A molecular survey of *Besnoitia caprae* in goats in the southwest of Iran

Running title: *Besnoitia caprae* in goats

Omid Sarkhosh [1], Asma Keyvanlou [2], Gholamreza Razmi [3]

1- Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

2- Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

3- Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. https://orcid.org/0000-0002-0754-1278.

Present address of Corresponding Author*:

Gholamreza Razmi

Ferdowsi University of Mashhad, Department of Pathobiology,

Faculty of Veterinary Medicine, P.O. Box: 91775-1793

Mashhad, Iran

Tel: +98 51 38788944

Fax: +98 51 38763853

razmi@um.ac.ir

Abstract:

Introduction: Caprine besnoitiosis is a significant disease that leads to economic losses in goat herds due to its chronic nature and progressive weight loss. The disease is caused by *Besnoitia caprae*, which results in the formation of subcutaneous cysts in various parts of a goat's body. The disease has been reported in goats from the Fars and Kerman provinces, Iran. Given the proximity of Fars to Khuzestan province and the movement of nomadic sheep herds between these regions, we aimed to investigate the frequency of *B. caprae* in goats in the Behbahan area in Khuzestan province.

Materials &methods: In early autumn of 2022, a comprehensive inspection was conducted on 200 goat carcasses, focusing on their skin and eyes for the presence of *Besnoitia* cysts in Behbahan slaughterhouse, and then ear samples were obtained from each goat carcass. The samples were placed in containers with ethyl alcohol and transported to the parasitology laboratory at the Faculty of Veterinary Medicine. The inner surfaces of the skin of the ear samples were scraped with a scalpel and transferred to microtubes. Subsequently, the samples were divided into 50 pooled samples. DNA was extracted from each sample using a commercial kit. Finally, the samples were analyzed using the PCR method.

Results: In this study, no *Besnoitia* cysts were found in goat carcasses. The PCR results indicated that 30% (15/50) were infected with *B. caprae*. Two positive samples were sent to a genetic company for sequencing. The samples sequenced were analyzed using BLAST software, revealing approximately 99% alignment with the recorded sequences of *B. caprae* in the gene bank.

Conclusions: The molecular findings of this study highlighted a high prevalence of *B. caprae* in goats in the Behbahan area, necessitating further research to assess the economic impact of besnoitiosis on the local goat population.

Keywords:

Frequency, Goat, PCR, *Besnoitia caprae*, Iran

1- Introduction:

Besnoitia belongs to the Sarcocystidae family and the subfamily Toxiplasminae. So far, many species of this protozoan have been reported in different animals [1].These species are specific to each animal. The life cycle of some *Besnoitia* species, including *B. darlingi*, *B. wallasii*, and *B. erectafelsi*, is well known, and their definitive host is the cat. However, in other species, including *B. caprae*, the definitive host and life cycle have not been identified[1;2; 3] . Mechanical transmission of *B. caprae* by blood-sucking insects has been proven [4]. So far, a relatively high prevalence of *B. caprae* has been reported from goats in Nigeria [5], Kenya [6;7], and Iran[8; 9;10]. Caprine besnoitiosis has a chronic form in goats in these countries, causing skin lesions such as hyperkeratosis, along with hair loss on the head, face, and testicles. These symptoms are seen in goats over three years of age. The disease leads to weight loss and leads to severe economic losses. Additionally, the most common clinical sign in infected goats is the presence of cysts in the conjunctiva of the eye [11;12]. Also, A study has mentioned the *B.caprae* infection in nomadic goats in some southern provinces, Iran [3]. Goats are content animals, and their walking power allows them to travel to large areas in search of forage and often consume plants that are undesirable or unusable for other animals. This genetic potential of goats has caused the goat population in Khuzestan province to be higher than that of sheep. Given the proximity of Khuzestan province to the infected province (Fars), and the migration of nomadic sheep herds in these two provinces, it seems that this infection also exists in goats in this province. Therefore, in this study, an attempt was made to determine the frequency of *Besnoitia* spp in goats in the Behbahan area, which is the closest to Fars province, which is located on the migration route of nomadic sheep herds, using molecular PCR methods.

2- Materials and Methods:

2_1 Location of sampling:

Behbahan area is in the southeast of Khuzestan and borders Kohgiluyeh &Boyer-Ahmad and Bushehr provinces. With an area of 3,715 square kilometers, this county is the second-largest county in Khuzestan province. Behbahan area is a semi-desert area, with annual rainfall of 366 milliliters and a hot and dry climate, with the highest temperature being 50°C in July and August, and the lowest temperature being 0°C in February.

