

Molecular detection of *Eimeria* species in backyard chickens in the Nakhchivan Autonomous Republic

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ABSTRACT

Coccidiosis poses a significant threat to the health and productivity of poultry worldwide. However, there is currently a lack of comprehensive scientific data on the prevalence and species diversity of *Eimeria* in the Nakhchivan Autonomous Republic. Despite the widespread rearing of backyard chickens in the region, no molecular-level research has yet been conducted to identify the circulating *Eimeria* species, leaving a critical gap in regional epidemiological knowledge. The aim of this study was to determine the prevalence of *Eimeria* species in backyard chickens in the Nakhchivan Autonomous Republic and to conduct a molecular analysis for the first time to investigate the presence of seven recognized *Eimeria* species in the study area. A total of 186 chicken faecal samples were collected from 65 backyard poultry farms in 58 locations from April to September of 2024. The study revealed that 48.9% (91/186) of the faecal samples were positive for *Eimeria* spp., and the mean OPG value was 1790.1. *Eimeria* positivity was 60% (63/105) in the group older than six months and 34.6% (28/81) the group younger than six months. The highest prevalence was recorded in the autumn (September) at 64.9%, followed by spring (46.4%) and summer (36.7%) months. The following species were detected in the molecular analysis: *E. acervulina* (83.5%, 76/91), *E. maxima* (71.4%, 65/91), *E. tenella* (61.5%, 56/91), *E. necatrix* (46.1%, 42/91), *E. mitis* (46.1%, 42/91), *E. praecox* (41.8%, 38/91), and *E. brunetti* (16.5%, 15/91). All positive samples exhibited multiple infections involving three to five *Eimeria* species. The study results revealed that *Eimeria* species were widespread in the region, suggesting the possibility of subclinical coccidiosis, which is characterized by low to moderate levels of oocyst shedding, despite the absence of acute infections. Since the disease leads to economic losses, further research is needed to develop sustainable prevention and control methods.

Keywords: Backyard chicken, Coccidiosis, Nakhchivan, Nested PCR

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Introduction

The poultry industry is one of the primary sources of animal protein worldwide. Over the past few decades, the increasing demand for affordable poultry products has led to expansion of both extensive and intensive poultry farming (14, 18, 42). Although large industrial farms dominate chicken production, some consumers who prefer more natural diets are shifting toward alternative farming

systems such as free-range and organic (26). Additionally, people in rural areas raise poultry in backyards to meet their protein needs and supplement their income by selling eggs and chicken carcasses.

Backyard chickens in Nakhchivan typically live in simple coops with poor hygienic conditions, a lack of bedding, and soil floors. Most of these chickens are fed grain in the coop. They are allowed to forage for food in

the morning to search for food and kept in the shelter at night. These unfavorable conditions, coupled with their ability to roam freely in the yard and common areas around the house alongside other chickens, expose them to a high risk of infectious agents, including parasites (3, 6, 25).

There are many pathogens of great importance in the poultry industry, and among these, parasites play a key role (28, 53). Coccidiosis is one of the most common parasitic diseases affecting poultry worldwide. It causes substantial economic losses in the poultry industry due to its ability to result in mortality and reduced productivity (4, 10, 34). A study (8) used global data to calculate the cost of coccidiosis in chickens, revealing a cost of about 10.4 billion pounds at 2016 prices. Although effective biosecurity measures, vaccination, and anticoccidial treatments have reduced the risk of coccidiosis in large-scale modern industrial poultry farms, the risk remains high in backyard chickens (14, 17, 46).

Worldwide, seven *Eimeria* species are recognized as infecting chickens: *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella*. Also, three cryptic *Eimeria* operational taxonomic units (OTUs) have been identified in chickens in Australia, Africa, South America and Europe (9, 24, 52). Based on their genotypic and phenotypic characteristics, these OTUs were subsequently named *E. lata*, *E. nagambie*, and *E. zaria* (9). *Eimeria* species differ in terms of their localization, pathogenicity, the morphological characteristics of their oocysts and the number of schizogonous generations they undergo (9, 42). Conventional approaches to identify *Eimeria* species involve analyzing oocyst and sporocyst morphology and developmental patterns, as well as evaluating the location and severity of intestinal lesions in chickens (33). However, due to intraspecific variation, the morphological method is not entirely reliable, since natural *Eimeria* infections usually involve more than one species, and several species have similar characteristics (12, 33). Molecular techniques have been reported to be useful in species identification, thereby overcoming the limitations of traditional methods (2, 20, 32, 46). The amplification and sequencing of target amplicons from nuclear loci, such as *18S rDNA*, *ITS1* and *ITS2*, are widely used for the typing and subtyping of *Eimeria* species (7, 10, 23, 42). Among these, the *ITS1* region is particularly valuable for molecular identification due to its ease of amplification, conserved regions and the high number of rRNA copies. Furthermore, its rapid evolutionary rate and significant sequence variation enable the differentiation of closely related species (7, 42, 50). In avian *Eimeria* species, the *ITS1* sequence is usually between 320 and 600 bp long. This inherent sequence heterogeneity, coupled with its genomic abundance, makes the region an excellent target for species-specific identification of *Eimeria* (23).

