

## **Original Research Article**

### **Small ruminants Eimeriosis in high altitude region of Iran**

#### **Abstract:**

Coccidiosis (*Eimeria* spp.) is a parasite disease in small ruminants that causes diarrhea and reduced economic benefits. The purpose of this epidemiological study was to use microscopical and molecular assays to detect coccidia prevalence and isolate species-specific *Eimeria* from sheep and goats kept in a common pen in the central Zagros region. This region is a mountainous and cold region which its southern counties are warmer than other districts. Throughout the course of several seasons, 1200 fecal samples were obtained from three age groups (less than six months, six to twelve months, and more than six months). Oocysts were counted using saturated saline floating and modified MacMaster techniques and the physical characteristics of sporulated *Eimeria* oocysts were used to identify them. *Eimeria* infection was shown to be more prevalent in sheep than in goats, with the highest prevalence in spring and the lowest in winter. Females and animals younger than 6 months were similarly found to be more susceptible to Eimeriosis than males and older animals ( $P < 0.05$ ). In comparison to the southern area, the western and eastern counties had the highest rates of oocyst excretion in sheep and goats, respectively. In addition, a total of fourteen *Eimeria* species were isolated, including the highly pathogenic *Eimeria granulosa* for sheep and *E. jolchijevifor* for goats. These findings explain how coccidia prevalence varies with age, season, and geographic direction, and use of PCR to rule out the possibility of co-infection between sheep and goats kept in the same stall.

**Keywords:** Central Zagros, Sheep, Goats, *Eimeria*, Coccidiosis.

#### **Introduction:**

Eimeriosis affects all livestock such as; cattle, sheep, goats, and poultry [1, 2]. Constable et al (2016) and Keeton and Navarre (2018) believe that overcrowding in winter and other stressors (weaning, weather, transportation, other diseases, and so forth), nutrition, and genetic susceptibility, increase the occurrence of coccidiosis. Seasonal variation rules in its prevalence, according to Saratsis et al (2011) and Yan et al (2021) studies [1, 3]. This intracellular parasite is host specific, and mixed infections with various pathogenic and/or

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nonpathogenic species can occur at any time during an animal's life, with younger animals being more vulnerable [2, 4, 5, 6].

It was also shown that healthy sheep and goats are generally immune to disease by the age of one year, although older animals can infect younger animals [2, 4]. Levine (1985) believed that animals following coccidia recovery are relatively immune to re-infection with the same species. However, immunity isn't solid and oocysts of even highly pathogenic species can be found in the feces of otherwise healthy animals [7].

Coccidiosis tend to be asymptomatic and self-limiting unless immune defenses are compromised [8] and bloody diarrhea, rectal tenesmus, inappetence and dehydration develops followed by destruction of intestinal epithelial cells [6]. Bloody diarrhea with tenesmus are less common in lambs, but pasty and/or watery diarrhea with dehydration are more common in kids [5, 6].

Catchpole et al (1976) emphasized that due to geographic area, 11 to 15 species of *Eimeria* can isolate from sheep, of which *E. ovinoidalis* is considered the most pathogenic one [9], but *E. ovinawas* recorded as the most infective species by Al-Saadoon and Al-Rubaie (2018) [10]. *E. arloingi*, *E. christenseni* and *E. ninakohlykimovae*, on the other hand, are highly pathogenic for goats, according to Kaufman (1996) [11]. These findings indicate the impact of the region, climate, type of maintenance and many other factors on the infectivity of *Eimeria spp.* in different region.

For these reasons, the aims of this study were as follow;

- 1- To find out which *Eimeria* species are the most pathogenic in sheep and goats in the central Zagros region.
- 2- To investigate transient co-infection between sheep and goats that were kept in a common stalls.
- 3- To determine whether Eimeriosis prevalence varies by season.
- 4- To investigate the impact of age and gender on *Eimeria* prevalence.

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## Material and methods:

### *Management of animals and Experimental Design*

The experimental protocols were approved by the guidelines of Shahrekord University's (Shahrekord, Iran) guidelines for animal care and usage. This research was carried out on 15 traditional flock from 9 different localities of the central Zagros region (from autumn 2020 to the summer 2021).

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### Sample collection

A total of 1200 fecal samples were collected from 740 sheep and 480 goats (300 per season), which are kept in stables during the winter and stay outside during the spring and summer. In addition, thirty positive animals (15 goats and 15 sheep) were kept in common stalls for a year to determine the possibility of cross infection. Animals were also divided into three age groups; less than six months (<6 M), 6 to 12 months (6-12 M) and more than 12 months (12 M+).