2-2 Sampling

From September to October 2022, A total of two hundred samples was collected from the goat carcasses in the slaughterhouse. Briefly, the age and sex of the slaughtered goats were recorded, and then the goats' carcasses were examined for the presence of cysts in the eyes and skin. The ear tissue containing cartilage was cut with a clean scalpel blade and placed in sampling containers containing 70% ethyl alcohol. The samples were kept under cool conditions and transferred to the parasitology laboratory of the Faculty of Veterinary Medicine, Ferdowsi University. In laboratory, 200 samples were divided into 50 groups of 4 according to age and gender. The inner surface of the skin samples of each ear was scratched with a scalpel and placed into a microtube

2-3 DNA extract and PCR:

The DNA of *B. caprae* was extracted using an animal tissue DNA extraction kit (Sinacolon Company, Tehran) according to the kit's instructions. The quality of the extracted DNA was observed by electrophoresis on a 1% agarose gel after staining. A PCR assay was carried out to detect the *B. caprae* gene with amplification of the ITS1 fragment. [14]. The simple PCR reaction was performed in a 25µl mixture containing 2µl of total DNA, 12.5µl of commercial premix master mix (Parstous Mashhad), 1µl of each primer, and 8.5µl of nuclease-free water in a thermocycler. The cycling condition was as follows: an initial denaturation step at 94 °C for 5 min followed by 35 cycles of 94 °C for 15 sec, 61 °C for 1 min, 72 °C for 1 min, and a final extension step at 74 °C for 10 min. The Oligonucleotide primers were the forward primer

Table 1: The sequences of primers of *B. caprae*

Primer name	Gene name	Sequence name	Primer sequences
Bes-F	18 S rDNA	ITS1	5`-CCT CCT CAC TCT GCT ATC ACG -3`
Bes-R	18 S rDNA	ITS1	5`-TTC CAC TGG TAA CGC CTC T-3`

The distilled water was added instead of the DNA sample in the negative control microtube. The positive control sample was provided by Dr Namvari from the Razi Vaccine & Serum Research Institute-Shiraz branch

The PCR products were subjected to electrophoresis on a 2% agarose gel in TBE ×1 buffer, stained with CyberGreen, and visualized using ultraviolet light. The size of the DNA fragments was compared with a standard molecular weight (100bp DNA ladder). The positive control sample had a band of 755 bp.

2-4 Sequencing

Among all PCR-positive samples, four samples with high DNA concentration were chosen. The DNA samples with primers were submitted for sequencing to a company in Iran (Topaz-Genekaush Co, Karaj, Iran). The nucleotide sequences were assembled and edited with the Mega software Version 11. The edited nucleotide sequences of isolates were aligned with previously sequenced *B. caprae* in GenBank (NCBI) using the Clausthal W method (Mega software Version 11). The phylogenetic analysis was carried out with the neighbor-joining process and bootstraps of 1000 replications.

2-5 Statistical analysis

The frequency of *Besnoitia* infection in slaughter goats was analyzed by age and sex using the *Chi-square* test.

3- Results

In this research, post-mortem inspection showed no *Besnoeitia* cysts beneath the skin and the eyes of all goat carcasses. Of the 50 pool samples of goat ear skin DNA, 15 pool samples (30%) reacted positively in the PCR test with *Basnoitia* specific primers (Fig.1).

