

Advanced and Rapid Serodiagnosis of Oestrosis (*Oestrus ovis*; Diptera: Oestridae) in Sheep Using Indirect and Dot-ELISA

Marwa M. Attia^{1,*}, Soliman M. Soliman² and Nagla M.K. Saleh³

¹Parasitology Department; ²Department of Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Cairo University, 12211 Giza; ³Zoology Department, Faculty of Science, Aswan University, Aswan, Egypt.

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Abstract

This study is aimed at evaluating performance of the indirect ELISA for the diagnosis of oestrosis versus the rapid easy assay of Dot-ELISA which could be directly used in the field. Two hundred and forty head of sheep were examined in a Cairo abattoir over the period from May 2017 to May 2018. Sera were collected from each examined sheep and preserved in -20 °C. The Anterior cone (AC) of third-stage larvae of *Oestrus ovis* were prepared. Hyperimmune sera were processed in rats using the anterior part of the larvae (AC); ELISA and Dot-ELISA tests were done after checkerboard titration. The lowest antigen concentration which gives positive results in ELISA was 10 µg protein, whereas the concentration used in the Dot-ELISA was 200 ng/µL, with the sera dilution being 1:100 in the two tests. The two tests were performed with known *O. ovis*-positive and negative sheep sera and known hyperimmunized rat sera against AC of *O. ovis*, as well as known sera for *Coenurus cerebralis*, *Dictyocaulus filaria* and *Haemonchus contortus* without an *O. ovis* infection. Two hundred out of the two-hundred and forty examined sheep were positive for *O. ovis* larvae at post mortem. High optical density (O.D.) values ranging from 0.9- 2 were estimated in the sheep infected with the second and third-stage larvae. On the other hand, O.D. values varying from 0.9 to 1.6 were recorded when sheep (95 sheep) were infected only with the third-stage larvae of *O. ovis*. The results of the current study confirm that Dot-ELISA had similar sensitivity and specificity to those by the indirect ELISA but is more rapid and has an easy assay. So, it could be applicable in the field directly for diagnosing oestrosis.

Keywords: *Oestrus ovis*, Nasal bot fly, ELISA, Dot-ELISA, Coenurosis, and Lungworm.

1. Introduction

Oestrosis has serious pathogenic effects on small ruminants (goats and sheep) as the adult flies deposit its first larval stage in and around the nose of ovine and caprine species. The first-stage Larvae wander in the nasal passages, sinuses, and ethmoid bones in which they molt to the second and third larval stages. Then, the full mature third-stage larvae excrete to the outside by sneezing onto the ground where they become pupa in the soil and complete their life cycle (Hall and Wall, 1995, Zumpt, 1965). The migration and development of the larvae inside their habitat (nasal cavities and sinuses) cause serious problems to small ruminant animals which may lead to several pathological conditions such as nasal discharges, frequent sneezing, and respiratory disorders resulting in serious economic losses (Pandey and Ouhelli, 1984; Dorchies *et al.*, 2000).

Different epidemiological studies on *O. ovis* larvae recorded their wide distribution all over the world. These parasites were reported present in different countries by (Jagannath *et al.*, 1989, Alahmed, 2000, Abo-Shehada *et al.*, 2000; Karatepe *et al.* 2014; Ozdal *et al.* 2016; Allaie *et al.* 2016) in India, Saudia Arabia, Northern Jordan, Turkey, Kashmir respectively. Different records also confirmed

their distribution in African countries (Biu and Nwosu, 1999, Amin *et al.*, 1997, Berrag *et al.*, 1996) such as Nigeria, Egypt and Morocco respectively. There are also records of their distribution in some European countries including Greece (Papadopoulos *et al.*, 2001), France (Yilma and Dorchies, 1991; Bergeaud *et al.*, 1994; Dorchies *et al.*, 2000), Italy (Caracappa *et al.*, 2000; Scala *et al.*, 2001), South-western Germany (Bauer *et al.*, 2002), Spain (Reina *et al.*, 2001; Alcaide *et al.* 2003). In other parts of the world, the infection was recorded in Mexico (Martinez *et al.*, 1999), in Argentina by (Trezeguet, 1996), and in the United Kingdom by (Bates, 1997; Goddard *et al.*, 1999).

Many cases of Ophthalmomyiasis in humans were recorded through the infection of the eyes by the first-stage larvae of *O. ovis* (Lucientes *et al.*, 1997; Pampiglione *et al.*, 1997; Prosl and Meyer, 2003; Fasih *et al.* 2014). Masoodi and Hosseini (2003) reported another case of pharyngeal oestrosis by the first-stage larvae.

