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Original Article

Characterization of *Eimeria* Species in Commercial Broilers by PCR Based on ITS1 Regions of rDNA

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Abstract

Background: Coccidiosis is an intestinal disease of chickens caused by various species of protozoan parasites within the genus *Eimeria*. Diagnosis and genetic characterization of different species of *Eimeria* are central to the prevention, surveillance, and control of coccidiosis. The aim of this study was to detect different chicken *Eimeria* species from several areas in Khuzestan, southwest Iran.

Methods: From February to September 2008, PCR assay as well as parasitological examinations was applied for the identification of field isolates of *Eimeria* parasites around Ahvaz, center of Khuzestan, southwest Iran. Data were analyzed by the Kappa statistic test.

Results: *Eimeria maxima*, *E. necatrix*, *E. tenella*, *E. acervulina* and *E. mitis* were detected in this study. The prevalence of *Eimeria* spp. was 31.5% (126 of 400) and *E. tenella* was the most prevalent species in Khuzestan. Based on the Kappa statistical test, a good correlation between the results of PCR and traditional biometrical methods was only observed for *E. maxima*.

Conclusion: The present study is the first on the prevalence of *Eimeria* species in Khuzestan, based on the molecular findings. We believe that traditional methods are not sufficiently reliable for specific diagnosis of *Eimeria* species in chickens and PCR based amplification of DNA sequence of parasite, could resolve this problem.

Keywords: *Eimeria*, Poultry coccidiosis, PCR, ITS1, Microscopic examinations, Iran

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Introduction

Protozoan parasites of the genus *Eimeria* (Coccidia: Eimeriidae) are highly successful organisms which inhabit and multiplies in the intestinal tract. These parasites cause chickens coccidiosis, an enteric disease of major economic importance worldwide (1). Economic importance of the disease is due to production losses and high morbidity resulting from an acute, bloody enteritis and mortality rates (2). However, intestinal lesions of the infection vary, depending on the species of coccidian. About 1800 *Eimeria* spp. affect the intestinal mucosa of different mammals and birds, but seven species of *Eimeria* including *E. tenella*, *E. necatrix*, *E. acervulina*, *E. maxima*, *E. brunetti*, *E. mitis*, and *E. praecox* are the causative agents of coccidiosis in chickens (3).

Diagnosis of coccidiosis is based on clinical features and gut pathology of host, parasite characteristics such as morphology at different stages of parasitism, and the pre-patent period (4, 5). Analysis of these characteristics is labor intensive for diagnosis and does not provide accurate data for identification of the *Eimeria* species (6).

Identification and genetic characterization of different species of *Eimeria* genus are central to prevention, surveillance, and control of coccidiosis. This is particularly important with regard to the appearance of a widespread anticoccidial resistance of *Eimeria* species and the problems associated with drug residues.

Due to difficulties in the morphologic identification of some of chicken *Eimeria* spp., diagnostic laboratories are increasingly utilizing DNA-based technologies for the specific identification of the parasite (7).

So far, there is limited knowledge on the epidemiology of *Eimeria* infections under different rearing conditions in Iran. In The

present study, together with morphometric diagnosis, PCR assay, based on the amplification of internal transcribed spacer 1 (ITS1) regions of ribosomal DNA (8) was used for identification of chicken *Eimeria* species in Khuzestan Province, southwest Iran.

Materials and Methods

Sample collection preparation

From February to September 2008, 400 samples of fresh fecal droppings were collected by cluster sampling from 40 broiler chickens flocks without previous exposure to anticoccidial vaccines. The sampled flocks were located in different areas within a radius of 20 to 100 km from the center of Khuzestan Province. Samples of each flock were collected from different locations in the poultry house. One hundred samples, containing a high number of *Eimeria* and representing all areas of the province were selected and preserved in 2.5% potassium dichromate at 28°C.

After sporulation of oocysts, potassium dichromate was removed from the master stock by repeated centrifugation and resuspension in water. Oocysts present in feces were then purified by a saturated saline solution and washed by centrifugation at 1000 x g for 5 min with distilled water. The sediment containing oocysts was resuspended in phosphate buffered saline (PBS) pH 7.4 with 0.1% Tween 80 and homogenized by vortexing and subsequent centrifugation at 800 x g for 5 min. Thereafter, a 6% solution of sodium hypochlorite was added, 20 minutes incubation was performed at 4 °C and the oocysts were finally washed 4 times, with distilled water (6).

Parasitologic examination

A modified saturated salt flotation technique was used to isolate oocysts for length measurements using a calibrated ocular micrometer at 400x magnification (4). Fifty random oocysts from each sample were examined by measuring their length and width with light microscopy, armed with calibrate ocular lens as well as determination of the oocysts shape and index (Length/Width). For more accurate diagnosis, sporocysts' diameters were also determined (9).