Consequently, *ITS1* amplification has been successfully employed in various studies, including the present investigation, for the accurate differentiation of *Eimeria* species (20, 29, 32, 37, 47).

Epidemiological investigations on the prevalence of *Eimeria* species are crucial for effectively preventing and managing coccidiosis (44). Additionally, efficient control of coccidiosis also requires precise identification of circulating *Eimeria* species (2, 21). The large number of small-scale backyard chicken farms in the Nakhchivan Autonomous Republic necessitates a more detailed investigation of the current situation regarding coccidiosis. Only one study (41) using microscopy has been conducted on coccidiosis in chickens in Nakhchivan, and no research at the molecular level has been conducted to investigate its prevalence among different species. This study aimed to determine the prevalence of *Eimeria* in backyard chickens in the Nakhchivan Autonomous Republic and to investigate, for the first time, the presence of seven recognized species molecularly in *Eimeria*-positive samples.

Materials and Methods

Study Area: The Nakhchivan Autonomous Republic is a landlocked exclave of the Republic of Azerbaijan. It is located in southwestern Azerbaijan and has an average altitude of 1450 meters above sea level. Approximately two-thirds of the region consists of mountainous areas over 1000 meters in altitude. Nakhchivan has a continental, semi-arid climate, featuring short, cold, snowy winters and long, dry, hot summers. Temperature and precipitation are unevenly distributed across the region. Summers are characterized by high temperatures (often >30°C), while winters are cold (occasionally below -10°C). Annual precipitation averages ~200–350 mm on the Araz plain and 500–800 mm in the uplands. The Nakhchivan Autonomous Republic comprises Nakhchivan province and seven districts: Babek, Julfa, Kangarli, Ordubad, Sadarak, Shakhbuz and Sharur (Figure 1).

Sample Collection: The minimum required sample size was estimated to be approximately 165 using Cochran's formula, based on a reported prevalence of 30% ($P = 0.30$) in the region (41), a 95% confidence level ($Z = 1.96$), and a margin of error of 7% ($d = 0.07$). To ensure greater statistical representativeness, 186 chicken faecal samples were collected, exceeding the calculated minimum requirement. For this study, a total of 186 chicken faecal samples were collected from 65 backyard poultry farms across 58 locations in Nakhchivan province and its seven districts between April 2024 and September 2024 (Figure 1). The sampling period (April–September 2024) was divided into three seasons: spring (April–May), summer



Figure 1. Map of Nakhchivan Autonomous Republic indicating the locations of the sampled backyard houses

(June-August) and autumn (September). Approval has been obtained from farm owners to collect faecal samples and to use the research results for scientific purposes. Information including the sampling date, number of samples, chicken age, and total number of chickens was recorded. The mean number of chickens reared on the farms was 17.7 (range: 5-47). The ages of the chickens were recorded as either younger than or equal to six months or older than six months based on information provided by their owners. Additionally, detailed information was obtained regarding the chickens' general health status and any recent treatments administered. No signs of coccidiosis were noticed in any of the chickens examined during the sampling process. The chicken coops were traditional, open-air structures with soil floors and no insulation. Drinking water was provided via bell drinkers. Manufactured feed was not used but the grain was distributed by hand. In addition, most of the chickens obtain food by scavenging, eating food scraps, and grazing on vacant land. The chickens roamed freely in the backyard or nearby open areas during the day and in coops at night. Chickens were randomly selected from each household. Immediately after defecation, faecal samples were collected directly from the ground, placed in special analysis containers, and then transported to the laboratory.

Microscopic Examination: Fülleborn's saturated salt flotation method (NaCl, specific gravity of 1.2) was used to examine *Eimeria* oocysts, and the samples were observed under a light microscope (Nikon Eclipse Ci, Japan). OPG was calculated using the standard McMaster technique in *Eimeria*-positive samples (43). OPG levels were categorized as negative (0), low (<1800), medium (1801–6000), or high (>6000) (58).