**Comment [E4]:** Please enumerate what are the species in goat and not present in sheep and vice versa.

**Comment [E5]:** Did collect the samples from apparent healthy animals or animals with clinical signs. If with clinical signs please mention them

### Isolation and identification of *Eimeria*

*Eimeria* oocysts were isolated from the fecal bulk using a flotation technique utilizing a saturated saline solution with a high specific gravity. The results were computed using the McMaster technique, as described by Rinaldi et al (2011) and samples containing more than 300 oocytes per gramme (opg) were evaluated for sporulation [12]. For this, a 2% potassium dichromate solution was combined with 4 grammes of feces and held at 28 to 30 degrees Celsius with daily aeration. Sporulated *Eimeria* oocytes were used for the species identification based on its morphological features (size, shape, color, presence or absence of micropyle and cap) and also for DNA extraction.

**Comment [E6]:** Did you do the morphological examination first? If yes you mean you could prove *Eimeria* infection so if that why did you do the molecular analysis?

### Extraction of genomic DNA

To eliminate non-oocyst DNA, 1.5 mL of 10% sodium hypochlorite solution was applied to each fecal sample and left on ice for 10 minutes. Harvested oocysts were resuspended in 1 mL of phosphate-buffered saline (PBS, pH 7.2), then transferred to a round bottom tube, homogenized with 250 mg of 0.5 mm glass beads, and vortexed five times for one minute each time to disrupt oocyst walls. After centrifuging the samples, the pellet was washed twice with distilled water. Finally, according to the commercial kit, 0.3 mL lysate was utilized, and extracted DNA was kept at -20° C until use.

### PCR reactions for internal transcribed spacer 1 (ITS-1) region

The quantity and quality of the purified DNAs were verified by a nanodrop spectrophotometer and electrophoresis using 1% agarose gel, respectively. The genus-specific primer sets used for sheep *Eimeria* were, Forward: 5'-TACCCAATGAAAACAGTTT-3', and Reverse: 5'-CAGGAGAAGCCAAGGTAGG-3' and primer which used for goats *E. arloingi* were, Forward: 5'-GGGTATCATCTATCCATTACATCC-3', and Reverse: 5'-GCACGCCTGATTGGTGTAGAAG-3', to amplify 100 and 500 bp DNA fragments for sheep and goats specimens, respectively. Thermal cycling conditions consisted of a pre-denaturing cycle at

**Comment [E7]:** This primer for what type of *Eimeria* in Sheep?

94°C for 5 min followed by 35 cycles at 94 °C for 45 s; 57 °C for 1 min; 72 °C for 1 min; and a final extension of 72 °C for 7 Min. Multiplex PCR master mix was prepared by using reaction mixture (Sinaclone PCR Kit, Iran) according to company instructions. Agarose gel electrophoresis was carried out to separate the PCR products and finally images was captured using a gel documentation.

Comment [E8]: were

### Statistical analysis

The data (Mean±SEM) was analyzed using the SigmaPlot 14 version (Systat Software Inc.) program, which included the t-test, One-Way Analysis of Variance (ANOVA), and Chi-square. The Bonferroni test was also used for multiple data comparisons at the  $P<0.05$  level.

### Results

The study was done over a 17000 square kilometers area in the central Zagros. Because of the region's size, 9 counties in distinct geographical orientations (north, south, center, east, and west) were chosen to determine the prevalence of *Eimeria*. The annual prevalence of Eimeriosis showed that sheep were more susceptible than goats, with the peak frequency in the spring (Table 1). Tables 2 to 4, reveal that females excrete the most oocysts ( $P<0.0001$ ), while sheep and goats excrete less ( $P<0.05$ ) in the southern and western counties, respectively.

**Table 1:** Seasonal differences in sheep and goats Eimeriosis (Mean±SEM)

	Spring		Summer		Autumn		Winter	
	Sheep	Goats	Sheep	Goats	Sheep	Goats	Sheep	Goats
Oocysts excretion	3767.7±330 <sup>b,c</sup>	2507.3±206.89 <sup>b</sup>	2892±247.3 <sup>b,c</sup>	1813.7±129.4	1503.7±315	1200±42.6	1388±153.45	1155.7±14
Total	6275 <sup>b,c</sup>		4705.7 <sup>b</sup>		2703.7		2543.7	

<sup>b</sup> Significant to autumn  $P<0.05$ .

<sup>c</sup> Significant to winter  $P<0.05$ .