PCR protocol

Sporozoites were released from oocysts and sporocysts wall by sonication and DNA was extracted from sporozoites by Stool Mini Kit extraction (Qiagen) according to manufacturer's recommendation. Single PCR assays targeting ITS-1 regions of ribosomal DNA were performed for each of the seven chicken *Eimeria* species. Forward and reverse species-specific primer sequences used in this study have been reported previously by Haug et al. 2007 (10) (Table 1). Amplification of the ITS-1 sequences of genomic rDNA was carried out in 20 µl reaction volumes containing 2 µl of DNA template, 8 pmol of reverse and forward primers, 3.0 mM MgCl₂, 2.0 µl 10 X PCR Buffer, 200 µM of each dNTP and 0.4 U Taq DNA Polymerase. Thermal program of PCR was as follows: denaturation step at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C or 65 °C for 30 s and extension at 72° C for 1 min. A final prolonged extension step at 72 °C for 3 min completed the PCR process. A commercial vaccine (Paracox[®] 8, UK) which included all the eight pathogenic *Eimeria* species of chicken was used for positive controls and fecal samples without any oocyst examined by floatation method used as negative control in PCR. To verify the results, 10 µl of each PCR product was electrophoresed in a 1.5% agarose gel, stained with ethidium bro-

mid, and visualized on a UV transilluminator. The PCR products were identified by size using a 100 base pair ladder.

Statistical analysis

The Kappa statistic test (κ) was used to test the level of agreement between the PCR and the parasitologic examination for the detection of *Eimeria* species. Kappa and its 95% Confidence Interval (CI) , was used further to measure the degree of agreement between the two procedures after taking into account the probability of agreement by chance alone. Strength of agreement based on κ was judged according to the following guidelines: <0.2=slight; 0.2–0.4=fair; 0.4–0.6=moderate; 0.6–0.8=good; >0.8=very good (11).

Results

Out of 400 specimens collected, 126 (31.5%) fecal droppings were positive for oocysts of *Eimeria* spp. in parasitological examination (*E. tenella*=31%, *E. maxima*=24.6%, *E. acervulina*=23%, *E. mitis*=12.7% and 8.73%).

The data showed that *E. tenella*, *E. necatrix*, *E. acervulina*, *E. mitis* and *E. maxima* exist in poultry farms of Khuzestan by both molecular examination, using species-specific PCR (Fig. 1 and 2) and parasitological methods. *E. tenella* was the most prevalent species and *E. brunette* and *E. praecox* were not detected at all. PCR analyses of 100 collected samples showed that all the identified species were present in different areas, around the center of Khuzestan Province.

Statistical analyses using Kappa test revealed a poor agreement between PCR and the traditional biometrical identification for diagnosis of detected *Eimeria* species, except for *E. maxima* and to some extent for *E. acervulina* ($\kappa = 0.35, 0.30, 0.56, 0.22$ and 0.84 for *E. tenella*, *E. necatrix*, *E. acervulina*, *E. mitis* and *E. maxima* respectively).

Table 1: Sequences of the PCR primer pairs used (10)

<i>Eimeria</i> species	Amplicon size (bp)	Primer sequence 5' to 3'
<i>E. maxima</i>	205	5'-GTGGGACTGTGGTGATGGGG-3' 5'-ACCAGCATGCGCTCACAACCC-3'
<i>E. acervulina</i>	145	5'-GGGCTTGGATGATGTTTGCTG-3' 5'-GCAATGATGCTTGCACAGTCAGG-3'
<i>E. brunetti</i>	183	5'-CTGGGGCTGCAGCGACAGGG-3' 5'-ATCGATGGCCCCATCCCGCAT-3'
<i>E. mitis</i>	330	5'-GTTTATTTCTGTCGTCGTCGTC-3' 5'-GTATGCAAGAGAGAATCGGGATTCC-3'
<i>E. praecox</i>	215	5'-CATCGGAATGGCTTTTTGAAAGCG-3' 5'-GCATGCGCTAACAACCTCCCTT-3'
<i>E. tenella</i>	278	5'-AATTTAGTCCATCGCAACCCTTG-3' 5'-CGAGCGCTCTGCATACGACA-3'
<i>E. necatrix</i>	160	5'-AGTATGGGCGTGAGCATGGAG-3' 5'-GATCAGTCTCATCATAATTCTCGCG-3'