The flotation technique was employed to isolate oocysts from *Eimeria*-positive faecal samples. The oocysts were then washed three times in 1x phosphate-buffered saline (PBS) by centrifugation at 1300xg for 10 min and stored at 4°C until DNA extraction.

DNA Extraction: Molecular techniques were used to identify species in *Eimeria*-positive samples. The oocysts were then subjected to three cycles of freezing and thawing. The cycles consisted of two minutes of freezing in a dry ice/ethanol bath and thawing at 100°C in a water bath. DNA was extracted from the lysed oocysts using a genomic DNA kit (EcoTech, Türkiye; Cat. No. E1075), in accordance with the manufacturer's instructions. The concentration and purity of the DNA samples were evaluated using a spectrophotometer (NanoDrop One; Thermo Fisher Scientific, Wilmington, DE, USA). The samples were then stored at -20°C for future analysis.

PCR Amplification: A nested PCR was performed to amplify the partial *ITS1* region of rDNA from *Eimeria* species. Amplification was carried out in a 20 µl reaction volume including 10 µl 2x PCR master mix (EcoTech, Türkiye), one µl of each genus-specific primer (BSEF and BSER) (49), three µl DNA and five µl sterile water. The PCR protocol involved an initial denaturation at 95°C for five min, followed by 40 cycles consisting of denaturation at 95°C for 15 sec, annealing at 45°C for 30 sec, and extension at 72°C for 30 sec, with a final extension step at 72°C for 10 min. One µl of the PCR reaction was utilized as a template for the second PCR amplification using species-specific primers (23). A similar PCR reaction mix and temperature profile, as described above, were utilized for the second PCR, with the annealing temperature set at 58 or 65°C, depending on the species. In each PCR, DNA from seven *Eimeria* species, sourced from the Department of Parasitology, Faculty of Veterinary Medicine, Atatürk University, was included as a positive control, and distilled water was used as a negative control. The PCR products were separated using agarose gel electrophoresis (1.5%), stained with SYBR Safe (Invitrogen) and photographed using an UV transilluminator (Quantum ST4 1100, Vilber Lourmat, France).

Sequence Editing and Phylogenetic Analysis: The selected PCR products with high DNA concentrations were sent to a commercial company (Medsantek, Türkiye) for bidirectional Sanger sequencing. After visual inspection, the raw nucleotide sequences were edited and aligned using the BioEdit 7.0 software. The edited sequences were then compared to the reference sequences in the GenBank database using the BLASTN program. The generated sequences were submitted to GenBank to obtain accession numbers. Sequences from this study and sequences from the GenBank database were used to construct a phylogenetic tree using the Maximum Likelihood method and the Kimura two-parameter model in MEGA XI (56).

Statistical Analysis: Statistical analyses were performed using SPSS (Version 21.0, Chicago, IL). Categorical variables were analyzed using the chi-square test to evaluate the molecular results. The numerical OPG counts were subjected to the Shapiro-Wilk test to assess normality of distribution. As the OPG values were not normally distributed, non-parametric tests were performed for all comparisons. The Mann-Whitney U test was used to determine the significance of differences in mean OPG

levels between two independent groups (age groups: ≤ 6 months and > 6 months). The Kruskal-Wallis test was utilized to evaluate differences in OPG levels across multiple independent groups (seasonal variations). P value of less than 0.05 was considered statistically significant.

Results

Individual faecal samples were collected from a total of 186 chickens. Of the 186 chickens, 81 were aged four to six months and 105 were aged seven to 36 months. All animals sampled appeared clinically healthy and their feces were normal in appearance. No protective vaccination or chemical treatment against coccidiosis had been administered to the sampled chickens.

Eimeria oocyst positivity was identified in 48.9% (91/186) of the samples. *Eimeria* species were found in all age groups, with positivity rates of 60% and 34.6% in groups older than six months and younger than six months, respectively ($P < 0.05$). *Eimeria* positivity was observed throughout the six-month sampling period. Prevalence was highest in autumn (September) at 64.9%, followed by spring (46.4%) and summer (36.7%) ($P < 0.05$) (Table 1). The Shahbuz district had the highest *Eimeria* positivity rate, at 56.2%, while the lowest was in Sharur, at 40% (Table 2). The OPG values ranged from 200 to 5100 (mean 1790.1). The majority of the samples (60/91; 65.9%) exhibited low OPG levels (< 1800), while 34.1% (31/91) displayed medium OPG levels (1801–6000). The mean OPG was 2055.3 in the under six-month-old group and 1672.2 in the over-six-month-old group ($P < 0.05$). The highest OPG mean was determined as 2104 in autumn (September) (Table 1). The relationship between OPG counts and season was evaluated, infection rate was highest in autumn (September) (64.5%, 20/31) with a moderate mean OPG (2937.5; range 1850–4500), whereas spring showed a lower infection rate (46.7%, 28/60) with a low mean OPG (1228.6; range 200–1800) ($P < 0.05$).

