Original Article

Molecular Detection of *Eimeria zaria* in Iran: Targeting the *ITS-2* Gene in Broiler Chicken Fecal Samples

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ABSTRACT

Background: Three newly identified *Eimeria* species (*Eimeria lata*, *Eimeria nagambie*, and *Eimeria zaria*) were first discovered in Australia. Initially recognized as unidentified genotypes (operational taxonomic units X, Y, and Z), these species have since been detected across multiple continents. Genomic analyses confirmed their distinction from classical *Eimeria* species, leading to their formal classification in 2021. Accurate characterization of these cryptic species requires advanced molecular tools and isolation of pure strains.

Objectives: Currently, controlling chicken coccidiosis continues to rely largely on vaccination and the use of anticoccidial medications. Since these drugs target *Eimeria* species differently and rarely provide similar effectiveness, and typically there is no cross-protection, accurate identification of regional species and strains is vital for selecting the right vaccines and treatments.

Methods: Fecal samples from 8 broiler farms in Mazandaran Province, Iran, were collected and confirmed to contain *Eimeria* microscopically. DNA was extracted from pooled oocysts of each farm, and species-specific polymerase chain reaction (PCR) targeting the internal transcribed spacer 2 gene was performed using designated primers.

Results: *E.lata* and *E. nagambie* were not detected, but *E. zaria* was detected in two farms from Sari and Behshahr cities.

Conclusion: The samples were selectively collected from Mazandaran Province in northern Iran, a region known for its dense poultry production and humid climate, leading to high coccidiosis prevalence. This area's significant role in the poultry industry makes it important for studying less-characterized *Eimeria* species. The findings reported in this study suggest the circulation of *E. zaria*, along with possibly two other species, in the region, emphasizing the need to revise *Eimeria* taxonomy and further investigate species diversity.

Keywords: Broiler, Coccidiosis, *Eimeria zaria*, Iran, Polymerase chain reaction (PCR)

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Introduction

Coccidiosis is one of the most detrimental parasitic diseases in poultry farming, caused by intracellular protozoa of the genus *Eimeria* (Schoch et al., 2020). Despite significant advances in diagnosis, control, and treatment, it remains one of the most harmful poultry diseases worldwide, with young birds being particularly susceptible. Depending on several determinants, including the species of *Eimeria*, the host's immune status, and environmental conditions, the clinical outcomes may range from asymptomatic cases to reduced productivity and high mortality due to severe intestinal tissue destruction (Blake et al., 2020; Swayne, 2020).

Seven species of *Eimeria* have been identified worldwide, with their morphological and biological characteristics, as well as nucleotide sequences, well-documented. *Eimeria praecox* and *Eimeria mitis* exhibit the lowest pathogenicity and often subclinically impair productivity. *Eimeria acervulina* and *Eimeria maxima* exhibit higher pathogenicity and are more commonly associated with clinical symptoms. The most severe lesions, frequently appearing as hemorrhages in the small intestine and ceca, are associated with *Eimeria necatrix*, *Eimeria brunetti*, and *Eimeria tenella*. These species differ in oocyst morphology, lesion location and appearance, the size of developmental stages within tissues, minimum prepatent periods, and immunogenicity. However, certain overlaps in these features can be misleading when relying on traditional criteria (Johnson & Reid, 1970; Swayne, 2020; Mesa-Pineda et al., 2021).

Two isolates, *Eimeria mivati* and *Eimeria hagani*, are sometimes referred to as the eighth and ninth chicken *Eimeria* species. However, supporting evidence for this classification is insufficient (Chapman, 2003; Vrba et al., 2011). The discovery and identification of three new *Eimeria* species, known to cause chicken coccidiosis, was prompted by reports of high mortality among breeder chickens on an Australian farm. In 2007, Morris et al. detected three unknown genotypes that did not match any previously known isolates. These genotypes were subsequently termed as operational taxonomic units (OTUs) X, Y, and Z (Morris et al., 2007). Surveys have confirmed the presence of these genotypes in Africa, North America, South America, and Asia (Fornace et al., 2013; Clark et al., 2016; Hauck et al., 2019; Jaramillo-Ortiz et al., 2023). Complementary studies using more precise methods such as next-generation sequencing and mitochondrial genome analysis have revealed significant genomic differences between these isolates and the classical *Eimeria* species (Godwin & Morgan, 2014; Hinsu et al., 2018; Hauck et al., 2019; Soares Júnior et al., 2023). In 2021, these isolates were formally named *Eimeria lata* (OTU-X), *Eimeria nagambie* (OTU-Y), and *Eimeria zaria* (OTU-Z) (Blake et al., 2021). These species exhibit distinct biological characteristics; however, information on their epidemiology, pathogenesis, and response to vaccination remains limited (Blake et al., 2021). Accurate detection and comprehensive insight into these cryptic species depend on precise molecular techniques and the isolation of pure isolates.

