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Original Article

Morphometric and Molecular Identification of *Eimeria* Species Isolated from Turkeys in Central Iran

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Introduction: Coccidiosis, caused by host-specific *Eimeria* species, is a major parasitic disease threatening the turkey industry. This study aimed to isolate and identify *Eimeria* species circulating in turkeys from central Iran using morphological and molecular methods.

Materials and Methods: A total of 500 samples (280 from slaughterhouse digestive tracts, 120 from litter of 6 industrial farms, and 100 from traditional farms) were collected from Alborz, Tehran, Zanjan, and Qazvin provinces. Samples were examined using direct smear and sucrose flotation. Oocysts from positive samples were purified, sporulated, and identified morphometrically. Species-specific polymerase chain reaction (PCR) targeting the mitochondrial *Cytochrome c oxidase subunit I* gene was used for molecular confirmation. Sequencing and phylogenetic analysis were performed for selected isolates.

Results: Only 12 samples (2.4%) were positive, all from asymptomatic turkeys on traditional farms. Morphological identification revealed four species: *Eimeria meleagrimitis* (n=8), *Eimeria meleagridis* (n=2), *Eimeria dispersa* (n=1), and *Eimeria gallopavonis* (n=1). PCR results were 100% concordant with morphometric identification.

Conclusion: This study confirms the presence of four *Eimeria* species in Iranian turkeys, with *E. meleagrimitis* being the most prevalent. The complete agreement between morphological and molecular methods validates this diagnostic approach. The isolation of these species from asymptomatic traditional flocks highlights their role as reservoirs, emphasizing the need for integrated control strategies.

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Introduction

Coccidiosis is one of the most economically detrimental diseases in the poultry industry worldwide, causing substantial annual losses due to increased mortality, poor feed conversion, reduced weight gain, and treatment costs [1]. While most research has focused on broiler chickens, coccidiosis in turkeys (*Meleagris gallopavo*) is of growing concern due to the rapid expansion of the turkey farming industry and the inherent susceptibility of this host [2]. The disease is caused by several species of the genus *Eimeria* (Apicomplexa: Eimeriidae), which are highly host-specific obligate intracellular parasites [3].

To date, seven *Eimeria* species have been reported to naturally infect turkeys: *E. meleagrimitis*, *E. adenoeides*, *E. gallopavonis*, *E. meleagridis*, *E. dispersa*, *E. innocua*, and *E. subrotunda*. These species vary significantly in pathogenicity and site of infection within the intestine [4]. *E. meleagrimitis* and *E. adenoeides* are considered the most pathogenic, causing severe lesions in the small intestine and ceca, respectively [5]. Accurate species identification is the cornerstone of any effective control program, whether it involves anticoccidial drugs or live vaccination strategies [6].

Traditional diagnosis relies on the morphological examination of oocysts (size, shape, and color) and the assessment of gross intestinal lesions. However, this approach has inherent limitations, including overlapping morphometrics between species, variability due to host and environmental factors, and the need for specialized expertise [7]. To overcome these challenges, molecular techniques, particularly polymerase chain reaction (PCR) targeting conserved genetic markers, have become essential. The mitochondrial *Cytochrome c oxidase subunit I (COI)* gene is a powerful "DNA barcode" for species-level identification and phylogenetic studies of *Eimeria* species due to its high evolutionary rate and lack of introns [8, 9].

In Iran, several studies have reported the prevalence of turkey coccidiosis [10-12]. However, comprehensive data combining rigorous morphological and molecular identification, alongside the establishment of a local strain bank, are scarce. Therefore, this study was designed to: (i) isolate and identify *Eimeria* species circulating in turkey flocks in central Iran using both morphometric and molecular (PCR/sequencing of the *COI* gene) methods, and (ii) store the purified, well-characterized pathogenic strains in a local bank for future research, vaccine development, and drug efficacy studies.

Materials and Methods

Study area and sample collection

This cross-sectional study was conducted between May 2023 and October 2024. A total of 500 samples were collected from turkeys reared in industrial and traditional systems across four provinces (Alborz, Tehran, Zanjan and Qazvin) in central Iran. Samples comprised 280 complete digestive tracts from a slaughterhouse in Zanjan, which were

collected randomly on different days of the week to ensure representativeness of birds from various regions, 120 litter samples from 6 industrial farms, and 100 samples from traditionally reared turkeys. Sampling targeted birds with or without clinical signs such as diarrhea, lethargy, and reduced growth.