Oestrosis is endemic in Egypt due to the hot and dry weather which constitutes good conditions for the fly and its larvae to grow. Unfortunately, the detection of *O. ovis* antibody levels is still poorly investigated in Egypt. So, the target of this study is to assess and compare the performance of ELISA in the diagnosis of *O. ovis* infection in sheep sera with the performance of the new and rapid

* Corresponding author e-mail: marwaattia.vetpara@yahoo.com; marwaattia.vetpara@Cu.edu.eg.

assay of Dot-ELISA used in the diagnosis of this parasitism directly in the field.

2. Materials and Methods

2.1. Collection of Samples

Two hundred and forty head of sheep were examined in a Cairo abattoir over the period from May 2017 to May 2018 (twenty sheep per month). The Sagittal section of sheep heads was performed, and the larvae were collected from the nasal passages and at the base of the horns (Figure 1). All the collected larvae were identified according to Zumpt, (1965). A total of fifty larvae from sheep were used in the serological analysis. Blood was collected from each examined sheep and centrifuged. The sera were collected in sterile vials and stored at -20°C until used. In addition to *O. ovis* sera, three parasites were chosen; two being the main causes of respiratory diseases and one causing a nervous manifestation in sheep. The sera of these sheep positively-infected with those parasites were chosen during postmortem in the abattoir; the sheep infected with only one parasite only, either with *Coenurus cerebralis* (*C. cerebralis*), *Dictyocaulus filaria* (*D. filaria*) and *Haemonchus contortus* (*H. contortus*) without *O. ovis* larvae infection. Negative control serum was obtained from one-month old sheep.

2.2. Antigen Preparation

The Anterior Cone (AC) of third-stage larvae (L3) were dissected freshly, relaxed in an iced PBS medium under a stereoscope microscope (LEICA M60, USA) for the cutting of the anterior part of L3 just after the cephalopharyngeal skeleton which had the larval cuticle and salivary gland. The antigen (AC) was preserved in 1 ml PBS (PH 7.2). The AC of L3 was ground using a homogenizer, and sonication was then done for five minutes at a 10-pulse rate 60-80 amplitude value using cole parner ultrasonic sonicator. The homogenates were centrifuged at 14,000 RPM for twenty minutes at 4°C as described by Innocenti *et al.* (1995) and Angulo- Valadez *et al.* (2008). The supernatant was collected and preserved for further analysis. The protein content of the prepared antigen (AC ag) was determined by the method of Lowry *et al.*, (1951). Then, the antigen (AC) was stored at -20°C until used in the two diagnostic tests (ELISA; Dot-ELISA).

2.3. Preparation of Hyperimmune Sera

Four male rats (*Rattus norvegicus albino*) of about a 150 gram in weight were raised for the preparation of hyperimmune sera versus AC according to Innocenti *et al.* (1995) with some modification. The rats were housed in two groups (two control negative and two immunized against AC ag). The housing was in a conventional rat cage with straw bedding. The rats were supplied with normally balanced rats' pellet and supplied with water *ad libitum*. The rats were raised in ambient temperature and humidity. The immunized rats were injected with 1mg of protein AC, mixed in 1ml of mineral oil subcutaneously (1st dose). Then, two subsequent intramuscular injections of 0.5 mg of protein antigens in the same volume of mineral oil were injected at a two-week interval. The rats were slaughtered for the collection of sera two-weeks after the last dose. The collected hyperimmune rat sera

were stored at the refrigerator until used. Ethical approval was obtained from the Animal Ethical committee of the Faculty of Veterinary Medicine, Cairo University under number: CU/II/F/105/18.

2.4. Sera Examined

The sera samples used in the ELISA and DOT- ELISA tests were the hyper-immunized rat sera against AC ag, negative control sera from rats and one-month old sheep, and sera from naturally infected sheep with *O. ovis*. As well as the three sera from sheep infected with other parasites such as *C. cerebralis* and *D. filaria*, *H. contortus* without *O. ovis* infection.

2.5. Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

In order to find out the optimum assay dilution of the antigen and tested sera which could be used under known dilution of conjugate and substrate materials, checkerboard titration was done according to Harlow and Lane (1988). A ninety- six well, flat- bottomed ELISA plates were incubated overnight with 100 μL / well of antigen at 10 μg /mL coating buffer (0.1 M Sodium carbonate, pH 9.6) in 4°C . Two hundred μL / well of the blocking buffer (Bovine serum albumin (BSA) - PBS) were added for two hours at 37°C . After three times washings with PBS- Tween-20; 100 μL of diluted sera 1:100 in PBS were incubated in two wells (each serum sample was put in two wells to take the average) and were incubated at 37°C for two hours. One hundred μL / well of horseradish peroxidase anti-sheep IgG; anti-rat IgG conjugate (Sigma, A-5420) diluted at 1:1000 were added to each well and incubated for one hour at room temperature. A hundred microliters of a substrate buffer containing (10 mg Ortho-Phenylene Diamine (OPD)) diluted in a citrate buffer (pH 5.0) and 30 % H_2O_2 were added to the plate well. Finally, the ELISA plate was stopped by adding 100 μL of 3N H_2SO_4 (stopping buffer), and the absorbance values or Optical density (O.D.) were obtained from an ELISA reader (Bio-Rad, USA) at 450. The sera were positive when the absorbance values were as or more than the cut off value equal to a double fold of the mean negative sera, The ELISA was done according to Tabouret *et al.* (2001); Angulo- Valadez *et al.* (2009; 2011) and Attia *et al.* 2019.

