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Research Paper

Morphological and Molecular Identification of *Eimeria* spp. Infecting Broiler Chicken Farms in Iran

Mojtaba Khoini[1], Afshin Bahman Shabestari[1], Rahmat Solgi[2*]

1. Department of Veterinary Parasitology, Ab.C., Islamic Azad University, Abhar, Iran.
2. Infectious Disease Research Center, Avicenna Institute of Clinical Sciences, Hamadan University of Medical Sciences, Hamadan, Iran.

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ABSTRACT

Introduction: The poultry industry in Iran plays a crucial role in the country's economy and food security. However, it faces numerous challenges, including parasitic infections such as *Eimeria* spp. This study aims to provide a comprehensive morphological and molecular characterization of *Eimeria* spp. infecting broiler chickens in Iran.

Materials & Methods: Fresh fecal samples were collected from chickens (18–45 days old) across 149 farms located in various regions of Iran. The fecal samples were subjected to standard parasitological techniques, including flotation and sedimentation methods, to identify *Eimeria* oocysts. DNA was extracted from the oocysts, followed by nested polymerase chain reaction (PCR) using specific primers targeting the *ITS1* gene of *Eimeria* spp.

Results: Out of the 149 poultry farms examined, 59.7% tested positive for *Eimeria* spp. Gheidar County showed the highest infection rate among the samples collected, standing at 81.8%. Molecular methods can successfully prove the morphological studies. The prevalence of these species varied, with *E. acervulina* being the most common (55.7%) in Zanjan Province, followed by *E. maxima* (48.3%), *E. mitis* (20.1%), *E. tenella* (20.1%), and *E. necatrix* (13.4%). Mixed infections with two or more *Eimeria* species were found in 64 out of 103(62.1%) positive samples. The most prevalent combination was *E. acervulina*, *E. maxima*, present in 23 out of 101(22.3%) positive samples.

Conclusion: Since vaccination is not currently employed to prevent coccidiosis in Iranian broiler production, the conclusions drawn from this study underscore the significance of implementing reliable chemoprophylactic control measures.

*** Corresponding Author:**
Rahmat Solgi, Assistant Professor.
Address: *Infectious Disease Research Center, Avicenna Institute of Clinical Sciences, Hamadan University of Medical Sciences, Hamadan, Iran.*
Tel: *+98 (921) 1975537*
E-mail: *rahmatsolgi@yahoo.com*

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1. Introduction

Coccidiosis is an infectious parasitic disease that impacts a diverse range of birds, such as chickens, turkeys, and other domestic fowl [1]. The disease is caused by protozoa belonging to the genus *Eimeria*, which invade the birds' intestinal lining [2]. Coccidiosis can quickly spread throughout a group of birds via contaminated feed, water, or bedding. Preventive measures, such as maintaining proper sanitation, administering vaccine, and following biosecurity protocols, are crucial for controlling the disease and ensuring the well-being and productivity of the birds [3]. Identifying and studying these parasites require the use of morphological and molecular techniques. Morphological identification involves examining physical traits of *Eimeria*, including the size, shape, and structure of their oocysts [4]. Furthermore,sequencing and molecular identification techniques can analyze the genetic material of *Eimeria* [5]. Combining these methods provides more reliable identification of *Eimeria* parasites [6]. The pathogenicity of various *Eimeria* species ranges from moderate to severe, underscoring the importance of determining species composition for effective control and prevention measures [7]. In Iran, where poultry farming is crucial for the economy and food security, *Eimeria* infections are a significant worry for poultry breeders [8]. Despite the widespread presence of these parasites, there is limited available information on the morphological and molecular characteristics of *Eimeria* species infecting domestic poultry in Iran.