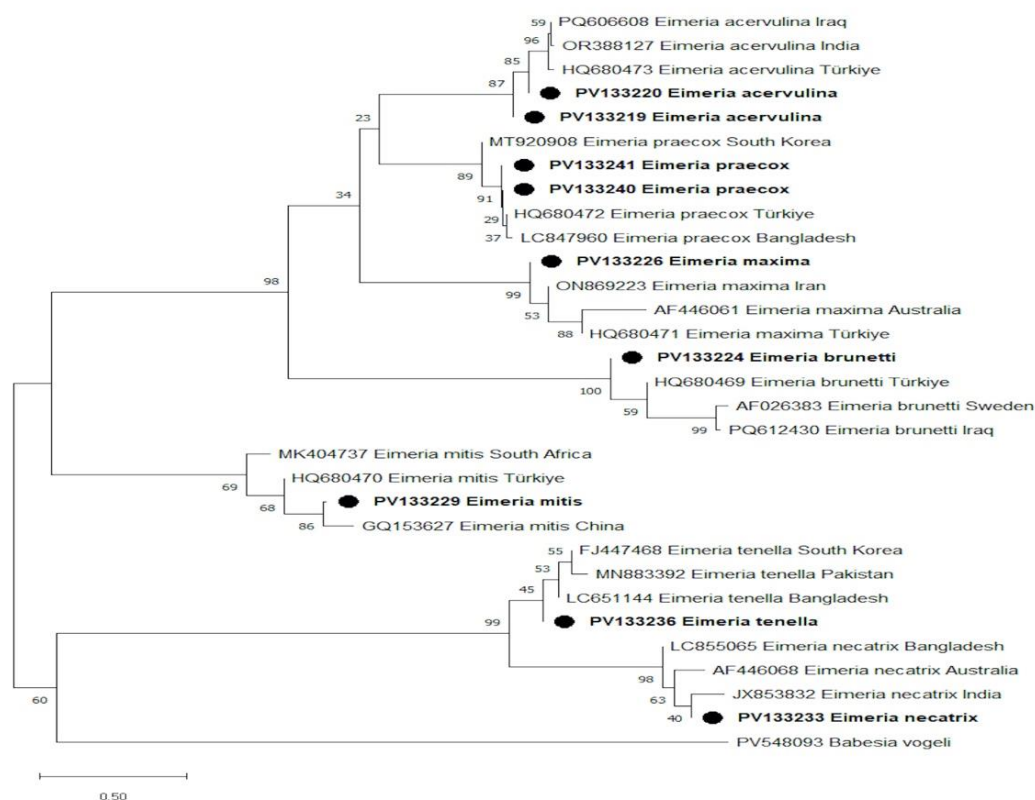
The PCR results revealed that the most prevalent *Eimeria* species in the study were *E. acervulina* (83.5%, 76/91), *E. maxima* (71.4%, 65/91) and *E. tenella* (61.5%, 56/91). *Eimeria necatrix* and *E. mitis* each had a prevalence of 46.1% (42/91), whereas *E. praecox* had a lower prevalence rate of 41.8% (38/91). The lowest prevalence, 16.5% (15/91), was observed for *E. brunetti*. All positive samples exhibited multiple infections involving three to five *Eimeria* species. The most common species in mixed infections were *E. acervulina* (29.7%), *E. maxima* (26.4%), and *E. tenella* (24.2%).

Table 1. *Eimeria* positivity and OPG values by age and season

	Variable	Total samples	Positive (%)	P	OPG mean (range)	P
Ages	≤ 6 months	81	28 (34.6)	0.001	2055.3 (200 - 4400)	0.002
	> 6 months	105	63 (60)		1672.2 (400 - 5100)	
Season	Spring	69	32 (46.4)	0.008	1543.7 (200 - 5100)	0.001
	Summer	60	22 (36.7)		1620.4 (200 - 4600)	
	Autumn	57	37 (64.9)		2104 (750 - 4500)	

