

Original Article

Serodiagnosis of *Przhevalskiana* spp. Infestation in Goats Using a Competitive ELISA

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ABSTRACT

Goat warble-fly infestation is one of the parasitic diseases caused by the larvae of *przhevalskiana* spp. It is known to be a major challenge for health and welfare in infested goats and causes high economic losses in livestock worldwide. The detection of goat grub was previously conducted by direct palpation of second and third stage larvae in the back and flank site of the live animals or visual evaluation of the carcasses in slaughterhouses. However, due to the small size of the first instar larvae of *przhevalskiana* (less than 1 mm during emerging from the egg), some of the infected cases were ignored and recorded as negative samples. Immunodiagnostic procedures as easy and cost-effective diagnostic methods provide early detection of myiasis in living animals (even when the larvae are still migrating or are undetectable in the animal body). This study was conducted to evaluate the competitive enzyme-linked immunosorbent assay (ELISA) system in order to detect the antibodies of *przhevalskiana* larvae in the goat sera. In order to prepare the larval antigen, 200 first instar larvae of *przhevalskiana* were collected from the subdermal region of the back and flanks of the infested goats in Khoramabad slaughterhouse, Khoramabad, Iran, from September 2017 to March 2018. Totally, 37 and 46 sera samples were taken from the infected and uninfected goats. The sensitivity and specificity at cut-off 3SD were obtained at 89.18% and 84.78%. Moreover, the measures of inter- and intra-assay coefficients of variability to express the precision or replicability of ELISA kit results were 5.33% and 2.81%, respectively.

Keywords: Antigen, Enzyme-Linked Immunosorbent Assay (ELISA), Goat, *Przhevalskiana*

Diagnostic Sérologique de l'Infestation aux Espèces de *Przhevalskiana* chez les Chèvres Utilisant l'ELISA par Compétition

Résumé: L'infestation des chèvres par des parasites (warble-fly) est l'une des maladies parasitaires causées par les larves de *przhevalskiana* spp. Ceci constitue un défi majeur pour la santé et le bien-être des chèvres infestées et cause des pertes économiques élevées dans le bétail à travers le monde. La détection des parasites de chèvre a été précédemment effectuée par palpation directe des larves des deuxième et troisième stades dans le dos et le flanc des animaux vivants ou par évaluation visuelle des carcasses dans les abattoirs. Cependant, en raison de la petite taille des larves du premier stade larvaire de *przhevalskiana* (moins de 1 mm lors de la sortie de l'œuf), certains des cas infectés ont été ignorés et enregistrés comme échantillons négatifs. Les procédures immunodiagnostiques, en tant que méthodes de diagnostic faciles et économiques, permettent une détection précoce de la myiase chez les animaux vivants (même lorsque les larves migrent encore ou sont indétectables dans le corps animal). Cette étude a été menée pour évaluer l'efficacité de la méthode immuno-enzymatique

ELISA afin de détecter les anticorps sériques des larves de *Przhevalskiana* chez la chèvre. De septembre 2017 à mars 2018, 200 larves de *Przhevalskiana* de premier stade ont été collectées dans la région sous-cutanée du dos et des flancs des chèvres infestées dans l'abattoir de Khoramabad, à Khoramabad (Iran). Au total, 37 et 46 échantillons de sérum ont été prélevés sur des chèvres infectées et non infectées. La sensibilité et la spécificité au seuil 3SD ont été obtenues à 89,18% et 84,78%. De plus, les mesures des coefficients de variabilité inter et intra-essai, exprimant la précision ou la reproductibilité des résultats du kit ELISA, étaient respectivement de 5,33% et 2,81%.