Although alternative or complementary strategies, such as the use of phytogenic additives, have shown promising results in recent years, the control of coccidiosis primarily relies on the use of anticoccidial drugs and immunization via trickle infection and or vaccination (Swayne, 2020; Ebrahimi, 2023). Anticoccidial drugs possess unique mechanisms of action, and nearly none are equally effective against all *Eimeria* species (Swayne, 2020). Furthermore, cross-protection among species is minimal (Rose & Long, 1962). Therefore, the identification of field and regional isolates is crucial for the development and selection of appropriate drugs and vaccines. This study aimed to detect and identify the presence of newly classified *Eimeria* species in broiler farms in northern Iran using ITS-2-based polymerase chain reaction (PCR) analysis.

Materials and Methods

Fecal sample collection

Fecal samples were collected from 8 *Eimeria*-positive commercial broiler farms located in Mazandaran Province, Iran. Sampling was conducted using 50 mL polypropylene tubes, following the methodology described previously (Kumar et al., 2014). Each tube was initially filled with 5 mL of a 2% (w/v) potassium dichromate solution. Starting from one corner of the poultry house, a 'W' shaped path was followed across the length of the facility to cover the entire area and minimize sampling bias systematically. Along this path, fresh fecal material was collected every 2 to 5 steps, continuing until the tube reached the 10-mL mark. Depending on the size and capacity of each farm, between 3 and 5 tubes were collected per site. The tubes were then vigorously shaken to ensure thorough homogenization of the contents. Subsequently, the samples were transported to the laboratory and stored at 4 ˚C until further analysis.

Oocysts isolation and DNA extraction

Following microscopic confirmation of *Eimeria* presence in the samples, oocysts from each tube were pelleted according to the procedure described by Kumar et al. (2014). Subsequently, all oocysts collected from separate tubes of the same farm were pooled and stored in 2 mL microtubes containing 2% (w/v) potassium dichromate solution at 4 ˚C. For DNA extraction, the method described by Jaramillo-Ortiz et al. (2023) was applied with slight modifications to accommodate available materials. A 200 µL aliquot of the original sample was transferred to a new microtube, and the oocysts were pelleted by centrifugation (~6000×g, 1 min) (Sigma Zentrifugen, Germany). The pellet was then resuspended in 2 mL of triple-distilled water, followed by centrifugation (~6000×g, 1 min) after each wash to remove residual potassium dichromate. The resulting pellet was suspended in 200 µL of distilled water, and approximately 200 mg of glass beads (0.5 to 1 mm diameter) were added to the microtube. Using the maximum speed on the beadbeater (brand not recorded), the tube contents were shaken for 3 minutes to disrupt the oocyst physically. The subsequent steps were carried out following the DNA extraction kit for stool samples protocol (MBST, 2025).

Eimeria species-specific PCR

Each reaction consisted of 1 µL of genomic DNA template, 20 pmol of forward and reverse primers (Table 1), and Taq 2x Master Mix (Ampliqon, Denmark), with molecular-grade water added to bring the total volume to 25 µL. The amplification steps were performed using a thermal cycler (SensoQuest GmbH, Germany), starting with an initial denaturation at 94 ˚C for 5 minutes, followed by 30 cycles of denaturation at 94 ˚C for 30 s, annealing at the variable temperature shown in Table 1 for 30 seconds, and extension at 72 ˚C for 1 minute. A final extension was performed at 72 ˚C for 10 min. PCR product sizes were checked by running samples on a 2% (w/v) agarose gel (Dena Zist Asia, Iran) prepared in 1x Tris-borate-EDTA (TBE) buffer, containing 0.01% (v/v) Safe Stain (YTA, Iran). Visualization was done using a KIACCD Gel Documentation System (Kiagene, Iran).