Table 2. *Eimeria* positivity and OPG values by location

Locations	Total samples	Positive (%)	P	OPG mean (range)	P
Babek	25	12 (48)	0.951	1516.7 (800-2400)	0.195
Julfa	24	12 (50)		1375 (400-3600)	
Kangarli	18	8 (44.4)		1525 (750-2600)	
Nakhchivan	20	10 (50)		2050 (200-4000)	
Ordubad	30	16 (53.3)		1956.2 (500-4000)	
Sadarak	12	5 (41.7)		1410 (800-2050)	
Shakhbuz	32	18 (56.2)		1627.8 (200-4500)	
Sharur	25	10 (40)		2785 (750-5100)	
Total	186	91 (48.9)		1790.1 (200-5100)	

**Figure 2.** Phylogenetic tree showing the relationship between *Eimeria* isolates in Nakhchivan Autonomous Republic and reference *Eimeria* species based on *ITS1* gene sequences. The branching pattern was evaluated by bootstrap test analysis with 1000 replicates. Bootstrap values are provided at the beginning of each branch.

The GenBank accession numbers for the sequences deposited in the present study are as follows: *E. acervulina* (PV133219-PV133222); *E. brunetti* (PV133224, PV133225); *E. maxima* (PV133226-PV133228); *E. mitis* (PV133229-PV133232); *E. necatrix* (PV133233-PV133235); *E. praecox* (PV133240-PV133242); *E. tenella* (PV133236-PV133239). The sequences of *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, and *E. tenella* were 100% identical to each other. However, *E. acervulina* isolates exhibited 99.3-100% similarity among themselves, while *E. praecox* isolates displayed 99.5-100% similarity. The phylogenetic analysis revealed that *ITS1* sequences of Nakhchivan *E. acervulina* isolates showed 100% identity with isolates from India (OR388127; KJ420580), United States (AY779476), Iraq (PQ612086), and Saudi Arabia (OR388129). *E. brunetti* isolates from Nakhchivan exhibited 98.3% identity with Sweden isolate (AF026383). The Nakhchivan *E. maxima* isolates exhibited 100% homology with those from the United States (AY779425; FJ230344), Bangladesh (LC855067), China (GQ153625; JN113573), Australia (AF446059), Iran (ON869223), and India (GQ856309). Nakhchivan *E. mitis* isolates were found to be 97.9% similar to the isolate obtained from a study conducted in the United States (FJ230365). Nakhchivan *E. necatrix* isolates showed 98% similarity with the Chinese isolate (FJ449693). Nakhchivan *E. praecox* isolates showed 98.6% similarity with isolates obtained from studies conducted in Sweden (AF065092), Bangladesh (LC855067), Australia (AF446072), China (GQ153629), and Türkiye (HQ680472). *Eimeria tenella* isolates showed 100% similarity with isolates obtained from studies conducted in Iraq (PQ606578; PQ612428), India (GQ856298; PP002176; PP131259), China (GQ153633; JX477100), Bangladesh (LC651144; LC651137; LC651139), and South Africa (MN727041). A phylogenetic tree was constructed using the sequences obtained in this study, as well as other relevant sequences sourced from the NCBI GenBank database (Figure 2). The Maximum Likelihood method was used with *Babesia vogeli* (PV548093) as an outgroup. The isolates obtained in this study formed clades with the corresponding reference sequences within species.

Discussion and Conclusion

Avian coccidiosis is a widespread disease found in all poultry species and in all types of rearing systems (12, 42). Epidemiological studies on coccidiosis prevalence are useful for controlling and preventing the disease (44). The only study (41) conducted on the prevalence of *Eimeria* in the Nakhchivan Autonomous Republic reported a 30.3% positivity rate in 960 chickens. There are also few studies on the prevalence of *Eimeria* in poultry in Azerbaijan, and these studies are based on microscopic diagnosis. Musaev

et al. (45) reported a 37.0% positivity rate for *Eimeria* spp. in the Absheron region, while Samadova et al. (48) and Mammadova et al. (36) found varying prevalence rates for *E. mitis*, *E. tenella*, and *E. acervulina*. The data from these previous studies provide essential baseline information on the presence of *Eimeria* species in the region. However, direct numerical comparison with the current findings is limited by methodological differences. Discrepancies in prevalence rates are expected, as molecular techniques, such as the PCR method employed in this study, offer substantially higher sensitivity and specificity than traditional diagnostic methods, such as microscopy. Despite these diagnostic variations, all these studies, including this one, reveal that *Eimeria* positivity is widespread in chickens in the Nakhchivan Autonomous Republic and Azerbaijan, and emphasize the importance of implementing control measures against coccidiosis.