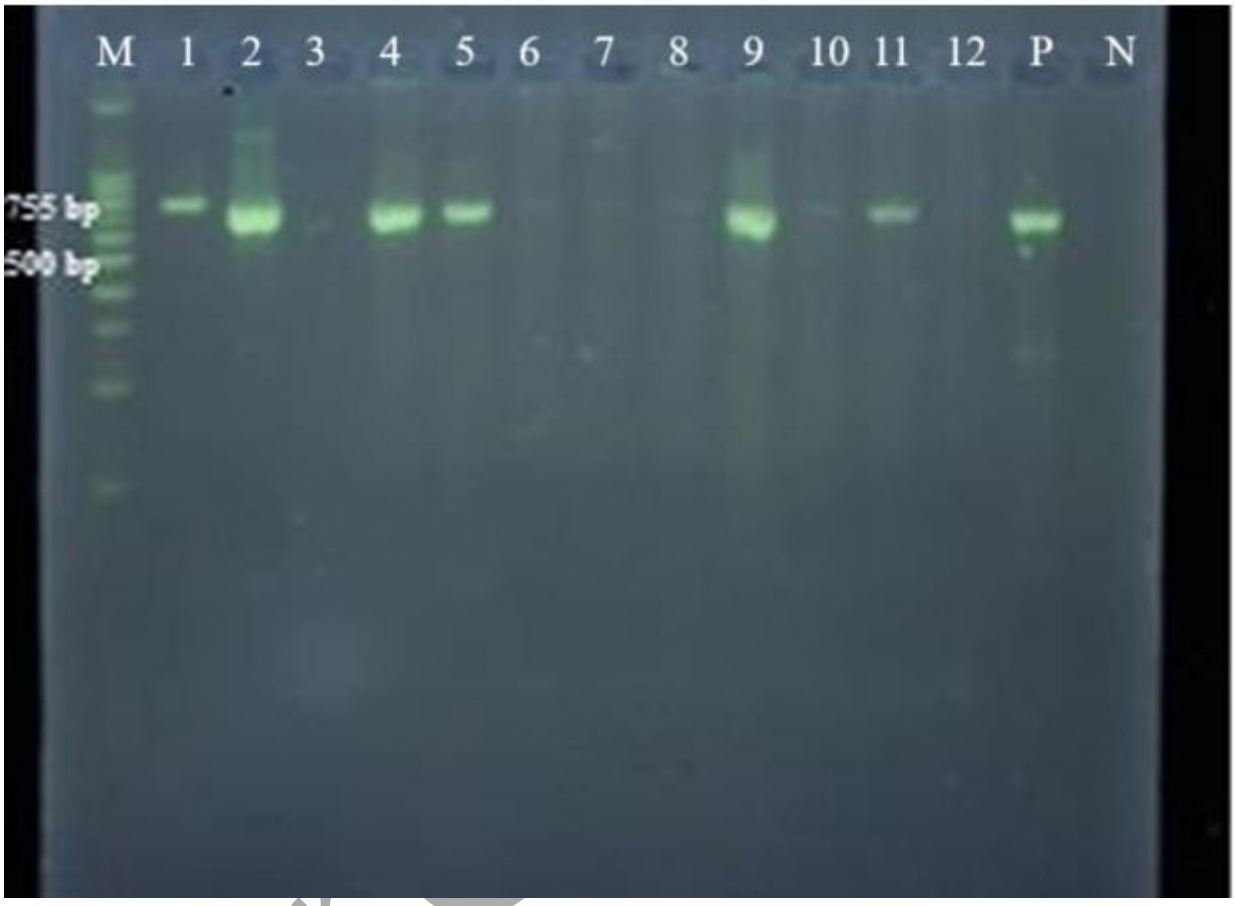

Figure 1- Detection of *Besnoitia* infection by PCR. M: 100 bp marker, well P: positive control with 755 bp band, well N: negative control, and wells 1-12 corresponding to sample DNA

The results indicated that the highest frequency of infection occurred in the age group exceeding four years, while the lowest in the 2–3-year age group ($P<0.05$) (Table 4). The statistical analysis did not indicate a significant difference in the frequency of *Besnoitia* infection between the two sexes ($p>0.05$) (Table 2).

Table 2. The frequency of *B.caprae* infection in slaughtered goats by age and sex

Risk factor	Results	Positive (%)	Negative	Total	P-value
Age (year)	< 2	0	1	1	P<0.05
	2-3	1 (8.30)	11	12	
	3-4	4 (23.52)	13	17	
	>4	10 (47.61)	11	21	
Total		15 (30)	35	50	
Sex	Male	11(28.20)	28	39	P>0.05
	Female	4 (36.36)	7	11	
Total		15 (30)	35	50	

The sequenced fragment was 99% identical to the sequenced fragment available in the GenBank under the identification number HM-008988 of *B. caprae* (Fars Province, Iran). Also, the phylogenetic tree of the sequenced sample was drawn in comparison with other sequenced samples of other *Besnoitia* species (Figure 9).

