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, AbharBranch, Islamic Azad University, Abhar, Iran

[2]Infectious Diseases Research Center, Birjand University of Medical Sciences, Birjand, Iran

Correspondence should be addressed to Rahmat Solgi; rahmatsolgi@yahoo.com

ABSTRACT

The poultry industry in Iran plays a crucial role in the economy and food security of the country. However, it faces numerous challenges, including the presence of parasitic infections such *as Eimeria* spp. The aim of this manuscript is to provide a comprehensive morphological and molecular characterization of *Eimeria* spp. infecting Broiler chickens in Iran. Fresh chicken feces samples (18–45 days old) were collected from a total of 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 and followed by nested PCR using specific primers targeting the ITS1 gene of *Eimeria* spp. 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%. The 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* which were present in 23 out of 101 (22.3%) positive samples .Since vaccination is not currently employed for preventing coccidiosis in broiler production in Iran, the conclusions drawn from this study underscore the significance of implementing reliable chemoprophylactic control measures.

Keywords: *Eimeria*, molecular characterization, Iran

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 triggered 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 like 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). Sequencing and molecular identification techniques can analyze the genetic material of *Eimeria* (5). There is a more reliable identification of *Eimeria* parasites by combining these methods (6). The pathogenicity of various *Eimeria*

species ranges from moderate to severe, underscoring the importance of knowing the 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 that infect domestic poultry in Iran.

2. Materials and Methods

2.1. Design and Collection of Study Animals

Fresh broiler chicken faeces (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 faeces were collected from various points within each broiler chicken house, including the four corners and center, using plastic bags. The oocyst samples exposed to 4 degree to sporulate poorly

.

2.2. Morphological identification

Two grams from each sample were added to 60 milliliters of saturated saline solution. After passing 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 technique, collecting the oocysts for incubation in 2.5% potassium dichromate. They were then allowed to sporulate at 27 ºC for 5 days (9). The oocysts that had formed spores were subsequently stored at 4 ºC for additional molecular examination. *Eimeria* identification was conducted by analyzing the oocysts and sporocysts, taking into account factors like their size, shape, wall composition, and color.

(10).

2.3. Genomic extraction

The oocysts, which had sporulated, were rinsed in TE buffer and then broken down with 0.5mm glass. The DNA was obtained using the Genomic DNA Kit from Qiagen in Hilden, Germany, following the standard protocol with some slight adjustments.

2.4. Molecular methods

The identification of *Eimeria* at the molecular level was carried out using PCR as described in a previous study (9) (Table 1).

Table 1 The primers used in the first PCR assay

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

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 minutes, followed by 35 cycles involving 98 ◦C for 30 ", 52.5-65◦C for 30", and 72 ◦C for 1'. A final extension step at 72 ◦C for 5' 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.

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 ($\chi 2$) for association was used to measure statistical significance.

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).

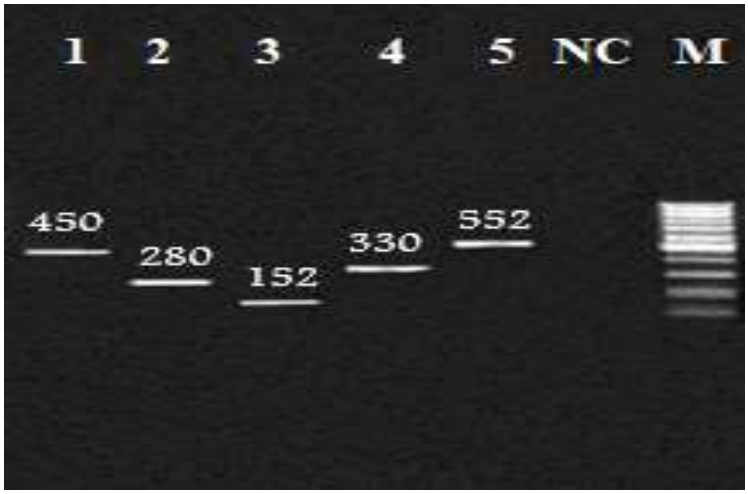

Figure1. Results were obtained by PCR followed by 2% agarose gel electrophoresis. Lines in M show a 100bp DNA marker. Samples include line 1, *E. necatrix*; Line2, *E. acervulina*; Line 3, *E. maxima*; Line4, *E. mitis* and Line5, *E. tenella*; NC, Negative control;M, Marker 100bp.

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.

Table 2 Prevalence of Eimeria spp. of chicken using PCR

Region/farm	***E. acervulina***	***E. maxima***	***E. mitis***	***E. necatrix***	***E. tenella***	**Total farm**	**p**

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%)	

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.

Table 3: Combinations of multiple infections of *Eimeria* species detected in 149 broiler farms in Zanjan provinces, 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%)

4. Discussion

Eimeria spp, a type of protozoan parasite, can result in notable financial losses within the poultry facilities due to decreased productivity, increased mortality, and additional expenses related to disease management (11, 12, 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 aim to investigate the prevalence of coccidiosis and the diversity of *Eimeria* species in local poultry in the 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. While in previous study, unlike our study, the highest prevalence was related to *E. tenella (13).* 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% (15). Analysis of *Eimeria* species present in poultry across the Zanjan region identified the presence of five distinct species: *E. acervulina, E. maxima, E. mitis, E. necatrix,* and *E. tenella*. *E. acervulina* 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% (16). Another study in the same area found a prevalence of *Eimeria* infection in poultry farms to be 21.53% (17). Additionally, in previous study in Iran (18), broiler chickens showed a high infection rate of coccidiosis (75%), which was much higher than 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). Alongside morphological analysis, molecular methods like polymerase chain reaction (PCR) and sequencing have become essential in accurately identifying *Eimeria* species. By targeting specific genetic markers based on conserved ITS1 regions of rDNA (20, 13), the chicken coccidian species can be identify more accurately (21). In the present study, the results of molecular methods were coinciding with morphological descriptions that were agreeing 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

Acknowledgment

We thank the Principal and staff members of department of microbiology of Bijand University of Medical Sciences for providing the necessary facilities to carry out the research work.

