

**Original Article**

## **Prevalence and Early Detection of Hypodermosis in Goats using a Competitive ELISA System in Lorestan, Iran**

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### **Abstract**

This study aimed to determine the prevalence and early detection of hypodermosis in goats by the investigation of *Przhevalskiana* larvae and sera collected from the infested animals. This study was conducted in Lorestan province, located in the South-West of Iran, from April 2017 up to April 2018. A total of 3350 goats slaughtered in Lorestan abattoirs were investigated by clinical-parasitological examinations in different periods. The larvae were collected from the back and flank regions of the slaughtered goats. The number of infested animals, gender and age, number of maggots present on the body of each animal, location, and larval stage of warble flies were recorded in this study. To detect an infestation in the early period, a total of 150 blood samples were randomly collected from the field animals in Lorestan, Iran. The morphological findings showed that out of 3350 goats examined, 706 (21.07%) goats were infested. Furthermore, three species of *Przhevalskiana*, including *P. Silenus* (n=726, 50.07%), *P. crossii* (n=440, 30.43%), and *P. aegagri* (n=284, 19.59%) were recognized as the causative agents of goat hypodermosis in this province. No significant difference was observed between genders and/or among the age groups ( $P>0.05$ ). The anti-*Przhevalskiana* antibodies in the serum samples were detected using ELISA from August up to mid-September (summer). Clinical diagnosis of infestation was usually performed from late October until mid-March (winter) by visual observations and direct palpation of warbles in the back and flank regions of the animals. It could be concluded that the use of ELISA can help to detect hypodermosis among goats in the early stages.

**Keywords:** ELISA, Goat, *Przhevalskiana*, Lorestan

### **Prévalence et Détection Précoce de L'hypodermose Chez les Chèvres à L'aide d'un Système ELISA Compétitif dans le Lorestan, Iran**

**Résumé:** Cette étude visait à déterminer la prévalence et la détection précoce de l'hypodermose chez les chèvres par l'enquête sur les larves de *Przhevalskiana* et les sérums prélevés sur les animaux infestés. Cette étude a été menée dans la province du Lorestan, située dans le sud-ouest de l'Iran, à partir d'avril 2017 à avril 2018. Au total, 3350 chèvres abattues dans les abattoirs du Lorestan ont été étudiées par des examens cliniques et parasitologiques à différentes périodes. Les larves ont été collectées dans les régions du dos et des flancs des chèvres abattues. Le nombre d'animaux infestés, le sexe et l'âge, le nombre d'asticots présents sur le corps de chaque animal, l'emplacement et le stade larvaire des mouches gazeuses ont été enregistrés dans cette étude. Pour détecter une infestation au début de la période, un total de 150 échantillons de sang ont été prélevés au

hasard sur les animaux de terrain du Lorestan, en Iran. Les résultats morphologiques ont montré que sur 3350 chèvres examinées, 706 (21.07%) chèvres étaient infestées. En outre, trois espèces de *Przhevalskiana*, dont *P. Silenus* (n=726, 50.07%), *P. crossii* (n=440, 30.43%) et *P. aegagri* (n=284, 19.59%) ont été reconnues comme les agents responsables de l'hypodermose caprine dans cette province. Aucune différence significative n'a été observée entre les sexes et / ou entre les groupes ( $P > 0.05$ ). Les anticorps anti-*Przhevalskiana* dans les échantillons de sérum ont été détectés par ELISA d'août à mi-septembre (été). Le diagnostic clinique d'infestation était généralement effectué de la fin octobre à la mi-mars (hiver) par des observations visuelles et la palpation directe des gastérophiles dans les régions du dos et des flancs des animaux. On pourrait conclure que l'utilisation de l'ELISA peut aider à détecter l'hypodermose chez les chèvres à un stade précoce.

