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Molecular identification of *Eimeria* species of broiler chickens in Turkey*

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Summary: The objective of this study was to determine whether seven *Eimeria* species involved in chicken coccidiosis are present in Turkey and to assess their prevelance in commercial flocks. Litter and faecal samples were collected from 1110 broiler flocks housed in 817 farms (about 12% of all broiler farms in Turkey) between September 2006 and September 2007. Coccidian oocysts were found in 624 (56.2 %) of the samples examined. Species-specific polymerase chain reaction (PCR) and nested PCR tests targeting the internal transcribed spacer-1 (ITS-1) sequences of the genomic rDNA were performed for all seven *Eimeria* species. The results of species-specific PCR assays confirmed the presence of *E. maxima*, *E. tenella*, *E. acervulina* and *E. praecox*, and nested PCR results showed the presence of *E. mitis* and *E. brunetti*. Although morphologic and morphometric observations revealed the presence of oocysts resembling *E. necatrix*; it was not confirmed by species specific or nested PCR. Nucleotide sequences of Turkish *Eimeria* isolates obtained by sequencing of the PCR products from 6 *Eimeria* species have been entered into the GenBank sequence database under accession numbers HQ680469 through HQ680474.

Key words: Broiler, *Eimeria* species, species-specific PCR, nested PCR.

Türkiye'de etlik piliçlerde görülen *Eimeria* türlerinin moleküler olarak belirlenmesi

Özet: Bu çalışmada tavuklarda coccidiosise neden olan 7 *Eimeria* türünün Türkiye'deki varlığı araştırılmıştır. Çalışma materyali olan altlık ve dışkı örnekleri, Eylül 2006-Eylül 2007 tarihleri arasında, broiler yetiştiriciliği yapılan 817 çiftlikteki toplam 1110 kümesten toplandı. İncelenen örneklerin 624 (%56.2)'ünde *Eimeria* oocystleri saptandı. Genomik rDNA'nın internal transcribed spacer-1 (ITS-1) bölgesini hedefleyen klasik ve nested PCR yöntemleri 7 *Eimeria* türü için uygulandı. Klasik PCR yöntemi ile *E. maxima*, *E. tenella*, *E. acervuline* ve *E. praecox* türleri saptanırken *E. mitis* ve *E. brunetti* ancak nested PCR yöntemi ile belirlendi. Morfolojik ve morfometrik incelemelerde *E. necatrix*'e benzeyen oocystler gözlenmiş olmasına ragmen, her iki PCR yönteminde de bu etken belirlenmedi. PCR yöntemi ile belirlenen 6 *Eimeria* türüne ait nükleotid dizi bilgileri HQ680469-HQ680474 numaraları ile Genbank veri tabanına kaydedildi.

Anahtar sözcükler: Broiler, *Eimeria*, klasik PCR, nested PCR.

Introduction

Avian coccidiosis is one of the most important diseases affecting the intensive poultry industry worldwide. Coccidia are almost universally found wherever chickens are raised and it is exceedingly rare to find a commercial chicken flock not affected. The infection causes tissue damage in the intestinal tract leading to interruption in digestive processes, blood loss, increased susceptibility to other diseases, subclinical enteric infection, and subacute mortality. The disease is controlled by the inclusion of anticoccidial drugs in the feed or vaccination (18, 26, 29, 31). There are seven commonly recognized species of chicken coccidia; *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella*. Each *Eimeria* species develops in a particular location within the chick digestive tract with some overlap seen between species. It is common to find six species (*E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. praecox* and *E. tenella*) in litter samples from a single flock during the first 6 weeks of growth (2, 17, 29).

The specific identification of *Eimeria* species and strains is important for diagnosis and control, as well as for epidemiology and population biology studies (21). Traditionally, *Eimeria* species have been identified by morphology and/or morphometry of their sporocysts and

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oocysts as well as their patterns of development, and assessing the site and extent of the pathological lesions in the intestine of chicken (1, 15). However, these methods are costly, time-consuming, require skilled personnel and can be unreliable under the circumstances of mixed field infections, particularly when the overlap in biological and morphological characters makes the unequivocal identification and differentiation of *Eimeria* species impossible (4, 28).