In the present study, the prevalence of *Eimeria* spp. was found to be 48.9%. This result falls within the global range of 25.8% to 85.7% reported for backyard chickens, yet it reveals distinct geographical patterns when categorized. In Asia, prevalence rates vary significantly, ranging from 20.5% in Myanmar to 58.3% in India (7, 51, 60). Our findings align closely with the 53.5% observed in Jammu and Kashmir (51). Studies in Africa and the Middle East report similarly diverse rates, ranging from 10% in Nigeria to 56% in Ethiopia and 31.8% in Tunisia (27, 31, 57). In contrast, notably higher prevalence rates have been documented in parts of Europe and the Americas, such as Greece (85.7%) and South America (84.2%) (2, 58). Regional discrepancies, including the 27% prevalence in neighboring Türkiye, reflect differences in sampling periods, host ages, sample sizes, diagnostic methods, management practices, geographic areas, and climates. Despite these discrepancies, the consistent detection of these parasites across all continents emphasizes that coccidiosis is a global challenge for backyard poultry. However, as Fornace et al. (19) noted, more research is needed to accurately determine the economic impact of this situation, as farm-level data remains insufficient to fully understand the burden of coccidiosis in non-commercial poultry systems.

A high prevalence of *Eimeria* was detected in backyard chickens in Nakhchivan, with many factors potentially contributing to this situation. The main control measures for coccidiosis include husbandry practices such as biosecurity, litter management, and ventilation; chemoprophylactic treatment; and vaccination (4, 42). However, implementing these measures in backyard poultry farming in developing countries is challenging due to financial limitations (44). Since biosecurity is generally low in backyard flocks, infections with pathogens such as *Eimeria* species are common (11, 42). Furthermore, the presence of chickens in backyards poses a significant risk

of developing clinical and subclinical coccidiosis, primarily attributable to their unrestricted outdoor access and natural scavenging behavior (3). Backyard chickens are generally raised in open housing with access to the outdoors, which allows them to forage freely and consequently increases their risk of infection with sporulated *Eimeria* oocysts (6, 28, 44). Several factors facilitate the ingestion of infectious *Eimeria* oocysts in backyard systems. These factors include cohabitation of different age groups, poor litter management (e.g., absence of litter or prolonged use), and lack of regular disinfection. Additionally, the use of contaminated feeders and drinkers that are infrequently cleaned significantly increases the risk of oocyst accumulation and transmission. Furthermore, as observed in this study, the use of preventive drugs for coccidiosis may not be widespread in poultry. On modern farms, allowing birds to roam freely helps spread oocysts over a wider area rather than concentrating them in a limited one. Free-range systems on smaller, backyard farms have lower stocking densities, which reduces the environmental oocyst load and consequently decreases oocyst shedding (30). This situation may result in the continuous ingestion of low doses of *Eimeria* oocysts, leading to subclinical infection, as observed in this study. Similarly, Ashenafi et al. (3) reported that repeated exposure to different *Eimeria* species may maintain immunity to certain *Eimeria* species in chickens and lead to subclinical coccidiosis. All animals sampled in this study appeared healthy. Their faeces appeared normal in both appearance and consistency on macroscopic examination; however, microscopic examination revealed that 91 of these animals were infected with *Eimeria* parasites. The oocyst counts showed that 60 of the animals had low oocyst loads (mean 1140.8 OPG) and 31 had moderate oocyst loads (mean 3046.8 OPG). The subclinical course of *Eimeria* infection in these chickens can be associated with the factors mentioned above, and these chickens can be considered reservoirs. As noted by Godwin et al. (21), *Eimeria* infections are common in backyard flocks, and considering the industry's shift toward free-range production systems, these flocks have the potential to serve as disease reservoirs.

The infection was observed during the six-month sampling period but the prevalence was significantly higher in the autumn (September) (64.9%) followed by spring (46.4%), and summer (36.7%). Similarly, a moderate level of OPG (mean: 2104) was recorded in autumn (September). In pasture or free-range poultry production systems, the effect of the season cannot be ignored (26). As suggested by Williams (59), the seasonal distribution of prevalence may be related to environmental factors, such as temperature, humidity, and oxygen, which affect the sporulation process of freshly excreted oocysts