2. Materials and Methods

2.1. Design and collection of study animals

Fresh broiler chicken feces (18–45 days old) were collected from a total of 149 poultry farms in Zanjan County (50 farms), Abhar County (35 farms), Gheidar County (33 farms), and Khoram Dareh County (31 farms) between June and December 2023. Each farm had a breeding stock of 5000 to 15,000 chickens. The feces were collected from various points within each broiler chicken house, including the four corners and center, using plastic bags. The oocyst samples were stored at to 4 ˚C, at which they sporulate poorly (or were kept at 4 ˚C to inhibit premature sporulation)

2.2. Morphological identification

Two grams of each sample were added to 60 milliliters of saturated saline solution. After the mixture was filtered through gauze and centrifuging at 1500 rpm for 10 minutes, a small drop of the sample was used to create a liquid film for observation under a light microscope. Samples that tested positive for oocysts were subjected to a flotation techniqueto collect oocysts for incubation in 2.5% potassium dichromate. They were then allowed to sporulate at 27 ˚C for 5 days [9]. The oocysts that had formed spores were subsequently stored at 4 ˚C for further molecular examination. *Eimeria* identification was conducted by analyzing the oocysts and sporocysts, taking into account factors such as their size, shape, wall composition, and color [10].

2.3. Genomic extraction

The sporulated oocysts were rinsed in TE buffer and then broken down using 0.5 mm glass. DNA was extracted using a Genomic DNA Kit (Qiagen in Hilden, Germany), according to the manufacturer's protocol.

2.4. Molecular methods

The molecular identification of *Eimeria* was carried out using polymerase chain reaction (PCR), as previously described in a previous study [9] (Table 1).

The PCR reaction mix was comprised of 12.5 µL of PCR master mix, 20 µM of each forward and reverse primers, 1 µL of DNA template, and nuclease-free water to make a total of 25 µL. The amplification process began with an initial denaturation step at 94 ˚C for 10 min, followed by 35 cycles involving 94 ˚C for 30 sec, 52.5-65 ˚C for 30 sec, and 72 °C for 1 min. A final extension step at 72 ˚C for 5 min concluded the process. The PCR products were then analyzed on agarose 1.5% agarose gel, were stained with ethidium bromide, and visualized under UV light.

Data collected from the study were analyzed using SPSS software, version 20. Values of $P<0.05$ were considered significant. The chi-square test (χ^2) for association was used to measure statistical significance.

Statistical analysis

Data collected from the study were analyzed using SPSS software, version 20. Values of $P<0.05$ were considered significant. Chi-square test (χ^2) for association was used to measure statistical significance.

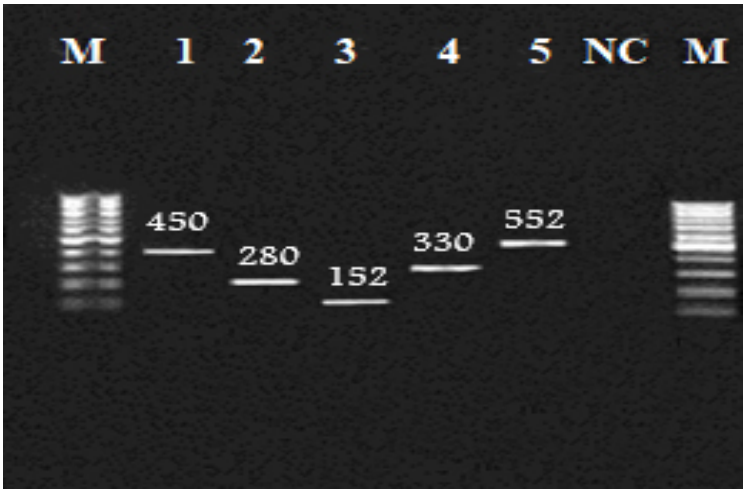

Figure 1. Results were obtained by PCR followed by 2% agarose gel electrophoresis

Note: Lines in M show a 100 bp DNA marker. Samples include Line 1: *E. necatrix;* Line 2: *E. acervulina;* Line 3: *E. maxima;* Line 4: *E. mitis* and Line 5: *E. tenella;* NC: Negative control; M: Marker 100 bp.