Mots-clés: Antigène, dosage d'immunoabsorption par enzyme liée (ELISA), chèvre, *Przhevalskiana*

INTRODUCTION

Myiasis disease caused by *przhevalskiana* spp. larvae are known as goat warble-fly infestation (GWFI). Goats reared in tropical, subtropical, and non-tropical areas are infected by warble flies of the *przhevalskiana* spp. It causes economic losses in animals, such as meat and milk losses (Oryan et al., 2009). Regarding previous and recent studies, *przhevalskiana* spp. cause severe skin damage in Iran (Rahbari and Ghasemi, 1997; Tavassoli et al., 2010). The infection in the early stages of the disease cannot be detected on the examination of hypodermosis in goats through touching the nodules under the skin of the backs and flanks of the livestock. Therefore, infections are commonly underestimated and neglected (Sinclair and Wassall, 1983). Some immunological methods have solved this problem and can significantly detect the infection even before the appearance of warbles on the back of animals (Sinclair and Wassall, 1983). There is a need to find methods in order to treat the infestation before economic losses incurred (Yadav et al., 2012). Immunological tests have been developed as one alternative method in order to perform clinical, parasitological, and post mortem examination to detect the infection (Navidpour et al., 2007). In the same line, some serological tests have been developed in order to diagnose the bovine hypodermosis created by *Hypoderma bovis* and *H. lineatum*. Moreover, an enzyme-linked immunosorbent assay (ELISA) test

prepared with HC antigen extracted from *H. lineatum* L₁ is commonly used in some countries for the serodiagnosis of hypodermosis (Otranto et al., 2005). The ELISA is a beneficial method for the serodiagnosis of the GWFI, and it can be used as baseline data in order to control and develop a future eradication program for this economically important parasite (Faliero et al., 2001; Jan et al., 2014). A competitive ELISA is intrinsically more specific than a sandwich ELISA since the positive findings are dependent upon the displacement of specific antibody/antigen binding (Webster et al., 1997) Therefore, considering the importance of this disease in goats, the present study aimed to develop a competitive ELISA system for the diagnosis of GWFI.

MATERIAL AND METHODS

Preparation of the Larval Antigen. In order to prepare the larva antigen, 200 first instar larvae of *przhevalskiana* were obtained from infested goats in Khorramabad slaughterhouse, Khorramabad, Iran, from September 2017 to March 2018. The obtained larva was washed in 200 ml sterile phosphate-buffered saline in pH=7.2 and in room temperature for 4 times as described by Webster et al. (1997). The samples were subsequently homogenized in Griffiths tubes containing carbonate buffer 0.1M in pH=9.6 and slowly stirred at 4 °C for 16 h. The homogenate samples were centrifuged in 28000 g at 4 °C for 15 min. Furthermore, Macro Lowry test was used to measure the protein

concentration absorbance at 750 nm by Bovine Serum Albumin (BSA) standards. The samples were maintained at -20 °C (Webster et al., 1997).

Preparation of the Hyperimmune Rabbit Sera. In order to prepare the hyperimmune rabbit sera, 5 six-month-old New Zealand white rabbits were subcutaneously inoculated with a total of 500 µg/ml of *przhevalskiana* antigen in 4 sites. The animals were administered with a 1:1 emulsion in Freund's complete adjuvant in the first instance and then administered with a 1:1 emulsion in Freund's incomplete adjuvant 14 and 28 days later. The blood samples were collected 11 days after the last inoculation through a cardiac puncture. Finally, the sera were separated and stored in small aliquots at -20 °C (Webster et al., 1997).

Collection of the Sera Samples from Goats. In order to prepare the positive and negative serum samples from September 2017 to March 2018, the blood samples were collected from infected slaughtered goats (by direct observation of different instars of *przhevalskiana* larvae in the subcutaneous tissues of a slaughtered goat) and non-infected goats. Finally, 37 and 46 positive and negative blood samples were taken, respectively, from the goats slaughtered in Khoramabad slaughterhouse. The sera samples were separated after blood clotting, centrifuged at 2000 rpm for 5 min, and stored in small aliquots at -20 °C.