**Table 2:** The effect of sex on oocyst excretion (Mean±SEM)

Sheep		Goats	
Male	Female	Male	Female

Comment [E9]: Why dose the number of oocysts produced in female higher than male ?please mention about scientifically.

Oocysts excretion	712.59	<b>1675.28*</b>	604.08	<b>1058.12*</b>
Total	<b>2387.87±572.97**</b>		1669.2±317.16	
P value	<b>0.01</b>		<b>0.05</b>	

\*Significant to other gender  $P < 0.05$ .

\*\*Significant compared to goats  $P < 0.05$ .

**Table 3:** Geographical differences of sheep Eimeriosis (Mean±SEM) in the central Zagros region

	North (3263.16±421.32)	South (542.86±44.76)	Center (3234.29±394.46)	West (3466.21±428.31)	East (2628.57±892.43)
North (3263.16±421.32)	-				
South (542.86±44.76)		-	<b>0.004</b>	<b>0.002</b>	
Center (3234.29±394.46)			-		
West (3466.21±428.31)				-	
East (2628.57±892.43)					-

**Table 4:** Geographical differences of goats Eimeriosis (Mean±SEM) in the central Zagros region

	North (1500±562.32)	South (502±51.48)	Center (2620±352.46)	West (842.86±53.92)	East (2827±217.68)
North (1500±562.32)	-				
South (502±51.48)		-			
Center (2620±352.46)			-		
West (842.86±53.92)				-	
East (2827±217.68)		<b>0.017</b>		<b>0.024</b>	-

Oocyst excretion was higher in lambs and kids under six months (<6 M) than 6 to 12 months (6-12 M) and also above 12 months (12 M<), indicating that younger lambs and kids are more susceptible to coccidiosis (Table 5).

**Table 5:** The effect of age on oocyst excretion in the central Zagros region

	<6 months	6-12 months	12 months<	
<b>Sheep</b>	<b>4968.42±132.75<sup>a, b</sup></b>	<b>2560±134.153<sup>b</sup></b>	1284.2±88.99	<i>P</i> <0.05
<b>Goats</b>	<b>4583.33±142.88<sup>a, b</sup></b>	<b>1543.88±145.7<sup>b</sup></b>	350±7.26	<i>P</i> <0.0001

<sup>a</sup> Significant to 6-12 months, *P*<0.05.

<sup>b</sup> Significant to more than 12 months, *P*<0.05.

### **Morphometric classification**

The shape and size of sporulated oocysts, and the presence or absence of micropyle and polar cap were used to identify the species using the methods given by Trejo-Huitron et al (2020) and Souza et al (2015) [13, 14]. Eimeriosis was found in 164 heads out of 1200 fecal samples, with an annual prevalence of 13.66 percent. Table 6, summarizes the results of the isolation of eight and six unique species from sheep and goats, respectively.

**Table 6:** The isolated *Eimeria* species from sheep and goats in the central Zagros region

	<b>Sheep Eimeriosis</b>	Frequency	Relative frequency	<b>Goats Eimeriosis</b>	Frequency	Relative frequency
1	<i>E. granulosa</i>	32	32%	<i>E. jolchijevi</i>	34	53.13%
2	<i>E. faurei</i>	24	24%	<i>E. caprina</i>	14	21.87%
3	<i>E. bakuensis</i>	16	16%	<i>E. christenseni</i>	6	9.37%
4	<i>E. ahsata</i>	12	12%	<i>E. arloingi</i>	4	6.25%
5	<i>E. crandalis</i>	6	6%	<i>E. caprovina</i>	4	6.25%
6	<i>E. weybridgensis</i>	4	4%	<i>E. ninakolyakimovae</i>	2	3.13%
7	<i>E. intricata</i>	4	4%	-	-	-
8	<i>E. ovinoidalis</i>	2	2%	-	-	-
<b>Total</b>	-	100	100%	-	64	100%

*Eimeria granulosa*, *E. faurei*, *E. bakuensis*, *E. ahsata*, *E. crandalis*, *E. weybridgensis*, *E. intricata* and *E. ovinoidalis* and also *E. jolchijevi*, *E. caprina*, *E. christenseni*, *E. arloingi*, *E. caprovina* and *E. ninakohlykimovae* were isolated from sheep and goats, that *E. granulosa* and *E. arloingi* infecting 32 percent of sheep and 53.13 percent of goats, respectively.

#### **Molecular assay**

All positive samples were tested for the *Eimeria* internal transcribed spacer 1 (ITS-1) region, and the infection was confirmed. This assay was also performed on 30 positive sheep and goats that were maintained in common stalls for a year to rule out transient co-infection between the two species.