and, consequently, their infectivity. Ventilation is necessary for effective oocyst sporulation. Simple coops or open areas where chickens can roam provide natural ventilation and thereby enabling sporulation. In general, temperatures between 25 and 30°C and a relative humidity of 60-80% are favorable for sporulation (4, 5, 42, 26). Many researchers have reported that coccidiosis is more prevalent in seasons with optimal humidity and temperatures (15, 21, 47, 51, 55). The optimal season for the sporulation of *Eimeria* oocytes varies depending on the climatic characteristics of the study area, with some researchers considering autumn (5, 7, 27, 28, 51) and others reporting spring (14) or the monsoon season (15) as the suitable time. The highest positivity rate was documented in autumn (September) in the current study. The rainy season in Nakhchivan, where the average annual rainfall is approximately 265 millimeters, lasts from March to June. In September 2024, light rainfall followed the August drought, and the average temperature was 23.2°C (20°C - 38°C). These conditions may affect the high prevalence observed in autumn (September). On the other hand, this result cannot be attributed solely to seasonal effects. While oocyst sporulation and survival are contingent on optimal humidity, temperature, and ventilation, elevated or diminished *Eimeria* prevalence cannot be ascribed exclusively to climatic factors (31). Other factors, such as housing structure, bedding material type and use, animal age and density, management, nutrition, and other stressors like dietary changes and immunosuppressive effects, can also influence differences in prevalence (4, 13, 15, 44).

The highest *Eimeria* prevalence, 56.2%, was recorded in Shahbuz, while the lowest, 40%, was recorded in Sharur. As mentioned above, *Eimeria* occurrence varies by season; this variation may be related to climatic differences between the two regions. Shahbuz is a more mountainous area with higher rainfall and optimal temperatures, whereas Sharur is at sea level with lower rainfall and higher temperatures.

Age is widely considered a significant factor influencing the severity and prevalence of coccidiosis. Although birds of all ages are susceptible to coccidiosis, younger individuals exhibit a markedly higher vulnerability to clinical infection compared with older birds (39). McDougald et al. (40) stated that most *Eimeria* species affect birds between three and 18 weeks of age, but they are most prevalent in birds between three and six weeks of age. This situation may be related to the immaturity of the immune system of young birds that have never encountered the pathogen, making them susceptible to infection even by lower numbers or less pathogenic strains of *Eimeria*, and to the small surface area of their intestines, which causes the same number of oocysts to concentrate in young birds, leading to large-scale lesions

(14, 15, 27, 28, 31). In the present study, the *Eimeria* positivity rate was higher in the older group (60%) than in the younger group (34.6%). While some studies (7, 27, 28, 31, 47) have reported higher prevalence in young chicks, the results of this investigation align with those reported by Debbou-Iouknane et al. (16) and Coroian et al. (14), who found higher prevalence in older birds. This shift in prevalence may be attributed to the management system of backyard poultry and the development of premunition-type immunity. Unlike in intensive commercial systems, where birds are kept in age-synchronized groups, backyard chickens of various ages often coexist. This leads to continuous environmental contamination and re-infection cycles. Older birds in such systems are exposed to oocysts in the scavenging area for a longer duration, which may result in higher cumulative prevalence and the establishment of a host-parasite equilibrium. Notably, despite this higher prevalence, the lower OPG counts observed in older chickens compared to acute infections in younger ones suggest that these chickens have developed acquired immunity. This allows them to act as subclinical carriers, maintaining the infection cycle without exhibiting severe disease. Furthermore, the absence of systematic coccidiostat use or vaccination programs in backyard settings could facilitate the persistence of subclinical infections into adulthood, whereas younger chicks might possess transient protection through maternal immunity during their initial weeks of life.

Eimeria acervulina, *E. maxima*, *E. necatrix*, and *E. tenella* represent the primary contributors to the global disease burden and economic impact (46, 59). McDougald et al. (38) and Bharti et al. (7) identified *E. tenella*, *E. maxima*, and *E. acervulina* as the three major economically significant pathogens. Jaramillo-Ortiz et al. (24) also reported that these three species are predominant in Europe, where they remain endemic. *Eimeria acervulina* and *E. maxima* cause malabsorptive pathologies and are less pathogenic than *E. tenella* and *E. necatrix*. However, depending on the ingested dose, strain-specific variation in virulence, and host factors such as age, breed, and immune status, morbidity and mortality can occur (1, 42). *Eimeria tenella* together with *E. necatrix* are considered the most pathogenic species, causing hemorrhagic coccidiosis with high morbidity and mortality (1, 34). Regarding the molecular characterization of *Eimeria* species in backyard poultry, *E. tenella* and *E. acervulina* are the frequently identified species and have been observed globally, as reported in commercial poultry flocks (28). The dominance of *E. acervulina* (83.5%) in this study is also reported by other studies (2, 14). *Eimeria brunetti* is the least common species (16.5%) in this study, a finding that has also been reported by Godwin et al. (21) and Prakashbabu et al (47).