Enzyme-Linked Immunosorbent Assay. Immuno module polysorb F12 strips (Nunc) were covered by *przhevalskiana* antigen in 1, 2.5, 5, 10, and 20 µg/ml in carbonate buffer 0.1M, pH=9.6 overnight at 4 °C (100 µl/well) as described by Webster et al. (1997). The wells were washed 3 times by Phosphate Buffered Saline Tween-20 (PBST) (phosphate 0.01M, sodium chloride 0.15 M, pH=7.2, and 0.05% Tween 20) and then blotted dry. In total, 250 µl PBST 5% normal horse serum (NHS) was added into each well for blocking and then incubated in a shaker for 1.5 h at 37 °C. The wells were washed 4 times by PBST and blotted dry. Goat serum at dilutions of 1/200 in competition with hyperimmune rabbit serum at 1/3000

in PBST 1% NHS (50 µl per well) was applied in order to test per sample. The samples were incubated on a shaker at 37 °C for 45 min, and then the wells were washed four times with PBST. Extra wells were considered as "rabbit serum only" and "goat serum only" control. Hyperimmune rabbit serum diluted in ratio of 1:3000 was added into all wells (50 µl per well). Goat serum was only considered as the control well, and then the samples were incubated at 37 °C for 30 min. Subsequently, the wells were washed before the addition of the conjugate. Conjugate serum, mouse anti-rabbit horseradish peroxidase, was diluted in ratio of 1/1000 in PBST 1% NHS and was added into all wells (100 µl per well) and then incubated on a shaker at 37 °C for 75 min. The wells were washed, and the reaction was observed through the inclusion of 100 µl 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), and optical densities were spectrophotometrically evaluated through assessing the absorbance at 450 nm.

Determination of the Cut-off. In order to determine the cut-off, initially, the percentage of inhibitions of the goat sample sera was calculated according to the following equation:

$$\text{Inhibition \%} = 100[1-(x-\text{Min})\div(\text{Max}-\text{Min})]$$

$$\text{Cut-off} = \text{Mean PI} \pm 3\text{SD}$$

Where "x" represents the absorbance values of each sample at 450 nm, "Min" refers to absorbance values with no inhibition, and "Max" denotes the absorbance values with 100% inhibition. Accordingly, the percentage of inhibition and mean percentage of inhibition of the goat sample sera were calculated and the cut-off between positive and negative samples was determined by calculating the mean percentage of the inhibition of the panel positive sera minus three standard deviations of the mean.

Determination of the Sensitivity and Specificity. The Tijssen (1993) formula was used to evaluate the sensitivity and specificity of the samples.

Sensitivity= True positive \times 100/true positive+false negative

Specificity= True negative \times 100/true negative+false positive

True positive was evaluated through the direct examination of different instars of *przhevalskiana* larvae in the subcutaneous tissue of slaughtered goats, whereas uninfected goats were considered as true negative.

Evaluation of the Enzyme-Linked Immunosorbent Assay Precision. In order to capture the precision or replicability of the immunoassay test results, two measures including inter- and intra-assay coefficients of variability (CV) were calculated in this study. Inter-assay variation was assessed by analyzing four control sera tested in quadruplicate in four separate assays conducted on different days. The intra-assay variation was assessed by testing the same control samples twice in quadruplicate on one day. The percentage of CV (%CV) was calculated according to the following equation:

$\% CV = \frac{\text{standard deviation of the samples' means}}{\text{mean of the samples' means}} \times 100$

The mean %CV of the samples was calculated as inter- and intra-assay. For the intra-assay, %CV of less than 10 is generally acceptable, whereas, for inter-assay, the %CV should be less than 15 (Webster et al., 1997).

RESULTS

Concentration of Larval Antigen. The extracted antigen from larvae was tested spectrophotometrically by absorbance at 750 nm and the Macro Lowry method using the BSA standards. The larval antigen concentration 2.5mg/ml was obtained and used for the ELISA test.