2.6. Determination of ELISA Parameters

Different ELISA parameters were calculated according to Tabouret *et al.* (2001); Angulo- Valadez *et al.* (2009; 2011) and Attia *et al.* 2019. The sensitivity and specificity, positive predictive value, and the negative predictive value were recorded.

2.7. Dot - Enzyme-Linked Immunosorbent Assay (Dot - ELISA)

Checkerboard titration was done for the assessment of the optimal condition of the different solutions of ELISA (AC antigen; the sera and the conjugate). Two hundred ng of the AC antigen was dotted onto nitrocellulose membrane discs (NC) with 0.22 μm pores (Bio-Rad Laboratories, USA) placed in ELISA plate and incubated for one at 37°C . The NC discs were blocked using blocking solution (BSA)-PBS for thirty minutes. After being washed with (PBS-T), for three times, positive and negative hyperimmune sera as well as the sera of sheep naturally infected with *O. ovis*, and three sera of three

other parasites mentioned in ELISA were diluted in PBS. One μL of 1:100 diluted sera was dotted onto NC. After one hour and three times of washing, the anti-rat IgG and anti-sheep IgG conjugated horseradish peroxidase (Sigma, A-5420) were diluted 1:1000 in PBS was dotted at $1\mu\text{L}$ /well. After one hour, and another three times of washing to NC. The used substrate (4-chloro-1-naphthol and methanol in PBS containing 30 % H_2O_2) was added onto NC. The developed violet color was stopped by washing the NC with distilled water. The violet color produced indicates a positive result, while if no color was developed it is considered as a negative result. The Dot- ELISA was carried out according to Kumar *et al.* (2008); Lakshmanan *et al.* (2016); Paller *et al.* (2017).

2.8. Statistical Analysis

The linear regression was assessed to detect the possible evaluation of antibody titers in relation to the number of larvae for each positive animal. Pearson's correlation was performed to detect the variation in different parameters. The statistical analysis was performed by SPSS, version 11.5.1.

3. Results

All the collected larvae were identified as *O. ovis* second and third-stage larvae at the base of the horn, in the nasal passages and the sinuses. *Oestrus ovis* larvae were yellowish to brownish in color with dark transverse bands on the dorsal surface according to the stage. Second-stage larvae were yellowish in color, while third-stage larvae were dark yellowish to brownish in color with dark transverse bands on the dorsal surface, Figure 1.

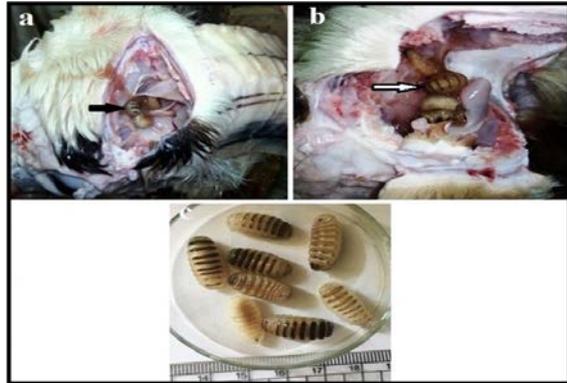


Figure 1. A, B, *O. ovis* larvae recovered from the sheep skull at the base of the horn and in the nasal sinuses. C. Collected *O. ovis* larvae note: it is oval in shape with a dark band on the dorsal surface.

The lowest antigen concentration that gives positive results after checkerboard titration for indirect ELISA was $10\ \mu\text{g}$ protein of AC antigen (ag), while it was $200\ \text{ng}/\mu\text{L}$ in Dot ELISA, with the sera dilution being 1:100 in the two tests. The two tests were performed with known *O. ovis* positive and negative sheep sera and known hyper-immunized rat sera against AC ag of *O. ovis*, as well as known sera for *C. cerebralis*, *D. filaria*, and *H. contortus* without *O. ovis* infection.