Comment [E10]: Dose crossinfection happen?

#### **Discussion**

Sheep and goats farming is particularly important commercially, culturally, and socially in the central Zagros region. This cold region is notable for its altitude of more than 2000 meters above sea level. Our observations led to the isolation of 8 and 6 *Eimeria* species from sheep and goats, respectively, with *E. granulosa* and *E. faurei* in sheep and *E. jolchijevi* and *E. caprina* in goats being the most prevalent. This subject is contradicting to previous studies. Catchpole et al (1976) isolated 11 *Eimeria* species from sheep [9], that *E. ovinoidalis* being the most virulent, while Kaufman (1996) declared *E. arloingi*, *E. christenseni*, and *E. ninakohlykimovae* to be extremely pathogenic for goats [11]. These data show that geographical district has an effect on the virulence of *Eimeria* species and this is the first report of *E. granulosa* as the most pathogenic species for Lori-Bakhtiari sheep in Iran. Yakhchali and Zareei (2008) and Yakhchali and Rezaei (2010) were reported that the most common sheep Eimeriosis in Malayer and Tabriz suburbs are belonging to *E. intricata* [15, 16]. Researches on goats Eimeriosis in other districts of Iran indicate that *E. arloingi* is more pathogenic [17, 18, 19], while in the central Zagros region, the predominant species is *E. jolchijevi* followed by *E. caprina*. It can be assumed that over the centuries, immunity to some species has occurred in the central Zagros region.

The annual prevalence of clinical coccidiosis was estimated to be 13.66 percent which lambs and kids under the age of 6 months excreting more oocysts than the other age groups. High oocyst excretion rate and severe pathogenicity of *Eimeria* in lambs and kids have already been confirmed by many researchers [2, 15, 16, 18, 19, 20]. Constable et al (2016) explained that artificial infection with *E. ninakohlyakimova* was very harmful to lambs, however natural infection with numerous species of *Eimeria* had no effect on their growth rate [4]. It has also been demonstrated that young animals who have never been exposed to coccidia are more sensitive to infection and have a high case-fatality rate. On the other hand, excessive stress in elderly animals can result to immunosuppression, which can lead to clinical coccidiosis and oocyst shedding [5]. Because coccidia immunity is specific but not solid, large quantities of oocysts may cause continuous reinfection and raise the level of environmental contamination. Furthermore, pathogenic and non-pathogenic *Eimeria* species can infect livestock at the same time [2]. As a result, in addition to oocyst excretion, clinical signs of coccidiosis should be addressed. Our findings revealed that 51.82 percent of positive cases (85 cases) had particular clinical signs, whereas others just excreted the oocysts.

Many studies believe coccidiosis is host specific [2, 4, 5], and our findings rule out the potential of transitory co-infestation between sheep and goats using molecular assay as well.

The incidence of coccidiosis was higher in yearling sheep and those under 6 months of age than in adults, which was consistent with other findings [3, 15, 16, 17, 21] but contradicted Al-Saadoon and Al-Rubai (2018) conclusion [10]. Silva et al (2012) have believed that acquired immunity is the main reason for decreasing oocytes excretion in adult sheep [22], however, rams may shed more oocysts during the breeding season in response to high testosterone levels.

This study showed that oocyst excretion was considerably higher in the spring and summer than in the autumn and winter, which is similar to Yan et al 's findings for the spring. In contrast, Constable et al (2016), Yakhchali and Rezaei (2010), and Yakhchali and Zareei (2008) believe that oocyst excretion is higher in late autumn and throughout the winter. Yan et al. (2021) also stated that the intensity of coccidia infection was lower in semi-arid, semi-humid, and cold areas with long winters and short summers than in similar areas with higher annual average temperatures and lower average precipitation [3]. The central Zagros region is a mountainous, semi-humid and cold region with average rainfall which its southern counties are warmer than others. But the height of the western (2365 meters), eastern (2432 meters) and central (2310 meters) counties are more than northern (1963 meters) and southern (1556 meters). In this study, the rate of oocyst excretion in sheep was higher in the central and western counties than in the southern ones. In goats, however, annual

oocyst excretion was higher in eastern counties than in the south and west. These findings imply that altitude plays a role in enhancing sheep oocyst shedding.

### **Conclusion**

It is concluded that several environmental conditions, such as high altitude, play an important role in sheep oocyte excretion in the central Zagros region. Coccidia prevalence was also shown to be higher in the spring, among females, and among the younger generation.

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