Enzyme-Linked Immunosorbent Assay. Various samples of goat serum and hyperimmune rabbit serum were tested by five concentrations of the extracted antigen from the first instar larvae of *przhevalskiana*. The results indicated that the dilutions of 1/200 goat serum and 1/3000 hyperimmune rabbit serum, as well

as the concentration of 5 μ g/ml antigen, were proper for the implementation of the ELISA test. Only rabbit serum control was considered as 100%. Based on these dilutions, and out of 83 goat serum samples, 37 positive and 46 negative samples were tested by the ELISA in order to detect the anti-*przhevalskiana* antibodies in the goat serum samples. Increasing the antibodies in positive sera and connecting them to the antigen leads to less binding of the labeled antibodies. Therefore, the optical density (OD) values of the positive samples were obtained less than OD values of the negative samples which resulted in a reverse standard curve in the competitive ELISA. The OD values of each sample were obtained at 450 nm, and based on the results, the mean percentage of the inhibition and cut-off value of the goat serum samples were also obtained in this study. When the percentage of the inhibition of goat serum sample was more than the cut off value obtained at dilution 1/200, it was considered to be positive. On the other hand, if the percentage of the inhibition of goat serum sample was less than the cut off value obtained at dilution 1/200, the sample was considered to be negative (Table 1).

Determination of the Cut-off. The mean inhibition percentage in positive goat serum samples in ratio of 1/200 dilution was obtained at 48.95%, and cut-off percentage for the pair of positive and negative samples was evaluated through measuring the mean percentage of inhibition of the panel positive sera minus three standard deviations of the mean. Based on that cut-off, the percentage for the pair of positive and negative samples in 3SD was obtained at 42.53% (Figure 1).

Sensitivity and Specificity of the Enzyme-Linked Immunosorbent Assay. Due to the collection of different samples of positive and negative goat sera in autumn and winter, some false negative and positive samples were observed with decreased sensitivity and specificity which can be related to differences in the sample collection season. Moreover, the small size of the first instar larvae of *przhevalskiana* (less than 1 mm during emerging from the egg) led to recording some infected cases as negative goat samples and were

reported as false negative in a serological study. Therefore, after testing the negative and positive sera and comparing the results (Kothgassner et al.) with cut-off, the sensitivity and specificity were obtained at 89.18% and 84.78%, respectively.

Enzyme-Linked Immunosorbent Assay Precision.

The inter-and intra-assay CVs to express the precision

or repeatability of ELISA kit results were 5.33% and 2.81%, respectively. It suggests the great accuracy and acceptable replicability of this method in testing the serum samples (Tables 2 and 3).

DISCUSSION

The tests in this study were conducted by the ELISA

Table 1. Percentage of inhibitions of goat sample serum at 1/200 dilution

46.33%	51.39%	24.65%	25.70%	49.11%	36.44%	48.35%	37.34%	52.61%	46.50%	39.62%
24.50%	21.31%	51 %	39.46%	36.28%	50.25%	21.39%	20.50%	48.37%	50.22%	47.24%
47.20%	36.76%	33.12%	50.25%	49.33%	37.50%	31.40%	53.37%	36.24%	25.56%	52.65%
51.85%	48.14%	21.15%	34.59%	47.15%	38.53%	49.55%	32.68%	50.75%	28.30%	46.13%
37.65%	28.10%	51.72%	21.43%	20.31%	48.19%	20.31%	21.53%	46.78%	38.50%	47.12%
28.45%	48.22%	20.23%	46.12%	47.58%	48.06%	37.55%	46.05%	21.80%	50.65%	39.52%
50.75%	30.35%	49.50%	20.10%	48.65%	46.20%	32.47%	29.78%	33.52%	51.70%	34.02%
E	D	C	B	A	21.60%	50.56%	30.11%	46.23%	34.23%	46.50%
42.53%	6.88%	2.14%	29.87%	48.95%						

A: Mean percentage of inhibition of positive goat serum samples at 1/200 dilution

B: Mean percentage of inhibition of negative goat serum samples at 1/200 dilution

C: Standard deviation of positive goat serum samples at 1/200 dilution

D: Standard deviation of negative

goat serum samples at 1/200 dilution

E: Cut-off 3SD serum sample

Table 2. Calculation of intra-assay precision in goat serum samples

Samples	Group 1	Group 2	Mean	SD	CV%
Sample1	24.56%	25.89%	25.27	0.87	3.44%
Sample2	37.44%	38.49%	37.96	0.74	1.94%
Sample3	39.34%	40.05%	39.69	0.5	1.25%
Sample4	21.51%	22.97%	22.24	1.03	4.63%
Intra assay					2.81%