**Mots-clés:** ELISA, Chèvre, *Przhevalskiana*, Lorestan

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## Introduction

Goat warble-fly infestation is a parasitic disease of goat that is caused by larvae of *przhevalskiana* species (spp.). Its larvae cause subcutaneous myiasis in native goats and with a lesser ratio in the sheep. Gazelles are reservoir hosts of the disease in most regions all over the world (Azizi et al., 2007). This type of subcutaneous myiasis causes the formation of subcutaneous abscesses and perforation of the skin in the animal. Such skin problems not only destroy skin and leather but also decrease economic animal production (Abo-Shehada et al., 2006; Yadav et al., 2012). Hypodermosis shows a global publication, and regarding previous studies, myiasis caused by *przhevalskiana* spp. has been reported in some regions of the world, such as Iraq, Saudi Arabia, Pakistan, Egypt, Greece, Turkey, Italy, and Albania (Oryan and Bahrami, 2012; Ahmed et al., 2016).

Goats reared in different areas are infected by warble flies of the *przhevalskiana* spp. Furthermore, it causes economic losses in livestock products, such as milk and meat (Oryan et al., 2009). Accordingly, there is a need to develop an appropriate diagnostic and control program for the prevention of the damage in animals' skin before such economic losses that are associated with an infestation (Otranto et al., 2005; Khan et al., 2012). Myiasis can directly be detected when subcutaneous nodules are palpable; however, the early stages of the disease cannot be detected by the method. The myiasis and infestations were commonly

underestimated and neglected, while indirect detection, such as ELISA, could be a beneficial procedure in the early diagnosis of this parasite (Faliero et al., 2001; Jan et al., 2014). The ELISA method has been reported as a simple, fast, and profitable technique that could be used as an appropriate alternative for the detection of the clinical condition and warble fly infestation (Otranto et al., 1999; Jan et al., 2014). Furthermore, ELISA has been used for goat grub in some European and Eastern countries (Otranto et al., 2004). Previous studies have identified the ELISA test as a reliable method for the detection of antigen C of *H. lineatum* and approved a correlation between the parasitological results and changes in the antibody contents (Otranto et al., 1999; Colwell et al., 2008). Regarding the different life cycles of this parasite based on the climate conditions in different areas and the importance of this disease in goats, this study aimed to investigate the early detection of antibody development in the goat hypodermosis infested-sera using a competitive ELISA system in Lorestan province, Iran.

## Material and Methods

**Study Area and Animals.** A total of 3350 slaughtered goats were obtained from different abattoirs located in such cities as Khoramabad, Alashtar, Borujerd, Kuhdasht, Delfan, and Pol Dokhtar in Lorestan province, Iran. They were then investigated weekly considering habitat, age, and gender from April 2017 up to April 2018. The age range was assessed by the investigation of the teeth, and the animals were

classified into the age groups of 1-2, 2-2.5, 2.5-3, and >3 years. The inner skin surface and subcutaneous tissue of the slaughtered and skinned goats were then investigated in this study. Furthermore, the number of maggots in each animal was recorded considering the different locations and larval stages.

**Collection of Larvae.** The larvae in different age stages of *przhevalskiana* flies were collected and counted from the subcutaneous tissues of the back and flank regions in the infested slaughtered goats. They were then separated by squeezing the subcutaneous nodules and stored in 70% alcohol as well as 5% glycerin. Following that, they were transferred to the laboratory for later identification.

**Experimental Laboratory Design.** The different larval stages and species were investigated, identified, and classified by key characteristics as reported by Zumpt (1965). The larval stage was classified based on the larval sizes of 2-7 (L<sub>1</sub> larvae), 9-13 (L<sub>2</sub> larvae), and 10-18 mm (L<sub>3</sub> larvae). The set of denticles above the mouth-dots were used to distinguish different larval species from each other. Denticles are arranged in a single and medially interrupted row in *P. crossii*. Moreover, they are extremely small and are in low numbers, quite irregular, and highly decreased in *P. silenus*. It should be mentioned that there were no denticles in the segment above the mouth-dots in *P. aegagri* (Zumpt, 1965).