Results

Among the identified species, *E. lata* and *E. nagambie* were not detected using the applied method. In contrast, a clear band at the expected size of 147 bp confirmed the presence of *E. zaria* in two farms (Figure 1). Positive samples were obtained from two farms located in the cities of Sari and Behshahr, situated in the central and eastern parts of Mazandaran Province, respectively (Figure 2).

Discussion

Despite a history of vaccination, persistent coccidiosis and increased mortality in a broiler-breeder farm in Victoria, Australia, prompted Morris et al. (2007) to conduct a molecular investigation of the causative protozoa. The primers used in this study targeted the internal transcribed spacer 2 (ITS-2) region of the ribosomal DNA specific to the family Eimeriidae and the genus *Eimeria*. Analysis of the capillary electrophoresis profiles of PCR products consistently revealed two patterns, pX and pY, that did not correspond to any of the 7 previously recognized species. Additionally, based on the authors' earlier work, another distinct genotype had also been identified. This 2007 study marked the first report of the presence of isolates with significant genomic divergence, later designated as OTU-X, OTU-Y, and OTU-Z (Morris et al., 2007). By sequencing the amplicons amplified using the specific primers from the study mentioned above, Cantacessi et al. (2008) identified 3, 3, and 4 distinct sequences of varying lengths corresponding to OTU-X, OTU-Y, and OTU-Z, respectively. The first investigation of *Eimeria* species diversity in chickens outside Australia, including the three cryptic genotypes, was conducted

Table 1. Parameters of species-specific primers used for detecting 3 newly identified *Eimeria* species

Target Species	Target Gene	Primer Name	Sequence (5′ - 3′)	Annealing (˚C)	Amplicon Size (bp)	Ref.
E. lata	*E. lata ITS-2*	OTU-Xf2	GGGTAGAGCCAGGGGTAGAG	58	1018	Blake et al. (2021)
		OTU-Xr2	CGTAGTCCCAAGTGCCAACT			
E. nagambie	*E. nagambie ITS-2*	OTU-Yf1	CAAGAAGTACACTACCACAGCATG	56	346	Fornace et al. (2013)
		OTU-Yr1	ACTGATTTCAGGTCTAAAACGAAT			
E. zaria	*E. zaria ITS-2*	OTU-Zf1	TATAGTTTCTTTTGCGCGTTGC	58	147	
		OTU-Zr1	CATATCTCTTTCATGAACGAAAGG			