The high prevalence of pathogenic species such as *E. tenella* (61.5%) and *E. necatrix* (46.1%) in asymptomatic chickens is a notable finding in this study. A similar finding was reported by Tomazic et al (58). In that study, *E. tenella* or *E. brunetti* was detected in asymptomatic cases, and the researchers emphasized the importance of molecular diagnosis to implement control measures even in asymptomatic chickens. Similarly, Khursheed et al. (28) reported that the highly pathogenic *Eimeria* species *E. tenella* and *E. necatrix* were the most frequently isolated in backyard poultry without clinical coccidiosis, suggesting that they can serve as reservoirs.

A notable finding of this study is the widespread presence of multiple *Eimeria* species in all positive samples, with simultaneous infection by three to five species being common. This high frequency of mixed infections suggests that the environment of the examined backyard flocks is persistently contaminated by numerous *Eimeria* species. In a backyard farming system, the absence of biosecurity measures and the cohabitation of birds of various ages facilitate the circulation of multiple species, allowing exposure to multiple species simultaneously. Furthermore, the high prevalence of highly reproductive species, such as *E. acervulina*, *E. maxima* and *E. tenella* (52), likely triggers this synergistic persistence.

It is crucial to determine the prevalence and species of *Eimeria* in a region in order to develop the necessary protection and control measures against the disease (2, 44, 46). Species-specific PCR was used for the first time in the Nakhchivan region to identify the seven recognized *Eimeria* species infecting backyard chickens. Similarly, many researchers (6, 21, 27, 28, 35) have performed molecular characterizations of *Eimeria* species in backyard poultry. Amplification of the *ITS1* region is widely used in molecular phylogenetics and taxonomy because of the high degree of variation among closely related *Eimeria* species. *ITS1* fragments are consistent within species but different between species due to convergent evolution, so cross-reaction is unlikely. The phylogenetic tree revealed that the *Eimeria* species prevalent in Nakhchivan chickens share the same genetic profiles and have a common ancestor. The Nakhchivan *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, and *E. tenella* sequences were 100% identical to each other. However, *E. acervulina* and *E. praecox* isolates exhibited 99.3-100% and 99.5-100% similarity, respectively. The high level of sequence identity (99.3–100%) between Nakhchivan isolates and those reported worldwide emphasizes the significant degree of genetic conservation among *Eimeria* species. These findings indicate that circulating strains are not limited to specific geographical regions, but rather represent a globally distributed genetic pool. This extensive overlap may suggest that *Eimeria* strains have

spread through the international poultry trade, facilitating the rapid dissemination of the same genotypes across continents. Genetic homogeneity is of considerable importance, as it implies that effective control measures developed elsewhere, such as vaccination strategies and anticoccidial protocols, may be directly applicable to poultry populations in Nakhchivan region, since the target pathogens share the same molecular signatures.

One methodological limitation of this study is that sampling could not be conducted in the same locations in all seasons. Regional environmental factors may also have influenced the data obtained. Nevertheless, our findings reveal a consistent seasonal trend in *Eimeria* prevalence that aligns with published data (5, 7, 27, 28, 51). Further studies should be conducted where sampling is performed at the same locations in every season to better elucidate the effect of seasonality.

This study provides a comprehensive molecular basis for understanding the prevalence of *Eimeria* species in backyard chickens in the Nakhchivan Autonomous Republic. Using a nested PCR approach with species-specific primers, all seven *Eimeria* species were identified, with *E. acervulina*, *E. maxima*, and *E. tenella* emerging as the primary drivers of observed subclinical infection rates. These findings address a significant geographical knowledge gap in poultry parasitology and emphasize the ongoing economic threat posed by coccidiosis in traditional farming practices. Future research should prioritize developing sustainable control measures and investigating the seasonal dynamics of these species to optimize management interventions in the region.

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Ethical Statement

This study did not require approval from an Institutional Animal Care and Use Committee/Ethics Committee.

Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

SB Conceptualization, Methodology, Investigation, Resources, Writing-Original Draft, **RK** Formal analysis, Methodology, Resources, Writing-Original Draft, Writing-Review&Editing, **EG** Formal analysis,

Methodology, Supervision, Writing-Original Draft, Writing-Review&Editing, **TG** Conceptualization, Methodology, Investigation, Resources, **İM** Conceptualization, Methodology, Investigation, Resources.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Generative Artificial Intelligence

The authors declare that generative artificial intelligence (AI) and AI-assisted technologies were not used in the writing of this manuscript.

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