**Preparation of the Larval Antigen.** To prepare the larval antigen, initially, the instar larvae of *przhevalskiana* were obtained from the back and flank regions of the infested goats slaughtered in different abattoirs in Lorestan province, Iran, from September to October 2017. The collected larvae were washed four times (4-5 min/once) by 200 ml sterile phosphate-buffered saline (PBS) in pH=7.2 as described by Webster et al. (1997). Larvae were then homogenized in a Griffiths tube containing carbonate buffer 0.1 M in pH=9.6 and slowly stirred in 4°C for 16 h. The homogenate samples were then centrifuged in 28000 g at 4°C for 15 min, and the supernatant soluble

containing larval antigens was removed and stored in small aliquots at -20°C for subsequent uses. To evaluate the protein concentration, the extracted antigen was assessed by the Macro Lowry method using the bovine serum albumin standards, and optical densities were obtained spectrophotometrically by absorbance in 750 nm (Webster et al., 1997).

**Preparation of the Hyperimmune Rabbit Sera.** To prepare the hyperimmune rabbit sera, five or six-month-old New Zealand white rabbits with almost equal weight were selected in this study. One of them was considered as a control, and others were subcutaneously inoculated with a total of 500 µg/ml of crude antigen or supernatant soluble isolated from homogenized larvae. The antigen was inoculated as a 1:1 emulsion in Freund's complete adjuvant in the first instance and then were inoculated as a 1:1 emulsion from Freund's incomplete adjuvant 14 and 28 days later. The blood samples were collected two weeks after the last inoculation through a cardiac puncture. The sera sample was then separated and stored in small aliquots at -20°C.

**Collection of the Sera Samples from Goat.** To collect the sera samples from goats, a total of 150 blood samples were randomly collected from the field animals between April and September 2017. The sera samples were then separated after blood clotting and centrifuged at 2000 rpm for 5 min. Furthermore, the goat serum positive and negative control sera were obtained from the infested goat after slaughtering and observing the larvae under the skin and a non-infested goat. The sera samples were kept in small aliquots at -20°C.

**ELISA Method.** Based on the method by Webster et al. (1997), immune module polysorb F12 strips (Nunc) were covered by L<sub>1</sub> antigen of *przhevalskiana* in 1, 2.5, 5, 10, and 20 µg/ml in carbonate buffer 0.1M and pH=9.6 for 16-24 h at 4°C (100 µl/well). The wells were washed triplicate using phosphate 0.01M, sodium chloride 0.15 M, pH=7.2, and 0.05% Tween 20 (PBST) and then blotted dry. In the blocking stage, 250 µl

PBST 5% normal horse serum (NHS) was included in each well and then incubated for 1.5 h at 37°C. The wells were washed four times by PBST and blotted dry. The different dilutions of goat serum in competition with various dilutions of the hyperimmune rabbit serum in PBST 1% NHS were used to test per sample (50 µl per well) and incubated for 45 min at 37°C. Extra wells were included as "rabbit serum only" and "goat serum only" control. Hyperimmune rabbit serum diluted was added into all the wells (50 µl per well). Goat serum only was regarded as a control well, and then the samples were incubated at 37°C for 30 min. The wells were washed four times using the PBST.

Following that, the conjugate serum and mouse anti-rabbit horseradish peroxidase was diluted in a 1:1000 ratio in PBST 1% NHS, added into all the wells (100 µl per well), and incubated for 75 min at 37°C. The wells were then washed five times, and the reaction was continued by adding 100µl of Chromogen BM blue (Roch) per well and incubated for 20 min in a dark place. The reaction was stopped after nearly 5 min by the inclusion of sulfuric acid 10% (50 µl per well). The results of the samples were read by a microplate photometer reader at 450 nm of optical density. Finally, the cut-off value for positive and negative samples was assessed based on calculating the mean percentage of inhibition of the panel negative sera plus two standard deviations (SD) of the mean.

**Statistical Analysis.** The data and the obtained information were analyzed in Office Excel 2016 and SPSS software (version16) through the Chi-square test. A p-value less than 0.05 was considered statistically significant.