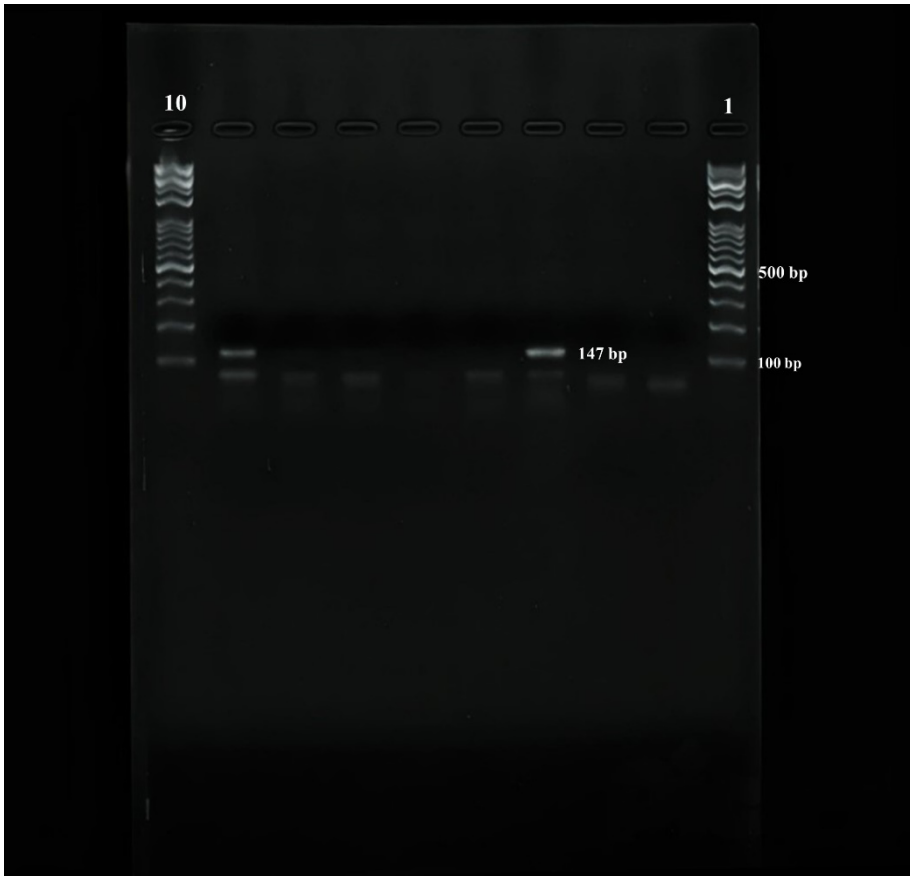

Figure 1. Detection of the 147 bp target amplicon using primers OTU-Zf1 and OTU-Zr1

Note: Lanes: 1 and 10, 0.1-10 kb ladder (SMOBIO, Taiwan); 2, negative control (distilled water); 3 and 5-8, negative samples; 4 and 9, positive samples for *E. zaria*.

by Fornace et al. (2013). Their study of samples from Africa revealed the presence of OTU-X and OTU-Z in small-scale poultry farms in Ghana, Tanzania, and Zambia. In 2014, Godwin and Morgan (2014), while developing a new molecular method for the identification of the seven recognized *Eimeria* species along with the three OTUs, detected and identified OTU-X, OTU-Y, and OTU-Z in Australia. Subsequently, they expanded their research on a larger scale to investigate the diversity and prevalence of these parasites. They once again confirmed the presence of the three cryptic genotypes in both industrial and backyard poultry flocks of Australia (Godwin & Morgan, 2015). In 2016, Jatau et al. (2016) examined samples from 12 chicken farms in the vicinity of Zaria, Nigeria. In addition to reporting the first detection of OTU-Z outside Australia, they also identified OTU-X and OTU-Y in the Nigerian poultry population. The first molecular survey of *Eimeria* species diversity in chickens, considering three new genotypes with a global distribution, revealed the presence of OTU-X and OTU-Z in Ghana, Tanzania, Nigeria, Uganda, Zambia, India, and Venezuela, as well as OTU-Y in Nigeria. Despite the number and geographic extent of sampling, none of these genotypes had been documented in the Northern Hemisphere at that time (Clark et al., 2016). Subsequently, OTU-Y and OTU-Z were documented in India (Hinsu et al., 2018). In 2019, Hauk et al. (2019) reported the presence of OTU-X, OTU-Y, and OTU-Z in chicken production farms in the United States, marking the first record of these genotypes in the Northern Hemisphere. Although previous detections were conducted outside this region, our study provides new regional data by identifying *E. zaria* in two Iranian broiler farms.

In 2021, Blake et al. published data demonstrating that the OTUs possess sufficient genetic and biological differences to be considered distinct species. They proposed the name *E. lata* for OTU-X, due to its wider oocyst. OTU-Y and OTU-Z were designated as *E. nagambie* and *E. zaria*, respectively, based on their locations of isolation Blake et al. (2021). Soares Junior et al. (2023) reported the presence of *E. lata*, *E. nagambie*, and *E. zaria* in alternative poultry production systems in Brazil. There was no evidence suggesting the existence

Figure 2. Approximate locations of the sampled farms

Note: Green markers indicate farms positive for *E. zaria;* red markers indicate farms with negative results.

of a new chicken *Eimeria* species in Europe until 2023. In the same year, Jaramillo Ortiz et al., (20023) investigated the presence of these species in poultry farms with fewer than 10000 chickens across Europe. Using species-specific primers targeting the *ITS-2* gene, they identified *E. zaria* in two samples from Italy and Greece (Jaramillo-Ortiz et al., 2023). The origins and determinants driving the spread of these species are not yet fully understood; however, a combination of global trade and transportation of poultry and their products, the movement of wild birds, and genetic recombination among different isolates has been proposed (Clark et al., 2016; Jaramillo-Ortiz et al., 2023).