Molecular techniques have some advantages over traditional methods in that they rely only on the genomic sequence of the *Eimeria* species. Several techniques based on the polymerase chain reaction using primers that specifically targeting different regions of the *Eimeria* genome have been described (6, 7, 20, 22, 25, 30). For molecular detection of *Eimeria* species in chickens, the DNA sequence of the first and second internal transcribed spacers (ITS-1 and ITS-2) of the nuclear DNA, which separate the ribosomal genes, is used most frequently. Besides its heterogeneity in both sequence length and base composition of the ITS sequence, the rDNA is a member of a multiple copy gene family and thus provides large numbers of potential PCR targets (3, 13, 14, 23, 27). Nevertheless, the practical implementation of these techniques in routine diagnostics and epidemiological studies has been limited (7).

The number of studies related to the presence of chicken *Eimeria* species in Turkey is limited. These studies have been conducted in few locations and species identification was determined on the basis of oocysts morphology, necropsy and/or histopathological findings. And as a result, nine chicken *Eimeria* species (*E. acervulina*, *E. brunetti*, *E. hagani, E. maxima*, *E. mitis*, *E. mivati, E. necatrix*, *E. praecox* and *E. tenella*) have been reported to found in Turkey (5, 11).

In this study, we aimed to identify the *Eimeria* species causing coccidiosis in broilers in Turkey using a molecular technique based on the PCR and determine the prevalence of coccidial infections in commercial broiler flocks in Turkey.

Materials and Methods

Study area and sampling: The study sample consisted of 1110 broiler flocks housed in 817 farms (about 12% of all broiler farms in Turkey) located in the regions in which the broiler industry is concentrated in Turkey (Figure 1). The farms were visited between September 2006 and September 2007. The flocks were selected by stratified random sampling from the total Turkish broiler population. The materials were collected from broilers that were fed prophylactic anticoccidial agents at some time during their lives. The age of broilers varied between 1 to 50 days. Each sampling consisted of faecal and litter collections made from 20 to 40 places throughout each poultry house, particularly around the drinkers and feeders. Signs of clinical coccidiosis such as diarrhoea or blood in the faeces, depression or inappetence were monitored in each sampling site.

Examination and processing of samples: The field samples were examined for the presence of *Eimeria* oocysts by standard sodium chloride flotation method. The levels of oocysts per gram of sample (OPG) were determined using a standard McMaster technique (19).

A modified saturated salt flotation technique was used to collect oocysts. Fifty random oocysts from each positive sample were initially identified based on morphometry and morphology of oocysts using a calibrated ocular micrometer at 400x magnification. A total of 35 oocyst pools were prepared based on the criteria of age (6 groups), province (19 groups), region (6 groups) and season (4 groups). Approximately 10x 10^8 oocysts were combined to form an oocyst pool from each positive sample. Isolated oocysts were suspended in 2% (w/v) potassium dichromate solution and stored at 4 °C until DNA extraction.

DNA preparation: DNA was isolated from approximately 100.000 oocysts from each sample. The potassium dichromate was removed by repeated centrifugation and resuspension in distilled water. The washed oocysts were then sterilised and prepared for isolation by sodium hypochlorite treatment (4 % available chlorine, 1 h, 4 °C). Oocysts were subjected to 3 freeze–thaw cycles of 2 min each in a dry ice/ethanol bath and a 100 °C water bath. DNA from the lysed oocysts was extracted with a QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions.