## Results

**Detection of the Genus and Species and the Abundance of Larvae.** A total of 2150 larvae of *przhevalskiana* fly in the different developmental stages were collected from the subcutaneous tissues of 706 infested goats slaughtered. In total, 700 larval samples were found to be in the first and second stage, and 1450 samples were in the third instar larvae

(Figures 1 and 2). After the investigation of the adult larvae (L3) and morphological features of the larvae based on the valid diagnostic key, three species of *Przhevalskiana* genus, including *P. Silenus*, *P. crossii*, and *P. aegagri* with 726 (50.07%), 440 (30.43%), and 284 (19.59%) infestations, respectively, were recognized as the causative agents of the goat hypodermosis in the region.

A significant difference was also observed among these infestations regarding different species of *Przhevalskiana* ( $P<0.05$ ). It was found that the *P. silenus* (50.07%) was the most effective agent in the infestation of goats in the region. The larvae of the *Przhevalskiana* species were observed in the second half of October 2017 in the subcutaneous tissues of the dorsal region in the slaughtered goats. However, they were usually found in the subcutaneous tissue of goats until early April 2018. The maximum amount of infestation was found in late December up to mid-January 2017. A total of 142 (31.55%) goats were infested out of 450 examined goats. The found larvae were in the second stage larvae of the *przhevalskiana*. The lowest levels of infestation were also found in 873 examined goats in April up to September 2017. No larval stages of the *przhevalskiana* were found in the subcutaneous tissue of the slaughtered goats. Additionally, there was a significant difference among the infestations in terms of different seasons ( $P<0.05$ ).

**Prevalence of the Infestation in Goats.** The results of the study showed that out of 3350 examined goats, 706 (21.07%) cases were parasitized, and 536 (75.92%) goats were female. There was no significant difference between male and female goats regarding infestation ( $P>0.05$ ). Regarding the age group, the infested goats by *przhevalskiana* spp. were in the age groups of 1-2 (n=138; 19.54%), 2-2.5 (n=226; 32.02%), 2.5-3 (n=163; 23.09%), and >3 years (n=179; 25.35%).

Furthermore, there was no significant difference among the different age groups in terms of infestation ( $P>0.05$ ). In all cases, the infestation was only observed in the dorsal region of the goats and rarely in the back-

neck (in severe cases of infestation). The prevalence rates of infestation with *Przhevalskiana* spp. in the examined goats in different abattoirs of Lorestan province, Iran, were obtained at 25.54% (Khoramabad), 13.66% (Alashtar), 12.5% (Borujerd), 28.78% (Kuhdasht), 12.83% (Delfan), and 30.06% (Pol Dokhtar) (Table1). It should be noted that there was no significant difference among the different areas of Lorestan province, Iran, regarding infestation ( $P>0.05$ ).

**Concentration of Larval Antigen.** The extracted antigen was evaluated by the Macro Lowry method to calculate the larval antigen density. The concentration of larval antigen was considered 2.5mg/ml and applied to the ELISA test.

**ELISA Test.** The different samples of goat sera and hyperimmune rabbit serum were tested in concentrations of 1, 2.5, 5, 10, and 20  $\mu\text{g/ml}$  of the  $L_1$  antigen of *przhevalskiana*. The results showed that the dilutions of 1/200 goat sera and 1/3000 hyperimmune rabbit sera, as well as 5 $\mu\text{g/ml}$  antigen concentration, were suitable for the ELISA test set up. Regarding these dilutions, a total of 150 serum samples were tested using the competitive ELISA for early detection of anti-*przhevalskiana* antibody development

in the goat serum samples. Based on the optical density values derived from the samples at 450 nm, the mean percentages of inhibition (PI%) in negative and positive samples were obtained at 30.16% and 50.37%, respectively. Moreover, the SD values of negative and positive samples were determined at 7.98% and 2.7%, respectively. Therefore, the cut off was considered a threshold, and the inhibition percentages higher and lower than that limit were regarded as positive and negative samples, respectively.

