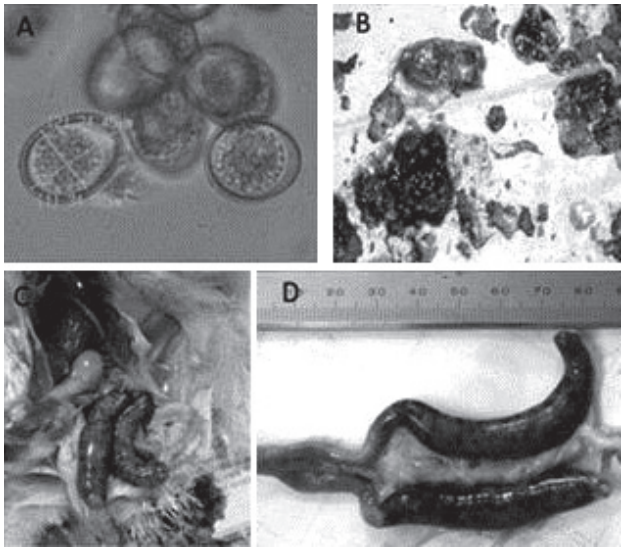

Figure 1. Oocysts morphological feature and clinical sign of *Eimeria* infection. A. Recovered oocysts by fecal flotation (×400); B. Fecal from 5 days of infection; C and D. Clinical sign of cecum from 5 days of infection.

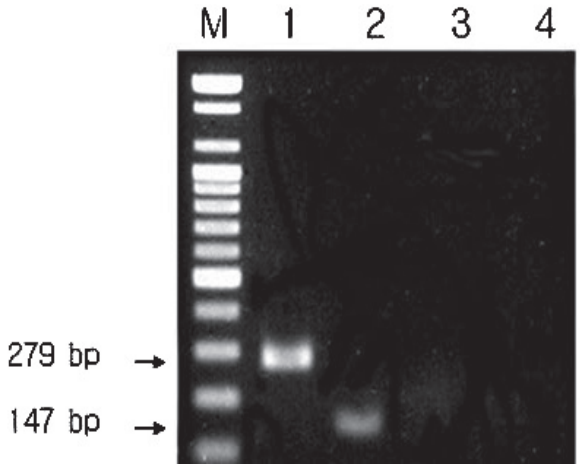

Figure 2. PCR amplicons of Eimeria spp. ITS-1 gene. Lane M, molecular size marker; lane 1, 279-bp PCR amplicons of *E. tenella*; GenBank accession no. AF446074; lane 2, 147-bp PCR amplicons of *E. acervulina*; GenBank accession no. AF446055; lane 3, 205-bp PCR amplicons of *E. maxima*; GenBank accession no. AF06594; lane 4, 270-bp PCR amplicons of *E. necatrix*; GenBank accession no. AF446070.

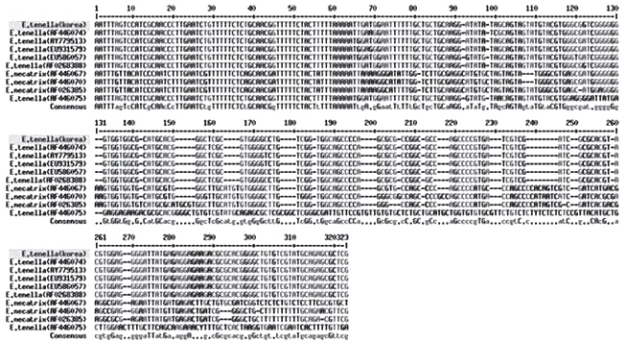

Figure 3. Average ratios of *E. tenella*/*E. acervulina* DNA density using PCR methods at 3-8th days PI.