Given the insufficient and incomplete data on morphological and biological characteristics for species differentiation based on macroscopic and microscopic observations, the detection of new strains of chicken *Eimeria* has relied primarily on molecular techniques, which often offer greater sensitivity and specificity (Blake et al., 2021; Soares Júnior et al., 2023). Identification of nucleotide sequences such as *ITS-1* (Fornace et al., 2013; Clark et al., 2016; Jatau et al., 2016; Soares Júnior et al., 2023), *18S rRNA* (Hinsu et al., 2018; Hauck et al., 2019; Blake et al., 2021; Soares Júnior et al., 2023), and *5S rRNA* (Blake et al., 2006; Fornace et al., 2013; Clark et al., 2016) genes is often combined with microscopic examination of oocysts to screen for the presence of *Eimeria* parasites. For species differentiation, sequences of ITS-2 (Morris et al., 2007; Cantacessi et al., 2008; Fornace et al., 2013; Godwin & Morgan, 2014; Clark et al., 2016; Jatau et al., 2016; Hauck et al., 2019; Blake et al., 2021; Jaramillo-Ortiz et al., 2023; Soares Júnior et al., 2023), cytochrome c oxidase I (Hauck et al., 2019; Blake et al., 2021; Jaramillo-Ortiz et al., 2023), and sequence characterized amplified region (Fornace et al., 2013; Clark et al., 2016; Hinsu et al., 2018; Jaramillo-Ortiz et al., 2023) markers are commonly used. Screening *Eimeria*-positive samples is particularly important because most molecular identification methods require multiple reactions and incur high costs per sample (Godwin & Morgan, 2014). Another limitation concerns the detection threshold: Under ideal conditions, the minimum DNA amount required for amplification of the *ITS-2* gene and detection on an agarose gel is approximately 5 to 10 pg, corresponding to about 5 to 50 oocysts (Woods et al., 2000).

Information regarding the efficacy of existing drugs, vaccine escape by these genotypes, and the impact of challenge trials on chicks remains limited. Although anticoccidial medications appear to have satisfactory efficacy, commercial vaccines may only partially reduce the replication of certain elusive isolates, without fully preventing their proliferation or clinical manifestations (Hauck et al., 2019; Blake et al., 2021). Conducting such

studies is challenging without access to pure isolates, morphometric and pathological evaluations, as well as long nucleotide sequence data. Cellular immunity, as a critical component of adaptive immunity, plays a more significant role than humoral immunity in protecting birds against coccidiosis (Kim et al., 2019). T cells are central to this protective response; however, their immunity induced by exposure to a single *Eimeria* species often provides limited or no cross-protection against heterologous species (Rose & Long, 1962; Joyner, 1969; Blake et al., 2011). This strict specificity is sometimes observed even among different strains within the same species, which explains why many commercial vaccines include multiple strains of *E. maxima* (Joyner, 1969; Blake et al., 2011; Soutter et al., 2020). Ideally, local and regionally circulating strains should be prioritized for research and vaccine development (Soutter et al., 2020). This result underscores the importance of continuously evaluating species diversity and searching for novel *Eimeria* species in poultry, a task that currently seems unlikely without the use of molecular methods. Our findings support this view, demonstrating the utility of ITS-2–based PCR in detecting under-recognized *Eimeria* species such as *E. zaria* in high-risk regions.

Conclusion

The evaluated samples were non-randomly collected from the Mazandaran Province in northern Iran. This province, due to its high poultry production density and humid, temperate climate, shows a high prevalence of coccidiosis among commercial poultry populations. An additional rationale for selecting this region was the high density of poultry operations belonging to the upper tiers of the broiler and layer production pyramid, which significantly contribute to the country's economy and food security. These characteristics make the region a strategic priority for investigating the presence of less-characterized and recently described *Eimeria* species. The present study is the first detection of *E. zaria* in Iran, suggesting the possible circulation of this species, potentially along with the other two, *E. lata* and *E. nagambie*, in Iran and the Middle East. These findings highlight the need for a revision of current taxonomic frameworks and further investigations into the species diversity of chicken *Eimeria* in this region.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization, supervision, funding acquisition and resources: Jamshid Razmyar; Data collection: Amirhossein Sharifi_Moghadam and Amin Riahi; Investigation, methodology and writing: Jamshid Razmyar, Amirhossein Sharifi_Moghadam, and Azam Yazdani; Data analysis: All authors.

Conflict of interest

The authors declared no conflict of interest.

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