Table 3. Calculation of inter-assay precision in goat serum samples

Time	Time1	Time2	Time3	Time4	Mean	SD	CV%
Sample1	24.65%	23.85%	24.12%	22.05%	23.66	1.12	4.73%
Sample2	37.44%	36.64%	39.11%	35.01%	37.05	1.7	4.58%
Sample3	39.34%	43.87%	41.31%	39.17%	40.92	2.19	5.35%
Sample4	21.51%	23.97%	22.14%	20.45%	22.01	1.47	6.67%
Inter assay							5.33%



Figure 1. The results of mean percentages of inhibition and cut-off percentage for the pair of negative and positive serum samples

method and the L₁ antigen of *przhevalskiana*. The development of the antibodies approved the correlation between the parasitological results and alterations in the antibody contents. In the current study, due to the small size of the first instar larvae of *przhevalskiana* and undetectable nature of them in the goats, parasitological examination of the body detected antibodies in the goat samples as negative, whereas the ELISA detected it as positive through the detection of *przhevalskiana* larvae antibodies in the goat serums. Previous studies have shown that antibody titer was increased by the migratory phase of the first instar larvae (L1) in case the samples were obtained at this time. The sensitivity and specificity of the test could be increased up to 100%. The antibodies were deleted from the circulation of the infected animals about 98 days after the disappearance of the infection (Sinclair and Wassall, 1983a; Boulard et al., 1996). In the current study, different samples of positive and negative goat sera were collected in autumn and winter; accordingly, there were some false negative and false positive samples with decreased sensitivity and specificity which can be related to differences in sample collection season. Moreover, the small size of the first instar larvae of *przhevalskiana* (less than 1 mm during emerging from the egg) led to recording some infected cases as negative goat samples and was reported as false negative in a serological study. In addition, the results of calculating the percentage of inhibition using the competitive ELISA showed a high level of dependence and specificity of the antibodies which can easily be used to detect the serodiagnosis of hypodermosis infected samples. This can be attributed to the combination of hyperimmune rabbit serum with samples and the competition of its antibodies with the antibodies in the serum of goats for binding the larval antigen of the first stage of the *Przhevalskiana* flies. According to the observations recorded by (Navidpour et al., 2009), the sensitivity and specificity of the competitive ELISA for the detection of *przhevalskiana* larvae antibodies in the goat sera were 81.25% and 87.57% at the cut-off 2CD and 84.37% and 80.95% at

the cut-off 3SD, respectively. Our findings approved the ability of the L₁ antigen of *przhevalskiana* in ELISA kit in order to detect the anti-*przhevalskiana* antibodies. Furthermore, the sensitivity and specificity at cut-off 3SD were 89.18% and 84.78%, respectively. The two measures of inter- and intra-assay coefficients of variability to capture the precision or replicability of ELISA kit results were 5.33% and 2.81%, respectively. These figures suggest the great accuracy and acceptable replicability of this method for testing the goat serum samples and studying in an epidemiological survey, especially when the disease has started. Regarding the life period of larvae and with the use of blood samples taken in the early months of infestation when the first instar larvae are still in the migratory stage and have not yet caused damage to the skin and before the animal slaughter, it is possible to detect the infestation early using the competitive ELISA. Following that, the seroepidemiology of the disease in the area can be investigated after parasite removal by the administration of a parasiticide.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contribution

Study concept and design: Bagheri, A.; Madani,
Acquisition of data: Bagheri, A.; Madani,
Analysis and interpretation of data: Madani, R.;
Navidpour, S.; Hoghooghi-Rad, N.
Drafting of the manuscript: Bagheri, A.; Madani,
Critical revision of the manuscript for important
intellectual content: Madani, R.; Navidpour, S.;
Hoghooghi-Rad, N.
Statistical analysis: Bagheri, A.; Madani, R.; Navidpour,
S.; Hoghooghi-Rad, N.
Administrative, technical, and material support: Bagheri,
A.; Madani, R.; Navidpour, S.; Hoghooghi-Rad, N.

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