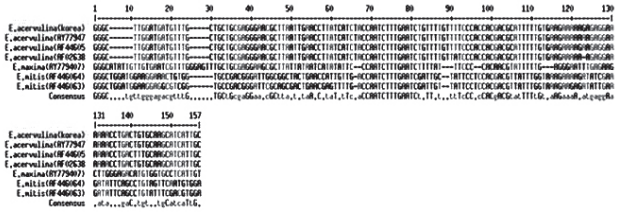

Figure 4. Comparison of the ITS-1 sequence of *E. tenella* from Korea and Eimeria spp. Additional accession numbers are shown. The sequences were retrieved from the GenBank database. The ITS-1 sequences listed are from *E. tenella* (this work, AF0268388, AF446074, EU931579, EU586057, AY779513 and AF446075) and *E. necatrix* (AF446067, AF446070 and AF446075).

num this was four times the rate of *E. acervulina* (Figure 4, 5). Therefore, a relatively friendly, but some parts showed that the infection takes place.

Sequence comparison and Phylogenetic analysis of ITS-1 sequence

A maximum parsimony tree was created using the ITS-1 sequences from Korean *Eimeria* spp. by comparing the available ITS-1 sequences of exotic isolates (Figure 6, 7). There was a clear species-wise clustering, irrespective of the geographical location, for all the ITS-1 sequences of *E. tenella*, and *E. acervulina* sequences formed two separate groups. Both *E. tenella* and *E. acervulina* infected in duodenum and cecum. But, there is a relative difference between their oocysts infection in duodenum and cecum. The 273-bp and 147-bp sequence of ITS-1 of *E. tenella* and *E. acervulina* in Korea strain were similar to that of *Eimeria* spp. from the GenBank database, and the phylogram constructed using the partial nucleotide sequences suggest similarity in *Eimeria* species. The similarity between *E. tenella* in Korea strain (FJ447468) and *E. tenella* (AF0268388, AF446074, EU931579, EU586057, AY779513) and *E. necatrix* (AF446067, AF446070, AF026385) were 98% and 89%, respectively. The similarity between *E. acervulina* in Korea strain (FJ447467) and *E. acervulina* (AF026384, AF446056, AY779476, AF446055) was 97%, whereas the similarity *E. acervulina* in Korea strain (FJ447467) and *E. maxima* (AY779407) and E. mitis (AF446064, AF446063) were 77% and 76%, respectively.

Discussion

Mainly distributed *Eimeria* species are *E. tenella*, *E. acervulina*, *E. maxima* and *E. necatrix*. This paper reported Korean local strain of chicken fecal *E. tenella* and *E. acervulina*. In this research protocol *Eimeria* spp were first identified by oocyst morphology, pathological sign and then conformed by PCR using ITS-1. The common species of the expected size (*E. tenella* 19.5 ~ 26.0 × 16.5 ~ 22.8 μm, *E. necatrix* 13.2 ~ 22.7 × 11.3 ~ 18.3 μm, *E. acervulina* 17.7 ~ 20.2 × 13.7 ~ 16.3 μm, *E. maxima* 21.5 ~ 42.5 × 16.5 ~ 29.8 μm) (Poultry science) and the size of *E. tenella* and *E. maxima* of our result were close to expected size.

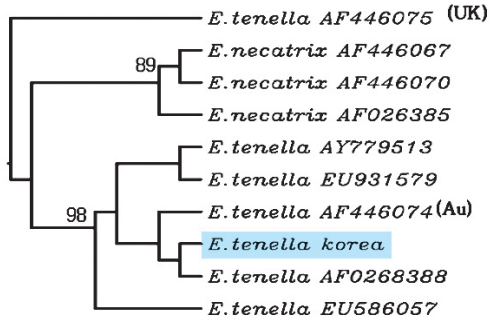

Figure 5. Comparison of the ITS-1 sequence of *E. acervulina* from Korea and Eimeria spp. Additional accession numbers are shown. The sequences were retrieved from the GenBank database. The ITS-1 sequences listed are from *E. acervulina* (this work, AF026384, AY779476, AF446055, and AF446056), *E. maxima* (AY779407) and E. mitis (AF446064 and AF446063).