PCR amplification: Amplification of the species-specific ITS-1 sequences of the genomic rDNA was carried out in 25 µl reaction volumes containing 2 µl DNA, 1 µl of 5 pmol/µl species specific reverse and forward primers (7), 21 µl sterile high-quality water and 1 PuReTaq Ready-To-Go PCR bead (GE Healthcare). The amplification was performed in a Veriti 96 well fast thermal cycler (Applied Biosystems). The reaction profile included an initial denaturation step at 95 °C for 5 min, then 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 or 65 °C for 30 s and extension at 72 °C for 30 s. A final prolonged extension step at 72 °C for 10 min completed the PCR process. Each sample (20 µl) was mixed with 6 µl loading buffer, and analysed by electrophoresis in 1.5 % agarose gels containing 0.5 µg/ml ethidium bromide. The PCR products were identified by size using a 100 bp ladder and comparison to a positive control DNA sample obtained from SWEPAR, Sweden. Ultra-pure autoclaved water served as negative control.

In order to amplify the ITS1 region of rDNA from *Eimeria* species with low oocyst numbers nested PCR

was performed using genus specific primers BSEF and BSER (24). A similar PCR reaction mix, as described above, was used for the nested PCR except that the PCR program comprised an initial denaturation step for 5 min at 95 °C, followed by 40 cycles, each consisting of 15 sec denaturation at 95 °C, 30 sec annealing at 45 °C and a 30 sec extension step at 72 °C with the final extension continued for 10 min. 1 µl of this PCR reaction was used as a template for second PCR amplification using species-specific primers as described above (7).

Cloning: The PCR products were analysed by agarose gel electrophoresis, purified using QIAquick Gel Extraction Kit (QIAGEN, Valencia, USA) and cloned into the pDrive Cloning Vector using a Qiagen PCR Cloning Kit (Qiagen, Valencia, USA) as described by the manufacturer. The isolation of plasmid DNAs was performed by Gene Jet Plasmid Miniprep kit (Fermentas) and detection of plasmids containing a cloned PCR product was determined by digestion of plasmid DNA with restriction endonuclease EcoRI (Fermentas) followed agarose gel electrophoresis. Plasmids containing a PCR insert were sequenced using T7 promoter primer at the Georgia Genomics Facilitity (The University of Georgia, Athens, GA).

Results

Faecal samples collected from 1110 broiler flocks in Turkey were examined to determine the presence of *Eimeria* oocysts using sodium chloride flotation method. No symptoms of clinical coccidiosis were observed in the broiler houses sampled. Coccidian oocysts were found in 624 (56.2 %) of all the samples examined (Figure 1). The OPG varied from 50 to 952.000. The age of chickens in *Eimeria* contaminated chick houses varied from 9 to 50 days.

Based on the morphologic and morphometric criteria, oocysts similar to *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix, E. praecox and E. tenella* were determined in each positive sample. A total of 35 oocyst pools were prepared based on the age (6 groups), province (19 groups), season (4 groups) and region (6 groups). Seven generally accepted *Eimeria* species were detected in all oocyst pools with *E. acervulina, E. maxima*, *E. praecox and E. tenella* (Figure 2) detected using species-specific primers in the primary PCR reaction, and *E. mitis* and *E. brunetti* (Figure 2) detected using a nested PCR strategy. We observed that some oocysts were similar to *E. necatrix* according to morphologic and morphometric criteria, but we were unable to detect this species by PCR.

The ITS-1 sequences length varies between 145 and 330 bp (*E. acervulina* 145 bp, *E. maxima* 205 bp, *E. praecox* 215 bp, *E. tenella* 278 bp*, E. brunetti* 183 bp, *E. mitis* 330 bp, *E. necatrix* 160 bp) in the chicken *Eimeria* species. The nucleotide sequences of 6 *Eimeria* species, obtained by sequencing of the PCR products of Marmara region oocyst pool, have been entered into the GenBank sequence database under accession numbers HQ680469 through HQ680474.