**Determination of the Cut-off.** Regarding the mean inhibition percentages of (PI%) negative samples plus 2SD, the cut-off value between positive and negative samples was estimated at 44.48%. In addition, this cut-off percentage was the threshold for a pair of positive and negative samples. Moreover, the cut-off value of 150 serum samples tested by the ELISA method showed that 35 (23.33%) and 113 (75.33%) samples were positive and negative, respectively. The presence of antibodies in the serum samples was also detected during August and mid-September 2017 in the summer. Additionally, the clinical diagnosis of the infestation is usually from October until mid-March (winter) by visual observations and direct palpation of warbles in the back and flank regions of the animals (Table 2).



**Figure 1.** Size of different larval stages of the *Przhevalskiana* flies ( $L_1$ : about 8 mm in length,  $L_2$ : 12mm in length, and  $L_3$ : larger than  $L_1$  and  $L_2$  larval stages about 18mm in length).



**Figure 2.** Nodules, ulcers, and abscesses containing third-stage larvae of *przhevalskiana* in the subcutaneous tissues of the infested slaughtered goats (a slaughterhouse in Khoramabad).

**Table 1.** Prevalence of hypodermosis infestation and different species of the *Przhevalskiana* isolated from the examined goats in different cities in Lorestan, Iran

Cities	Examined animals	Non-Infested	Infested	Prevalence (%)	Number of larvae species isolated		
					<i>P. silenus</i>	<i>P. crossii</i>	<i>P. aegagri</i>
<b>Khoramabad</b>	595	443	152	25.54%	127	79	55
<b>Alashtar</b>	476	411	65	13.66%	98	58	39
<b>Borujerd</b>	600	525	75	12.5%	117	65	35
<b>Kuhdasht</b>	594	423	171	28.78%	140	84	57
<b>Delfan</b>	483	421	62	12.83%	95	68	36
<b>Pol Dokhtar</b>	602	421	181	30.06%	149	86	62
<b>Total</b>	3350	2644	706	21.07%	726	440	284

**Table 2.** Number and percentage of infestation of goat serum samples tested by ELISA according to the time of sampling (From April up to September 2017)

Months	Number of collected sera	Number of positive sera tested by ELISA	%Percentage
April	15	-	-
May	15	-	-
June	15	-	-
July	15	-	-
August	40	13	32.5%
September	50	22	44%
<b>Total</b>	150	35	23.33%

## Discussion

The prevalence of *przhevalskiana* spp. in goats has been reported in different countries, such as Italy (49.1%) (Gaglio et al., 2016), Greece (54.2%) (Papadopoulos et al., 1997), Saudi Arabia (6.8%) (El-Azazy, 1997), and Jordan (10%) (Abo-Shehada et al., 2006). In Iran, there are reports from different regions, including Khuzestan (36.56%), Esfahan (51.36%), Fars (93%), Masjed-Soleyman (5.3%), Western Azerbaijan (13%), and Eastern Azerbaijan (4.2%) (Tavassoli et al., 2010). The results of this study indicated the prevalence of this disease in the examined goats in different abattoirs located at cities in Lorestan province, Iran, such as Khoramabad (25.54%), Alashtar (13.66%), Borujerd (12.5%), Kuhdasht (28.78%), Delfan (12.83%), and Pol-Dokhtar (30.06%). The present study obtained higher values, compared to those reported in Saudi Arabia and Jordan (El-Azazy, 1997; Abo-Shehada et al., 2006), as well as some areas in Iran, such as Eastern Azerbaijan and Masjed-Soleyman (Azizi et al., 2007; Tavassoli et al., 2010). On the other hand, the findings showed lower values, compared to the results of the studies conducted in Italy and Greece (Papadopoulos et al., 1997; Gaglio et al., 2016), as well as other regions in Iran, such as Fars, Esfahan, and Khuzestan (Rahbari and Ghasemi, 1997; Navidpour et

al., 2007b).

Some factors, such as low moisture, loose soil, as well as hot and dry weather, stimulate the growth of larvae and pupae of flies. It could be stated that the low prevalence of *Przhevaeskiana* spp. in northern Lorestan (Alashtar, Delfan, Borujerd), compared to southern Lorestan (Pol Dokhtar), as well as the center and south of Iran (Esfahan and Fars) could be attributed to cold weather, high soil moisture, and type of soil (clay loam) in these regions.