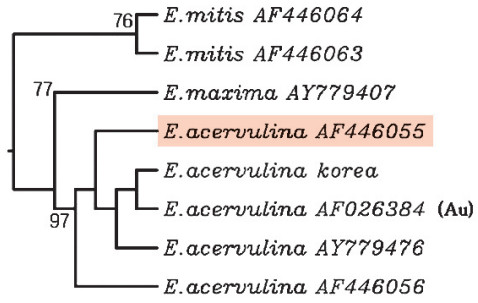

Figure 6. Phylogram of Eimeria tenella and other Eimeria spp. as inferred by neighbor joining analysis of partial nucleotide sequence from ITS-1. Additional accession numbers are shown.

Pathological lesion is seen in *E. acervulina* at the upper side of the duodenum, and in case *E. tenella* infection at the site of the appendix that has been reported. Doran and Farr (1962) reported *E. acervulina* infection of the proximal part of the 1 ~ 2 inches below the appendix infection takes place (Swinkels et al 2007). *E. tenella* showed the first and second generation asexual reproduction to be appeared in cecum (Daszak 1999) .

Yun et al (2000) and del Cacho et al (2004) are shown the condition of the infected cecum. *Eimeria* species appears the difference to depend on the location of infection (Tierney and Mulcahy, 2003).We collected cecum sample to confirm the extent of infection rate in 3-8 days.

For infection of *Eimeria*, Yun et al (2000) was carried out to 102-106 oocyst. In this paper, the result of infection in the case of $1x10^4$ oocysts of approximately 3 million, depending on chicken breeds were different. Swinkels et al (2007) shown *E. acervulina* infection is formed by experiments is $5x10^4$ oocysts, in the case of to infect one that the degree of immunity from the duodenum was measured on day 5 there were about 10 million oocysts. We experimentally infected at $1x10^5$, $1x10^4$. Oocyst discharged from fecal measured by the McMaster (Dunn and Keymer 1986, Haug et al 2007) per 1g contains. Approximately 48h after infection asexual reproduction takes place in most of cases and the invasion takes place before and after the 9th day (Del Cacho et al 2004).Therefore, in this experiments, starting with the first 5 days to 10 days oocysts was calculated. Most of the oocysts were discharged 6-8 days of infection (960,000, 860,000, and 560,000) (Figure 3), bloody stools were observed until days 5 to 7. Chicken breeds, ambient temperature, environmental stress may increase the condition. However, all coccidiosis oocysts emissions not appear for about a week.

We analyzed the part of sequence of *E. tenella* and *E. acervulina* (Figure 6-9). *E. tenella* Korea strain results were very similar to the kind of Australia. *E. tenella* (AF0268388, AF446074, EU931579, EU586057, and AY779513) showed a similarity of 98%. But UK strain of *E. tenella* AF446075 were some of the only similarity. In addition, *E. necatrix* (AF446067, AF446070, and AF026385) showed a similarity of 89%. *E. acervulina* of Korea strain have high similarities with Australia strain (AF026384, AF446056, AY779476, and AF446055) and showed that more than 97% similarity. *E. maxima* (AY779407) is 77%, E. mitis (AF446064, AF446063) showed a similarity of 76%. *Eimeria* of the chicken part of the ITS-1 sequence of the other animals did not indicate a similarity with *Eimeria*. (Bornstein et al 2008) using the ITS-1 were analyzed similarity of Isospora orlovi (Kalacha in Kenya) with Isospora orlovi (Dubai) and was compared with Isospora belli. Dubai species showed similarity of 84% with Isospora belli. This study confirmed the presence of *E. tenella* and *E. acervulina* from broiler chicken in Korea.

Conclusion

We confirmed that Korean strains appeared substantial similarity compared to ITS-1 of *Eimeria* spp. Thus the diagnosis of morphology, clinical sign, site of infection and molecular method examine in the present study appears useful for discriminating among similar *Eimeria* species.

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