In comparison with previously published sequences Turkish isolate of *E. mitis* was more similar (% 66) to Swedish (AF065093) and Australian (AF446065, AF446062) sequences; *E. brunetti* was more similar (%98) to Swedish (AF026383) sequence; *E. maxima* and *E. praecox* were more similar (%17, % 98) to Australian (AF446059, AF446071) sequences than American, British, Chinese and Indian; but *E. tenella* and *E. acervulina* isolates of Turkey were formed separate branches when compared to American, British, Chinese, Indian, Australian and Swedish sequences.

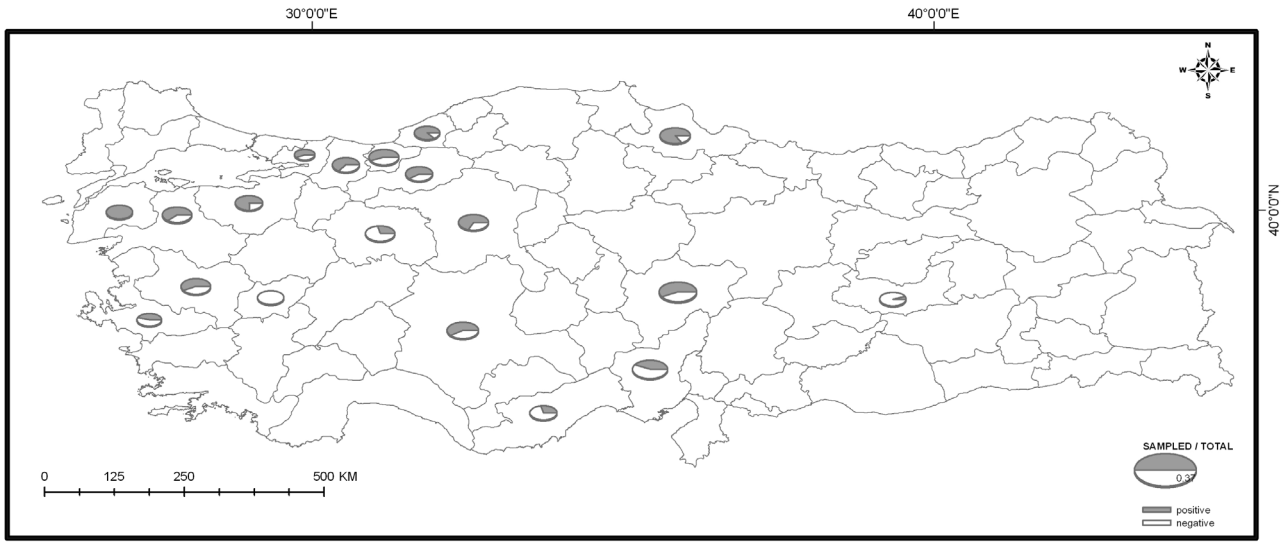

Figure 1. Map detailing farm location where samples were taken and percentages of farms positive for *Eimeria*.
Şekil 1. Örneklerin alındığı çiftliklerin lokalizasyonu ve *Eimeria* pozitif çiftliklerin yüzdesi.