Parasitological examination

Fecal and intestinal content samples were examined microscopically using both direct smear and sucrose flotation (Sheather's solution, specific gravity 1.20) techniques [13]. Positive samples were mixed with 2.5% potassium dichromate ($K_2Cr_2O_7$) and incubated at 25 °C for 7-14 days to allow oocyst sporulation. Oocysts were purified using flotation and centrifugation. Morphological identification was performed on at least 20 sporulated oocysts per sample using an optical microscope (Nikon Eclipse Ci, Japan) equipped with a digital camera. Length and width were measured using ImageJ software (version 1.53t, National Institutes of Health, USA), and the shape index (length/width ratio) was calculated. Species were identified according to standard keys [4, 14].

Oocyst purification and strain banking

After morphological identification, a portion of the purified, sporulated oocysts from each positive sample was transferred to a 2.5% potassium dichromate ($K_2Cr_2O_7$) solution and stored at 4 °C for short-term maintenance (up to 6 months). For long-term preservation, the remaining purified oocysts were cryopreserved in liquid nitrogen (-196 °C) using a standard protocol with 10% glycerol as a cryoprotectant. This established the native strain bank at the Razi Vaccine and Serum Research Institute.

DNA extraction and PCR

Genomic DNA was extracted from approximately 1×10^5 purified oocysts using a modified phenol-chloroform method as described by Boom et al. [15]. The quality and quantity of extracted DNA were assessed by spectrophotometry (Nano Drop, Thermo Scientific).Species-specific PCR assays were performed targeting the *COI* gene using previously validated primers [9]. Primer sequences, expected amplicon sizes, and annealing temperatures are detailed in Table 1. The PCR reaction mixture (25 µL) contained 12.5 µL of Master Mix (Amplicon, Denmark), 10 pmol of each primer, and 2 µL of DNA template. The PCR cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 39 cycles of 94 °C for 30 s, annealing at a gradient temperature of 50–62 °C for 45 s, and 72 °C for 60 s, with a final extension at 72 °C for 10 min.

Sequencing and Phylogenetic Analysis

PCR products were purified and sequenced bidirectionally (Sanger dideoxy method) by a commercial company (Pishgam Biotech, Iran). Sequences were edited and assembled using Chromas and Sequencher software. Homology searches were performed using BLASTn against the GenBank database. Multiple sequence alignments were conducted using CLUSTALW2 and BioEdit. A phylogenetic tree was constructed using the Maximum Likelihood (ML) method with 1000 bootstrap replicates in MEGA X software [16].

Table 1. List of species-specific primers targeting the mitochondrial *Cytochrome c oxidase subunit I* gene used for molecular identification of Eimeria species isolated from turkeys in Iran

Target Species	Primer Name	Sequence (5' → 3')	Amplicon size (bp)	Annealing temp. (°C)
E. meleagrimitis	E.mel-COI-F	CTCAAGTTTCCTATCCTCAG	554	50
	E.mel-COI-R	GCGTACCAGATATCTAAGGAG		
E. meleagridis	E.melg-COI-F	CCTCAGTAGATTTAATTGTC	1012	58
	E.melg-COI-R	TTAGAAGATTAGGGAATATAA		
E. dispersa	E.dis-COI-F	ACAGCTATTATGTTAATTGGT	451	55
	E.dis-COI-R	GCATACCAAGTATCTAATGAA		
E. gallopavonis	E.gal-COI-F	AGAGTGAATTGTGTATCACTATTA	861	62
	E.gal-COI-R	GAGATAATACGAAATGGAAGTGG		

Table 2. Morphometric characteristics of sporulated oocysts of *Eimeria* species isolated from turkeys in central Iran

Species	Number of isolates	Oocyst shape	Length (µm) (Mean ± SD)	Width (µm) (Mean ± SD)	Shape index (L/W) (Mean ± SD)
E. meleagrimitis	8	Ovoid to subspherical	19.1 ± 0.9	16.4 ± 0.7	1.16 ± 0.04
E. meleagridis	2	Subspherical	18.5 ± 0.8	16.5 ± 0.6	1.12 ± 0.03
E. dispersa	1	Broadly ovoid	26.2	20.3	1.29
E. gallopavonis	1	Elongated ovoid	26.5	17.5	1.51