3. Results

3.1. Morphological identification

Out of the 149 poultry farms that were examined, 59.7% tested positive for *Eimeria* spp. Gheidar County showed the highest infection rate among the samples collected, standing at 81.8%. In contrast, the prevalence was relatively lower in Khoram Dareh at 38.7%. The prevalence rates in Zanjan and Ahar were recorded at 56% and 62.8%, respectively. There was a significant difference between the prevalence of *Eimeria* sp. and different geographical areas ($P<0.05$). The morphological characteristics of the isolated *Eimeria* species indicated the presence of five distinct species in the samples analyzed, namely *E. tenella*, *E. necatrix*, *E. mitis*, *E. maxima*, and *E. acervulina*.

3.2. Molecular identification

The molecular data confirmed the morphological studies. The study found that all positive samples showed multiple infections by two to four different species of *Eimeria*. A molecular examination performed in poultry farms in Zanjan revealed the presence of *E. tenella*, *E. necatrix*, *E. acervulina*, *E. mitis*, and *E. maxima* (Figure 1).

Notably, the prevalence of these species varied, with *E. acervulina* being the most common (55.7%) in Zanjan Province, followed by *E. maxima* (48.3%), *E. mitis* (20.1%), *E. tenella* (20.1%), and *E. necatrix* (13.4%), as shown in Table 2.

Mixed infections with two or more *Eimeria* species were found in 64 out of 103(62.1%) positive samples (Table 3). The most prevalent combination was *E. acervulina*, *E. maxima*, which were present in 23 out of 101 (22.3%) positive samples.

4. Discussion

Eimeria spp, a type of protozoan parasite, can result in notable financial losses within poultry facilities due to decreased productivity, increased mortality, and additional expenses related to disease management [11-13]. In Iran, *Eimeria* infections are frequently observed in domestic poultry, highlighting the necessity for a thorough characterization of the various species affecting these birds. The main goal of this study was to evaluate the occurrence of *Eimeria* species in broiler chickens in Zanjan Province, Iran. The current study aims to investigate the prevalence of coccidiosis and the diversity of *Eimeria* species in local poultry in Zanjan Region, where such infections had not been previously examined.

In this study, fecal specimens were collected from 149 domestic poultry facilities situated in four distinct urban areas within Zanjan Province, across various seasons. The findings revealed that out of the 149 processed samples, 89 tested positive for *Eimeria* oocysts, indicating a prevalence rate of 59.7%. Notably, the prevalence of these species varied, with *E. acervulina* being the most common (55.7%) in Zanjan Province. However,in a previous study, and unlike our study, the highest prevalence was related to *E. Tenella* [13, 14].

Table 1. The primers used in the first PCR assay

Species	Sequences	Size	Annealing
E. mitis	F-GTTTATTTCCTGTCGTCGTCTCGC R-GTATGCAAGAGAGAATCGGGATTCC	330	65 °C
E. tenella	F-GTTGCGTAAATAGAGCCCTCT R-GTTCCAAGCAGCATGTAACG	552	52.5 °C
E. maxima	F-GTTGCGTAAATAGAGCCCTCT R-ACCAATGCAGAACGCTCCAG	152	52.5 °C
E. acervulina	F-GTTGCGTAAATAGAGCCCTCT R-CAAAAGGTGGCAATGATGCT	281	52.5 °C
E. necatrix	F-GTTGCGTAAATAGAGCCCTCT R-GATCAGTCTCATCATAATTCTCGCG	450	52.5 °C

Table 2. Prevalence of *Eimeria* spp. of chicken using PCR

Region/Farm	No. (%)						P
	E. acervulina	*E. maxima*	*E. mitis*	*E. necatrix*	*E. tenella*	Total Farm	
Zanjan/50	28(56)	25(50)	15(30)	0	15(30)	28(56)	0.002
Ahar/35	22(62.8)	20(57.1)	0	19(54.2)	10(14.2)	22(62.8)	
Gheidar/33	21(63.6)	17(51.5)	15(45.4)	6(18.1)	0	27(81.8)	
Khorramdareh/31	12(38.7)	10(32.2)	0	0	0	12(38.7)	