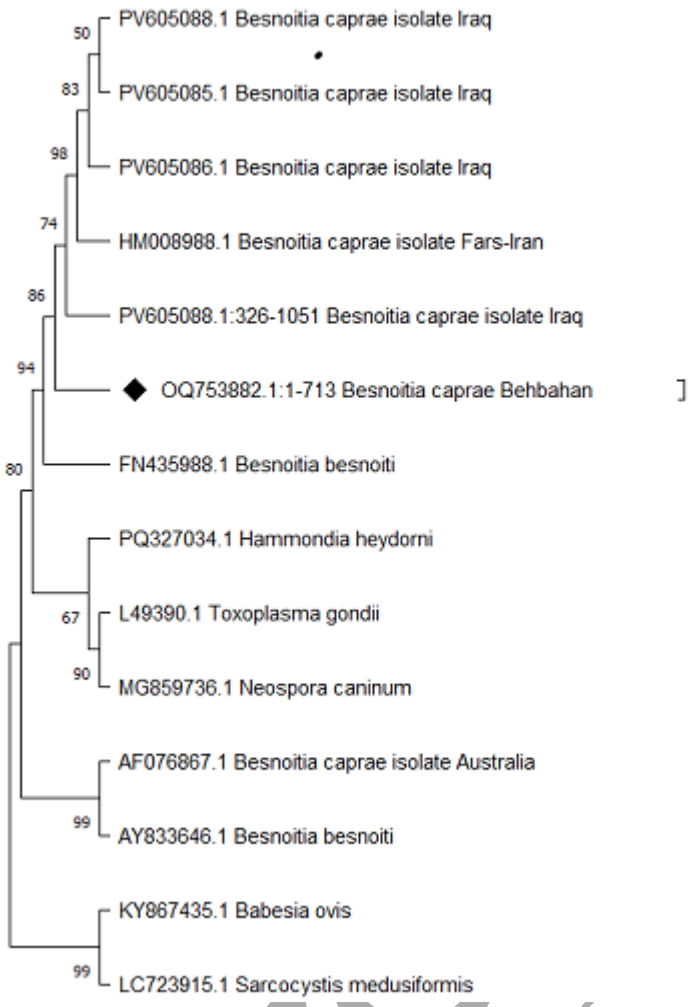

Figure 2- The phylogenetic tree of the isolate identified in this study with the sequenced samples of *Besnoitia* species in Iran and other countries based on the ITS1 gene with the neighbor-joining method, with a bootstrap of 1000 replications. *Babesia ovis* and *Sarcocystis medosiformis* were outgroups

4- Discussion

In this study, despite the absence of skin lesions and subcutaneous cysts in goat carcasses, a relatively high infection rate was determined in goats in the Behbahan slaughterhouse using PCR. So far, limited studies have been conducted on the prevalence of *Besnoitia* infection in goats in Iran and Kenya [3;7]. A study reported the frequency of *B. caprae* infection in goats at the range of 20 to 50% in Fars province and 5 to 35% in Bushehr, Khuzestan, Kohkiluyeh, and Boyer Ahmad provinces [3]. Caprine besnoitiosis is characterized by a prolonged chronic disease course, with symptoms appearing late in the progression of the illness [3;6]. Asymptomatic goats that are infected and grazing with the herd facilitate the transmission of the infection via blood-sucking insects [3]. The reported high frequency in this study may be related to sampling during the season of arthropod activity. A histopathological study involving forty symptomatic and one hundred asymptomatic goats with *Besnoitia* infection revealed that all symptomatic goats had several cysts measuring 0.4 to 0.9 mm in diameter in the eyes and subcutaneous tissues. In contrast, a few cysts were found in biopsy samples of 12 % asymptomatic goats [14]. In a similar study, no clinical and gross lesions were observed in the skin of the goats from abattoirs in Nigeria; only an incidence of 0.7% of caprine besnoitiosis was recorded in skin samples of the neck area using histopathological examination [5]. Furthermore, other histopathological studies established a correlation between the pathological lesions found in subcutaneous areas of the head and lower limbs with *Besnoitia* cysts infection [10;11]. In this study, the frequency of *Besnoitia* infection showed no significant difference in males and females, while the frequency of infection in goats over four years of age was significantly higher than in other younger ages. The similar results of the study also mentioned that *Besnoita* infection is observed more frequently in goats over three years of age than in other ages[14]. The nucleotide sequence of the positive samples was approximately 99% to the sequenced fragment of *Besnoitia caprae* from goats in Fars province, Iran. The phylogenetic analysis of this research utilizing the ITS1 gene showed that the isolates found in the goats were genetically similar to those categorized as *B. caprae* in Iran and shared a common ancestor with *Besnoitia*, *Toxoplasma*, *Neospora*, and

Hammondia. [14]. According to the results obtained, it can be concluded that *B. capreae* infection is very prevalent among goats in the Behbahan area. Conclusion: The relatively high frequency of *Besnoitia* infection was determined in goats in the Bebahan slaughterhouse by PCR. It is necessary to conduct similar molecular studies across southern Iran to establish strong epidemiologic data that would allow the implementation of effective transnational control programs

Funding:

This study was supported by grant 3/54170 from the Vice President of Research and Technology of Veterinary Medicine, Ferdowsi University of Mashhad, Iran.