Results

Morphological identification

Of the 500 samples examined, only 12 (2.4%) were found to be positive for *Eimeria* oocysts. All positive samples originated from traditionally raised turkeys that displayed no clinical signs. No oocysts were detected in samples from industrial farms. Based on morphological characteristics, the positive samples were identified as: *E. meleagrimitis* (n=8, 66.7%), characterized by ovoid to subspherical oocysts (mean size 19.1 × 16.4 µm, shape index 1.16); *E. meleagridis* (n=2, 16.7%), subspherical oocysts (mean 18.5 × 16.5 µm); *E. dispersa* (n=1, 8.3%), broadly ovoid with striated wall (mean 26.2 × 20.3 µm); and *E. gallopavonis* (n=1, 8.3%), elongated ovoid with a distinct micropyle (mean 26.5 × 17.5 µm) (Table 2).

Molecular identification and phylogeny

Species-specific PCR targeting the *COI* gene successfully amplified fragments of expected sizes for all 12 positive samples. The PCR results were in 100% agreement with the morphological identification (Table 3), confirming the presence of the four aforementioned species.

BLAST analysis revealed 100% sequence identity with other *E. meleagrimitis* isolates (e.g., KC346351.1, HG793050.1). Phylogenetic trees using Maximum Likelihood (Fig. 1) showed similarity among *Eimeria* spp. isolates in this study and with some other *Eimeria* spp. in animals from different parts of the world that were deposited in GenBank.

Table 3. Comparison of morphometric and molecular identification of *Eimeria* species.

Sample ID	Morphological diagnosis	PCR result (*COI* gene)	Amplicon size (bp)
VN1-VN8	*E. meleagrimitis*	*E. meleagrimitis*	554
VN9-VN10	*E. meleagridis*	*E. meleagridis*	1012
VN11	*E. dispersa*	*E. dispersa*	451
VN12	*E. gallopavonis*	*E. gallopavonis*	861

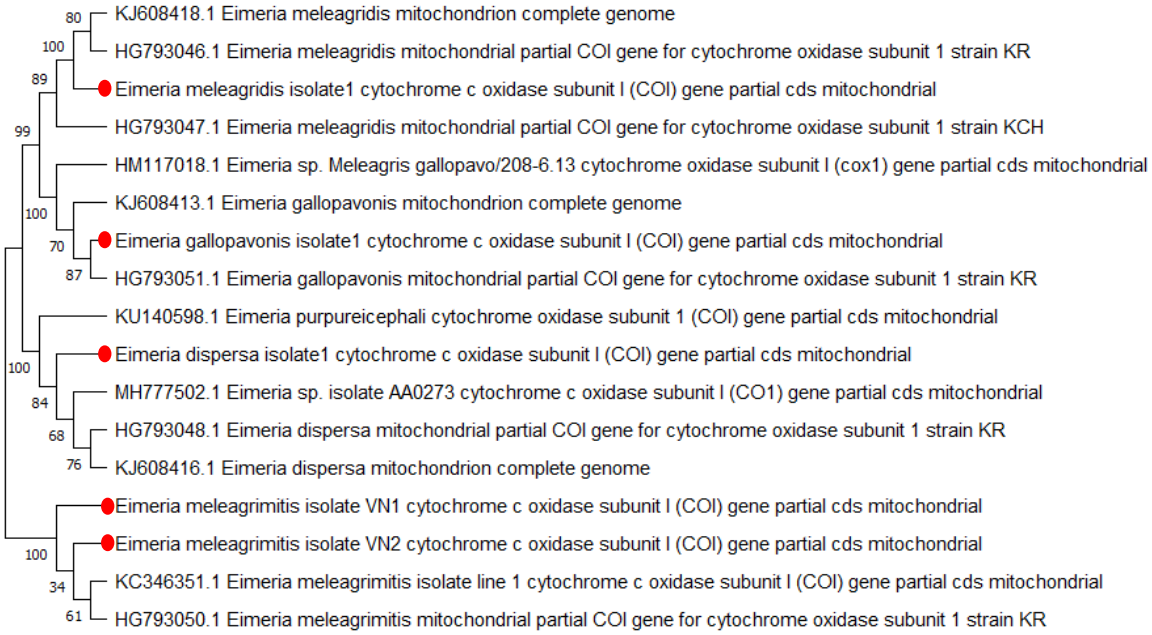

Fig. 1. Phylogenetic connections were acquired from 5 affirmative sequences in this investigation with sequences from other investigations utilizing the Maximum Likelihood technique based on their *Cytochrome c oxidase subunit I* gene sequences reinforced by 1000 bootstrap replicates. The sequences obtained in this study are indicated by red circles. Bootstrap values (≥70%) are shown at the nodes.