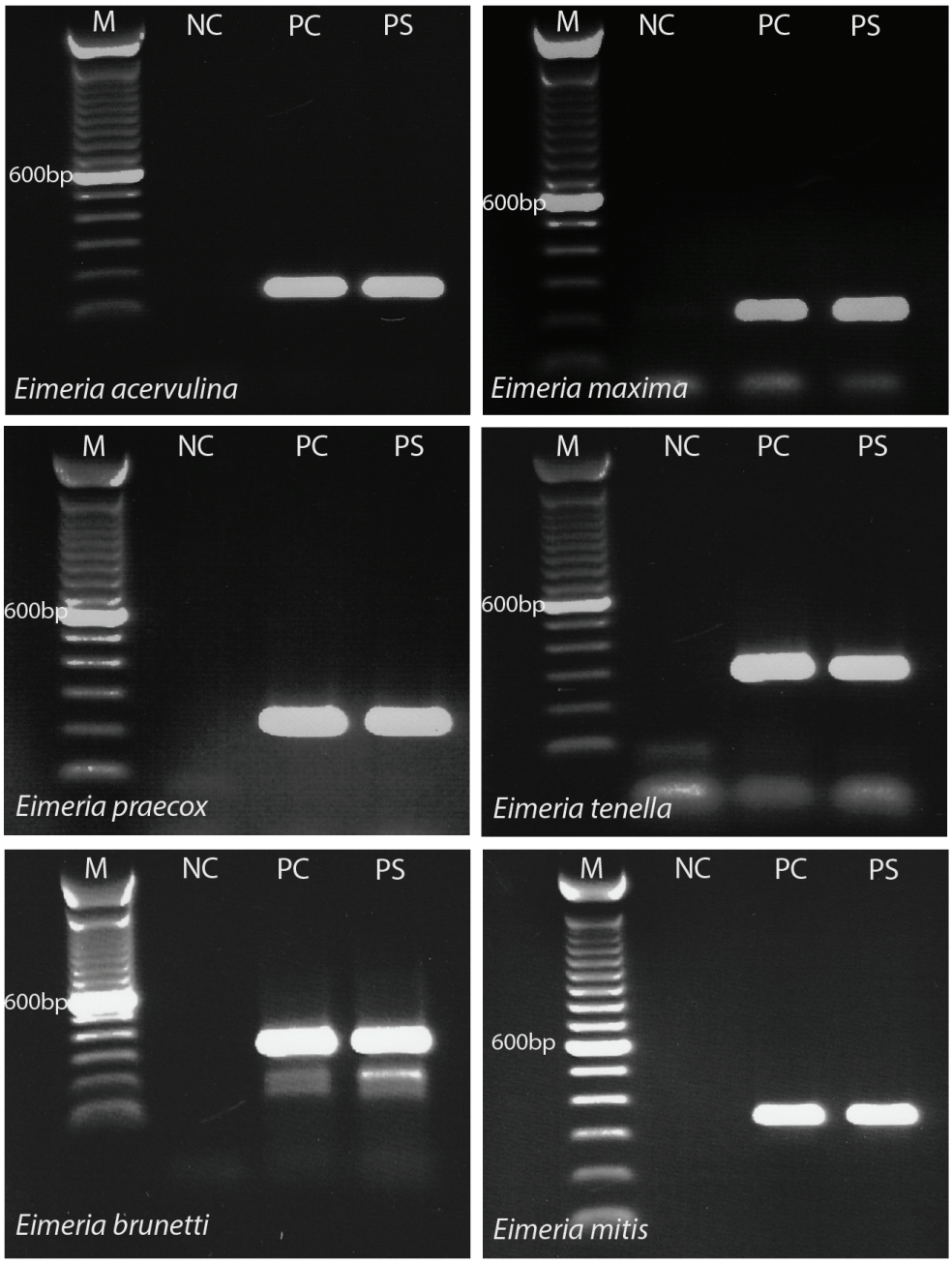

Figure 2. Agarose gel electrophoresis of *Eimeria* species-specific and nested PCR products from one sample. M: 100 bp molecular weight marker; NC: Negative control, PC: Positive control, PS: Positive sample.
Şekil 2. *Eimeria* pozitif bir örneğin tür spesifik ve nested PCR ürünlerinin agaroz jel elektroforez görüntüleri. M: 100 baz çiftlik moleküler ağırlık markırı; NC: Negatif Kontrol; PC: Pozitif Kontrol, PS: Pozitif örnek.