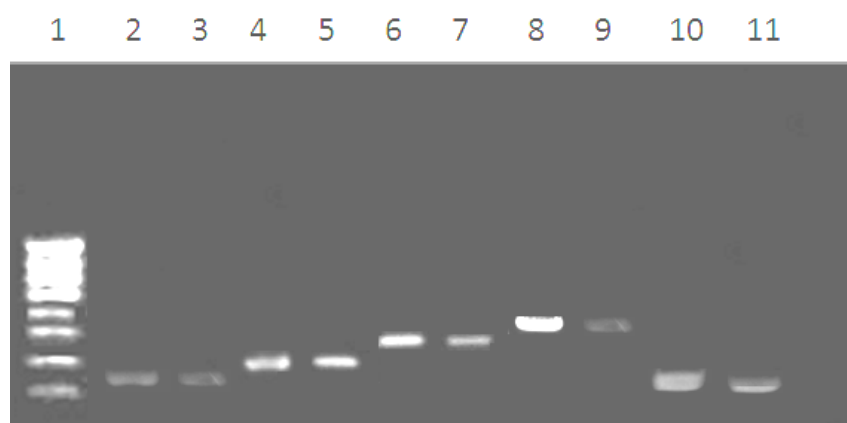


Fig. 1: Agarose gel electrophoresis of *Eimeria* species-specific PCR products. Polymerase chain reaction based on amplification of the species-specific ITS-1 sequences of the genomic rDNA. Positive reactions: *E. acervulina* (145bp) (2-Control, 3-Sample), *E. maxima* (205 bp) (4-Control, 5-Sample), *E. tenella* (278 bp) (6-Control, 7-Sample), *E. mitis*(330bp)(8-Control, 9-Sample) and *E. necatrix*(160bp)(10-Control, 11-Sample). 1= 100 bp ladder

Discussion

The specific diagnosis of *Eimeria* infections in chickens is clearly central to a better understanding of epidemiology and dynamics of the disease in intensive and extensive chicken establishments. This is particularly important for planning an effective prevention and control program of coccidiosis. Traditionally, diagnosis has been achieved by detecting *Eimeria* oocysts excreted in the feces of chickens by measuring oocyst and sporocyst dimensions or assessing the site and extent of the pathological lesions in the intestine of chickens. Although the microscopic examinations can absolutely show the negative fecal samples, such traditional methods have generally had major limitations in the specific diagnosis of coccidiosis and identification of *Eimeria* species. These approaches are unreliable, particularly when multiple species of *Eimeria* simultaneously infect a single host and there is overlap in the size and shape of oocysts and the sites of infection in the intestines (5).

During recent years, there have been significant advances in the development of molecular-diagnostic tools. Several PCR based assays targeting different regions of the *Eimeria* genome have been described, such as the 5S rRNA (, the small subunit rRNA (12, 13), the sporozoite antigen gene EASZ240/160 (14) and ITS-1 (8, 15-17) and ITS-2 (18-20) genomic regions. Since the ITS regions are less conserved than the rRNA genes, detecting variations in this region of DNA sequence, makes the design of primers straightforward and reduces the risk of cross reactions among different species (21). Apart from an accurate identification of *Eimeria* species, molecular methods can also be helpful in epidemiological study of the parasite, an aspect that has been less investigated to date.

At yet, there has not been any documentary report related to the occurrence and epidemiological pattern of the pathogenic *Eimeria* species of domestic chickens, in Khuzestan. Therefore, the results of the present study are the first on the prevalence of *Eimeria* species in the region, based on the molecular findings.

Out of 400 specimens collected, 126 (31.5%) samples were positive for *Eimeria* spp. Nowzari et al. in a large study including 5 provinces of Iran showed that *E. maxima*, *E. mitis*, *E. brunetti*, *E. tenella* and *E. acervulina* were distributed all over Iran. They identified that *E. mitis* and *E. brunetti* for the first time by PCR (22). *E. brunetti* has been found uncommon in broiler flocks (15). This is in accordance with our finding in Khuzestan by PCR. *E. necatrix* has also been considered as uncommon in broiler flocks, but we identified this species in 11 samples of seven farms. In our study, *E. tenella* was the dominant species. This finding suggests that in poor management conditions, poultry houses may encounter acute coccidiosis in Khuzestan due to highly pathogenic species, *E. tenella*.

Razmi et al, reported that prevalence of sub-clinical coccidiosis was 38% in Mashad, northeast of Iran and *E. acervulina* was the most prevalent species in broiler chicken farms (23). In north-west of Iran, Tabriz, five *Eimeria* spp., *E. acervulina*, *E. tenella*, *E. necatrix*, *E. maxima* and *E. mitis*, were identified by morphometric study and *E. acervulina* was the most prevalent species (23.58%) (24).

Poor evaluation of parasitologic method to identify the *Eimeria* species has been reported by Anita Haug et al., previously. They reported that PCR and morphometric identification were in complete agreement in only 49% of the cases (25). The present

study also showed that there was poor agreement between PCR and traditional identification for diagnosis of *Eimeria* species. However, there was a good agreement between PCR and morphometric diagnosis only for *E. maxima*. This finding can be due to the large size of the organism. Hence, as showed by low agreement with Kappa test, traditional methods are not sufficiently reliable for specific diagnosis of *Eimeria* species in chickens. Moreover, occurrence of multiple infections in a single bird and the fact that, *Eimeria* species with low oocysts frequency in the mixture maybe missed, indicates that PCR based amplification of DNA sequence of parasite, could resolve this problem and overcame the limitation in analysis of small amounts of oocysts in mixed infections. On the other hand, this protocol can even identify strains of *Eimeria* species, characterized by different drug-resistance phenotypes (15, 26).

In conclusion, we believe that traditional methods are not sufficiently reliable for specific diagnosis of *Eimeria* species in chickens and PCR based amplification of DNA sequence of parasite, could resolve this problem.

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