Discussion

This study presents the first integrated morphometric and molecular characterization of *Eimeria* species in turkeys from central Iran, culminating in the isolation and banking of native strains. The most striking finding was the low overall prevalence of detectable oocyst shedding (2.4%) and the exclusive detection of infection in clinically healthy, traditionally managed flocks. This observation strongly suggests that traditional rearing systems act as a silent reservoir for *Eimeria* species. Continuous low-level exposure to oocysts in these environments likely promotes the development of protective immunity without overt clinical disease [17]. Conversely, the absence of detectable oocysts in industrial farms may reflect the successful application of anticoccidial drugs, stringent hygiene protocols (e.g., dry litter, all-in/all-out systems), or very low-level shedding below the detection threshold of microscopy

[18]. Among the identified species, *E. meleagrimitis* was the most prevalent (66.7%). This finding is consistent with global reports where *E. meleagrimitis* is recognized as one of the most prevalent and pathogenic species in turkeys [4, 5]. The presence of *E. gallopavonis* and *E. meleagridis*, both considered pathogenic, alongside the less pathogenic *E. dispersa*, indicates a diverse parasite community circulating in the region [19]. The isolation of *E. dispersa*, known to have a broader host range (including chickens and quail), raises questions about potential cross-species transmission in mixed or non-biosecure farming systems [20]. This finding underscores the need for strict biosecurity measures, especially in regions where different poultry species are reared in close proximity.

The complete concordance between morphological and molecular methods (100%) validates our approach. While microscopy remains a cost-effective and rapid field tool, its limitations are well-known [7]. Our study confirms that for distinct species like *E. meleagrimitis*, experienced observers can achieve high accuracy. However, the use of PCR and *COI* sequencing is indispensable for definitive diagnosis, resolving ambiguous cases, and conducting phylogenetic analyses [8, 9].

The phylogenetic analysis revealed a close relationship between our Iranian *E. meleagrimitis* isolates (GenBank: PV769790, PV769791) and those previously reported from other parts of the world, such as Europe (e.g., KC346351.1) and Asia (e.g., HG793050.1), showing 100% sequence identity in the *COI* gene. This high degree of genetic conservation suggests a low level of geographical variation within this species, which is advantageous for the development of broadly effective diagnostic tools and vaccines. However, further studies using more variable genetic markers are needed to confirm this finding.

The primary practical implication of this study is the establishment of a bank of well-characterized, native *Eimeria* strains at the Razi Vaccine and Serum Research Institute. These strains are a crucial national asset for: (I) evaluating the efficacy of commercial anticoccidial drugs and detecting emerging drug resistance; (II) standardizing challenge models for vaccine development and testing; and (III) conducting detailed pathogenicity studies. The identification of traditional flocks as reservoirs highlights the necessity of extending biosecurity and monitoring programs beyond industrial farms. A holistic, integrated control strategy combining strategic anticoccidial use, improved litter management, and potentially targeted vaccination is essential for sustainable turkey production in Iran [6, 17].

Limitations of the study include the small number of positive samples, the lack of quantitative PCR (qPCR) to assess infection intensity, and no assessment of potential drug resistance in the isolated strains. Future studies should focus on full-genome sequencing of these isolates and experimental challenge studies to determine their precise pathogenicity. Additionally, the seasonal dynamics of the infection were not assessed in this study, which could provide further insights into the epidemiology of turkey coccidiosis in the region.

Conclusion

This study successfully isolated and identified four *Eimeria* species—*E. meleagrimitis*, *E. meleagridis*, *E. dispersa*, and *E. gallopavonis*—from traditionally raised turkeys in central Iran. The 100% correlation between morphometric and PCR/COI sequencing data confirms the accuracy of our diagnostic pipeline. The establishment of a local strain bank provides a vital research tool for the Iranian poultry industry to combat coccidiosis effectively.

Ethical Considerations

The Ethics Committee of Razi Vaccine and Serum Research Institute, Karaj, Iran approved the study protocol.

Funding Statement

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Conflict of Interest

The authors declared no conflict of interest.

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Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Authors' Contributions

V.N and, F.J were involved in the conception of the research idea and methodology design, supervision, performed data analysis and interpretation and in the methodology and data analysis; V.N, F.J and Hp prepared and critically revised the manuscript for publication and revision. All authors read and approved the final manuscript.

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