In a study carried out in West Azerbaijan, three species of *Przhevaeskiana*, including *P.crossii* (47.8%), *P. aegagri* (35.5%), and *P. silenus* (16.6%) were reported as causative factors of goat hypodermosis (Tavassoli et al., 2010). Similarly, in Khuzestan, three species of *P.crossii* (59%), *P. aegagri* (27.34%), and *P. silenus* (13.66%) were reported as causative factors of goat hypodermosis (Navidpour et al., 2007b).

In the same line, according to the studies carried out in Khuzestan and West Azerbaijan, *P.crossii* was recognized as the most important causative agent of goat hypodermosis with infestation rates of 59% and 47.8%, respectively (Navidpour et al., 2007b; Tavassoli et al., 2010). In this study, *P.silenus* with an infestation rate of 50.07% was found to be the major factor of infestation in the tested animals. This could be

explained by the difference in *Przhevaeskiana* flies fauna in different regions that could be attributed to climate conditions and seasonal variations in those areas. In the previous studies, the prevalence rate of the infestation in young goats was higher than that in the older goats that could be attributed to rigid and thick skin in the older goats, compared to the young goats. Accordingly, larvae cannot easily penetrate the thick skin that is due to the induction of immunity after the numerous exposure to infestation (Otranto et al., 1999; Oryan et al., 2009; Gaglio et al., 2016).

The results of the recent studies showed no significant difference among goats with different age groups, and between males and females regarding infestation by hypodermosis, which was consistent with the results of previous findings (Tavassoli et al., 2010; Jan et al., 2014). The first, second, and third stages of larvae are observed from mid-October to late November, late November to late January, and from February to April, respectively. In the current study, the larvae were observed from mid-October to April as subcutaneous lesions in the dorsal region of the infected goats.

Since this disease causes economic losses due to the reduction of livestock and leather production, it is beneficial to detect the infestation in the primary stages of the disease by a competitive ELISA system before the appearance of warbles on the subcutaneous tissue of the goats. Our findings approved the ability of the L<sub>1</sub> antigen of *przhevalskiana* in the ELISA kit to detect the anti-*przhevalskiana* antibodies in goat sera samples. After the detection of anti-*przhevalskiana* antibodies in the tested serum samples using ELISA, goat hypodermosis disease was diagnosed from August to mid-September in the summer. Clinical diagnosis of the infestation is usually conducted from late October until mid-March (winter) by visual observations and direct palpation of warbles in the back and flank regions of the animals. Our findings are consistent with those obtained from a study conducted by Jan et al. (2014). In the ELISA test, as a reliable method, the use of antigen C of *H. lineatum* approved the correlation between the parasitological results and alterations in the antibody contents (Otranto et al., 1999). Moreover,

increased antibody level was associated with the emigration of the larvae with a maximum in September to November. This maximum coincided with the arrival of the larvae to their final sites on the back region of the goats. Additionally, the decreased antibody level was associated with the maturity of the larvae and their appearance from December to February (Navidpour et al., 2007a). Concerning the high rate of infection with hypodermosis in goats in different parts of the country, it is recommended to have sufficient knowledge about the biological cycle of the parasite in different regions before larvae migration and the appearance of warbles on the back of animals that damage their skin. It can be recommended to use the competitive ELISA method as a reliable test with high sensitivity and specificity for early detection. The use of an appropriate control and/or eradication program for the prevention of the damage in the skin can significantly reduce the economic losses caused by this disease in goats.

#### Authors' Contribution

Study concept and design: R. M. and Sh. N.

Acquisition of data: R. M.

Analysis and interpretation of data: R. M. and Sh. N.

Drafting of the manuscript: A. B.

Critical revision of the manuscript for important intellectual content: R. M., Sh. N. and A. B.

Statistical analysis: A. B.

Administrative, technical, and material support: R. M. and Sh. N.

#### Ethics

All the used procedures were approved by the ethical committee standard.

#### Conflict of Interest

The authors reported that they had no conflict and financial interests.

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