Conflict of interests:

The authors declare no conflict of interest.

Data availability:

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics and animal experimentation

Not applicable

Author contribution

Conceptualization: G.R.R., Methodology: O.S., A.K Investigation: O.S, A.K, G.R.R, Writing - original draft preparation: G.R.R; Writing - review and editing: G.R. Funding acquisition: G.R.R, Supervision: G.R.R.

Acknowledgments: The authors give special thanks to Dr. Namavari at the Razi Vaccine & Serum Research Institute -Shiraz branch, for providing the positive control PCR. We would like to express our gratitude to Dr Alireza Sazmand for their assistance with the laboratory examination.

References

[1] Wallace GD, Frenkel JK. Besnoitia species (Protozoa, Sporozoa, Toxoplasmatidae): recognition of cyclic transmission by cats. Science (80-). 1975;188(4186):369–71.

[2] Smith DD, Frenkel JK. Besnoitia darlingi (Protozoa: Toxoplasmatinae): cyclic transmission by cats. J Parasitol. 1977;1066–71.

[3] Oryan A, Silver IA, Sadoughifar R. Caprine besnoitiosis: an emerging threat and its relationship to some other infections of ungulates by Besnoitia species. Res Vet Sci. 2014;97(1):1–7.

[4] Kasigazi S. Caprine besnoitisis: studies on the experimental intermediate hosts and the role of the domestic cat in transmission. Vet Parasitol. 1994;52(3–4):207–10.

[5] Sambo SJ, Ibrahim NDG, Esievo KAN, Hambolu JO, Makoshi MS, Tekdek LB. First incidence of caprine besnoitiosis in Kaduna State of Nigeria. J Anim Vet Adv. 2007;6(5):673–5.

[6] Mehlhorn H. Besnoitia. In: Parasitic Protozoa of Farm Animals and Pets. Springer; 2017. p. 169–85.

[7] Bwangamoi O, Carles AB, Wandera JG. An epidemic of besnoitiosis in goats in Kenya. Vet Rec. 1989;125(18):461.

[8] Bazargani TT, Gharagozlou MJ, Ebrahimi A. A survey on besnoitiosis in goats in Baft, Kerman. J Vet Fac Univ Tehran. 1987;41(3/4):19–44.

[9] Cheema AH, Toofanian F. Besnoitiosis in wild and domestic goats in Iran. Cornell Vet. 1979;69(3):159–98.

[10] Oryan A, Namazi F, Namavari MM, Sharifiyazdi H, Moraveji M. Comparison of the pathogenesis of two isolates of Besnoitia caprae in inbred BALB/c mice. Vet Res Commun [Internet]. 2010;34(5):423–34. Available from: https://doi.org/10.1007/s11259-010-9415-0

[11] Mirzaie M, Derakhshanfar A, Pourjafar M. Caprine besnoitiosis in the southwest of Iran. Vet Arh. 2007;77(5):435–9.

[12] Oryan A, Azizi S. Ultrastructure and pathology of Besnoitia caprae in the naturally infected goats of Kerman, East of Iran. Parasitol Res. 2008;102(6):1171–6.

[13] Ellis JT, Holmdahl OJM, Ryce C, Njenga JM, Harper PAW, Morrison DA. Molecular Phylogeny of Besnoitia and the Genetic Relationships Among Besnoitia of Cattle, Wildebeest and Goats. Protist [Internet]. 2000 Dec 1 [cited 2025 Aug 8];151(4):329–36. Available from:

https://www.sciencedirect.com/science/article/abs/pii/S1434461004700310

[14] Oryan A, Sadeghi MJ. An epizootic of besnoitiosis in goats in Fars Province of Iran. Vet Res Commun [Internet]. 1997;21(8):559–70. Available from: https://doi.org/10.1023/A:1005918931296