Discussion

In this study, we confirm the presence of 6 *Eimeria* species through PCR/seqeuncing in Turkish broiler chickens during the 2006/07 growing season. Jordan and Pattison (9) reported that all important *Eimeria* species appear to be distributed throughout the world; *E. acervulina* and *E. maxima* are the most prevalent, and *E. tenella* is the commonest of the highly pathogenic species. In this study we detected *E. acervulina*, *E. maxima*, *E. praecox* and *E. tenella* with species spesific primers in the primary PCR reaction but we could only detected *E. mitis* and *E. brunetti* when we used genus spesific primers to amplify the target sequence first. This suggests a lower density of *E. mitis* and *E. brunetti* oocysts in the oocyst sample population. Future experiments need to be performed with quantitative PCR to determine the relative concentration of each species in their respective pool. Because these are pooled samples, our data can't determine whether each farm is infected with all 6 *Eimeria* species.

Coccidian oocysts were found in 624 (56.2%) of all the samples examined and 6 species were detected in all oocyst pools. Each of these flocks had been medicated with prophylactic anticoccidials suggesting that these

birds may be exhibitting subclinical coccidiosis with a high prevalence (56.2%). These results support the protective effect of anticoccidial use in the flocks but despite drug use there is a high level of *Eimeria* contamination in these poultry houses and suggests that upon disuse of anticoccidial drugs outbreaks of clinical coccidiosis will occur readily.

Epidemiological studies indicate that each of the seven *Eimeria* species has a worldwide distribution and many individual farms harbour up to six species. The infection prevalences may vary from less than 10% to more than 90% in broilers worldwide (8, 10, 12, 18, 21). Our findings of *Eimeria* species in commercial broilers were in agreement with these conclusions. Most of the chick houses (624/1110) were contaminated with coccidia in Turkish broiler farms. All PCR tests showed the same result; there wasn't any differences in age, province, season and region groups in terms of presence of *Eimeria* species.

The detected oocyst measurements and morphological features indicated oocyst profile resembling *E. acervulina*, *E. brunetti, E. maxima*, *E. mitis*, *E. necatrix, E. praecox* and *E. tenella.* After PCR, we determined six species except *E. necatrix*. Williams (29) reported that it is usual to find *E. acervulina*, *E. brunetti, E. maxima*, *E. mitis*, *E. praecox* and *E. tenella* oocysts appearing in litter samples during the first 6 weeks of the life of a flock; *E. necatrix* tends to appear up to 12 weeks or more such as with breeder pullets or layer hens. This data is in accord with our findings. The samples of our survey were taken from broilers aged up to 50 days since standard broilers are reared only to about 6 or 7 weeks of age.

In Turkey earlier studies releated with diagnosis of chicken coccidiosis were based on oocyst morphology, pathological and histopathological findings. *Eimeria acervulina, E. brunetti, E. hagani, E. maxima, E. mitis, E. mivati, E. necatrix, E. praecox and E. tenella* (5, 11) were identified at these studies which were included a limited number flocks. The different findings of these surveys and ours are releated with the traditional diagnostic methods they used and also some of them sampled layers in addition to broilers. Traditional methods based on measurements and morphological features of the sporulated oocysts or assessing the site and extent of the pathological lesions in the intestine have limitations due to overlap of characteristics among different species when they simultaneously infect a single host (16). Woods et al. (30) reported that to distinguish species with "similar" oocysts such as *E. acervulina* and *E. mitis*, *E. praecox* and *E. tenella* or *E. brunetti* and *E. maxima* based on oocyst shape and size may be unreliable as these features can be very similar between the species. Sun et al. (27) noted that *E. maxima* and *E. mitis* are the two species that can be identified on the basis of oocyst size, shape or appearance. In our study we could only suggest that we have oocysts "resemble" *E. acervulina*, *E. brunetti, E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella* when we considered the measurements and morphological features of the oocysts since they were so close visually.

The specific diagnosis of *Eimeria* species in chickens using a PCR based approach is central to a better understanding of the epidemiology and dynamics of disease which underlies the effective prevention and control of coccidiosis. Although PCR-based methods can be costly and not practical for field studies, they have advantages of identifying all of the species in a sample. In this study, we confirmed 6 *Eimeria* species being in Turkish broiler industry by using species specific and nested PCR for the first time in Turkey.

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