In a study on poultry farms of Sistan in 2018, five species of *Eimeria*, including *E. tenella*, *E. maxima*, *E. acervulina*, *E. necatrix*, and *E. mitis*, have been reported and the prevalence of poultry infection to *Eimeria* species were reported to be 20.96% [8]. Analysis of *Eimeria* species present in poultry across Zanjan Region identified the presence of five distinct species: *E. acervulina*, *E. maxima*, *E. mitis*, *E. necatrix*, and *E. tenella*. *E. acer-*

Table 3. Combinations of multiple infections of *Eimeria* species detected in 149 broiler farms in Zanjan Province, West Iran

Eimeria Species Combinations	Prevalence (n=149)
E. acervulina	11/149 (7.3%)
E. acervulina + *E. maxima*	23/149 (15.4%)
E. maxima + *E. necatrix*	15/149 (10%)
E. acervulina + *E. necatrix*	6/149 (4%)
E. acervulina + *E. maxima* + *E. mitis*	9/149 (6%)
E. acervulina + *E. maxima* + *E. mitis* + *E. tenella*	15/149 (10%)
E. acervulina + *E. maxima* + *E. necatrix* + *E. tenella*	10/149 (6.1%)

vulina had the highest infection rate (55.7%), while *E. tenella* had the lowest rate (13.4%) across all regions in Zanjan provinces. A study conducted previously in Iran also reported the presence of five *Eimeria* species in poultry farms, *E. tenella*, *E. maxima*, *E. acervulina*, *E. necatrix*, and *E. mitis*, with an overall prevalence of 55.96% [15]. Another study in the same area found the prevalence of *Eimeria* infection in poultry farms to be 21.53% [16]. Additionally, in previous study in Iran [17], broiler chickens showed a high infection rate of coccidiosis (75%), which was much higher than that of the current study. The current study in Zanjan revealed a prevalence of 59.7%, indicating a need to increase coccidiosis control measures in the province.

Moreover, a study in Brazil identified eight *Eimeria* species in free-range chickens, with *E. necatrix* having the highest prevalence at 25%, followed by *E. mitis* (18.3%), *E. mivati* (17.3%) *E. tenella* (12.4%), *E. brunetti* (9.9%), *E. acervulina* (9.1%), *E. praecox* (4.8%) and *E. maxima* (1). Highly skilled individuals are necessary for accurately identifying *Eimeria* species, as there is a notable overlap in characteristics across the various species [2, 13, 18]. Alongside morphological analysis, molecular methods like PCR and sequencing have become essential in accurately identifying *Eimeria* species. By targeting specific genetic markers based on conserved ITS1 regions of rDNA [19, 13], the chicken coccidian species can be identify more accurately [20].

5. Conclusion

In the present study, the results of molecular methods were coinciding with morphological descriptions, which agreed with previous findings reported. The present study presented findings on the occurrence of *Eimeria* species in poultry through ITS1-PCR in Zanjan, Iran. It confirmed the existence of *E. tenella*, *E. acervulina*, *E. mitis*, *E. necatrix*, and *E. maxima* in poultry farm in Zanjan, Iran, relying on morphological features and validated by molecular PCR. F103.

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Compliance with ethical guidelines

This study was approved by the Institutional Animal Ethics and Research Committee of the Department of Veterinary Parasitology, Abhar Branch, Islamic Azad University, Abhar, Iran.

Data availability

The data that support the findings of this study are available upon request from the corresponding author.

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Authors' contributions

Conceptualization, study design, experiments, data interpretation, review and editing: All authors; Data acquisition: Mojtaba khoini and Rahmat Solgi; Writing the original draft: Rahmat Solgi.

Conflict of interest

The authors declared no conflict of interest.

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