Authors' Contribution:

Study concept and design: M Kh, A B Sh, RS

Acquisition of data: M Kh, RS

Analysis and interpretation of data: M Kh, A B Sh, RS

Drafting of the manuscript: RS

Critical revision of the manuscript for important intellectual content: M Kh, AB Sh, RS

Ethical approval Ethical approval for the present study was obtained and approved by the Institutional Animal ethics and Research committee of the Department of veterinary parasitology, Abhar Branch, Islamic Azad University, Abhar, Iran

Competing Interest: there is no any competing of Interest

Data Availability: The data that support the findings of this study are available on request from the corresponding author.

References:

1- Partovi, R., Seifi, S., Alian, S., Nikpay, A. Appraisal of Dietary Prebiotic Supplementation on Meat Properties and Carcass Characteristics of Broiler Chickens After Experimental Infection with Eimeria Species. Iranian Journal of Veterinary Medicine, 2021; 15(3): 346-357.

2. Györke A, Pop L, Cozma V. Prevalence and distribution of Eimeria species in broiler chicken farms of different capacities. Parasite. 2013;20.

3. Shirley MW, Smith AL, Tomley FM. The biology of avian Eimeria with an emphasis on their control by vaccination. Advances in parasitology. 2005;60:285-330.

4. Haug A, Gjevre A-G, Thebo P, Mattsson JG, Kaldhusdal M. Coccidial infections in commercial broilers: epidemiological aspects and comparison of Eimeria species identification by morphometric and polymerase chain reaction techniques. Avian pathology. 2008;37(2):161-70.

5. Carvalho FS, Wenceslau AA, Teixeira M, Carneiro JAM, Melo ADB, Albuquerque GR. Diagnosis of Eimeria species using traditional and molecular methods in field studies. Veterinary parasitology. 2011;176(2-3):95-100.

6. Matsubayashi M, Shibahara T, Matsuo T, Hatabu T, Yamagishi J, Sasai K, et al. Morphological and molecular identification of Eimeria spp. in breeding chicken farms of Japan. Journal of Veterinary Medical Science. 2020;82(5):516-9.

7. Tewari A, Maharana B. Control of poultry coccidiosis: changing trends. Journal of Parasitic Diseases. 2011;35:10-7.

8. Shahraki F, Shariati-Sharifi F, Nabavi R, Jamshidian A. Coccidiosis in Sistan: the prevalence of Eimeria species in native chicken and its histopathological changes. Comparative Clinical Pathology. 2018;27:1537-43.

9. Huang Y, Ruan X, Li L, Zeng M. Prevalence of Eimeria species in domestic chickens in Anhui province, China. Journal of Parasitic Diseases. 2017;41:1014-9.

10. Soulsby EJL. Helminths, arthropods and protozoa of domesticated animals1982.

11. Györke A, Kalmár Z, Pop LM, Şuteu OL. The economic impact of infection with Eimeria spp. in broiler farms from Romania. Revista Brasileira de Zootecnia. 2016;45:273-80.

12. Fornace KM, Clark EL, Macdonald SE, Namangala B, Karimuribo E, Awuni JA, et al. Occurrence of Eimeria species parasites on small-scale commercial chicken farms in Africa and indication of economic profitability. PLoS One. 2013;8(12):e84254.

13. Partovi, R., Seifi, S., Alian, S., Nikpay, A. Appraisal of Dietary Prebiotic Supplementation on Meat Properties and Carcass Characteristics of Broiler Chickens After Experimental Infection with Eimeria Species. Iranian Journal of Veterinary Medicine, 2021; 15(3): 346-357. doi: 10.22059/ijvm.2020.303786.1005095

14. Nasiri, V., Jameie, F., & Morovati Khamsi, H. (2024). Detection, Identification, and Characterization of Eimeria spp. from Commercial Chicken Farms in Different Parts of Iran by Morphometrical and Molecular Techniques. *Acta Parasitologica*, *69*(1), 854-864.

15. Shahraki, F., Shariati-Sharifi, F., Nabavi, R., & Jamshidian, A. (2018). Coccidiosis in Sistan: the prevalence of Eimeria species in native chicken and its histopathological changes. Comparative Clinical Pathology, 27, 1537-1543

16. Nematollahi A, Moghaddam G, Pourabad RF. Prevalence of Eimeria species among broiler chicks in Tabriz (Northwest of Iran). Mun Ent Zool. 2009;4(1):53-8.

17. Yakhchali M, Fakhri M. Prevalence of Eimeria species in free-range chickens of villages of Khoy suburbs, Iran. Veterinary Research & Biological Products. 2017;30(1):206-11.

18. Mehrabi M, Yakhchali M. Study on frequency and diversity of Eimeria species in broiler farms of Hamedan suburb, Iran. 2014.
19. Haug A, Thebo P, Mattsson JG. A simplified protocol for molecular identification of Eimeria species in field samples. Veterinary parasitology. 2007;146(1-2):35-45.
20. Hamidinejat H, Shapouri MS, Mayahi M, Borujeni MP. Characterization of Eimeria species in commercial broilers by PCR based on ITS1 regions of rDNA. Iranian journal of parasitology. 2010;5(4):48.
21. Kumar S, Garg R, Moftah A, Clark EL, Macdonald SE, Chaudhry AS, et al. An optimised protocol for molecular identification of Eimeria from chickens. Veterinary parasitology. 2014;199(1-2):24-31.