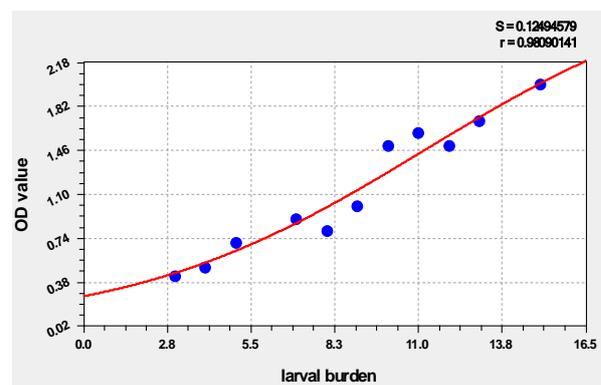
Indirect-ELISA was used for the diagnosis of anti- *O. ovis* antibodies in the collected sera versus anterior cone (AC) antigen of third-stage larvae. Two hundred out of the two-hundred and forty examined sheep were positive for *O. ovis* at post- mortem. The sera of non- inoculated rats

and one-month old sheep were used as a negative control while the hyperimmune rat serum inoculated with AC ag of third-stage larvae was used as a positive control.

The results displayed in Table 1 show that the optical densities (O.D.) varied according to the stage and number of larvae infecting sheep. High O.D. values ranging from 0.9- 2 were estimated in sheep infected with the second and third- stage larvae while O.D. varying from 0.9 to 1.6 were recorded when sheep (95 sheep) were infected only with the third-stage larvae of *O. ovis*. Sheep harboring only second stage larvae; O.D. value ranged between 0.5 and 0.7, Figure 2.

Table 1. ELISA Optical densities (O.D.) of sheep infected with *O. ovis* larvae in relation to larval stage and mean larval burden.

Stage of larvae	No. of infected sheep	Mean larval burden	Optical densities \pm S. E
2 nd only	25	5-20 (av.10)	0.5-0.7 (av. 0.59) \pm 0.095
3 rd only	95	10-30 (av. 20)	0.9-1.6 (av.1.078) \pm 0.132
2 nd and 3 rd	80	20-30 (av. 25)	0.9- 2.0 (av. 1.55) \pm 0.036
Total	200		



Logistic Model: $y=a/(1+b*\exp(-cx))$ Standard Error: 0.1249458
Correlation Coefficient: 0.9809014

Figure 2. Effects of the mean larval burden of *Oestrus ovis* larvae on the mean optical densities obtained by indirect- ELISA

The results displayed in Figure 3 indicate that the optical densities (O.D.) are significantly correlated with the months of the year. From May to August, no significant correlation was estimated as the O.D. values ranged from (1.5 to 1.3); throughout this period, the third-stage larvae were collected. There was a significant decrease in O.D. values ($P < 0.05$) and mean larval burden during the months from August to September. No significant correlation was found between September and October. However, between October and November, there was a significant decrease and the O.D. value ranged from 0.9 to 0.5 ($P < 0.01$). While between November, December, and January, there was no significant correlation ($P > 0.05$) between these months. There was a significant increase ($P < 0.05$) in January and February, and no significant correlation was found between the other months.

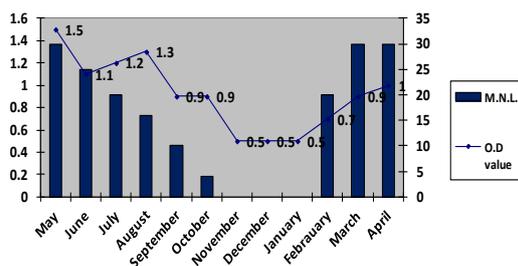


Figure 3. Effects of the mean number of *Oestrus ovis* larvae on the mean optical densities obtained by indirect ELISA during different months of the investigation period from May 2017 to May 2018; M.N.L.: Mean number of larvae.

Different parameters of indirect ELISA and Dot-ELISA are presented in Tables 2 and 3.

Table 2. Positive and negative values obtained from Indirect ELISA and Dot ELISA

Values	Test	
	Indirect ELISA	Dot-ELISA
True Positive	198	199
False Negative	2	1
False positive	0	0
True Negative	40	40

Table 3. The diagnostic sensitivity, specificity, positive and negative predictive values as well as accuracy of the two tests for the diagnosis of oestrosis in sheep

Test	Indirect-ELISA		DOT-ELISA	
	Value	95% CI	Value	95% CI
Sensitivity	99%	96.43%- 99.88%	99.5%	97.25% - 99.99%
Specificity	100.00 %	91.19% - 100.00%	100.00 %	91.19%- 100.00%
Positive likelihood Ratio	-	-	-	-
Negative likelihood Ratio	0.01	0.00- 0.05	0.01	0.00-0.04
Disease Prevalence	83.33%	78.00%- 87.82%	83.33%	78.00%- 87.82%
Positive Predictive Value	100.00%	-	100.00%	-
Negative Predictive Value	95.24 %	83.43% - 98.76%	97.56%	84.99%- 99.65%
Accuracy	99.17%	97.02% - 99.90%	99.58%	97.70%- 99.99%

Based on data of the present study, cross reactions were not detected in ELISA and Dot-ELISA by AC ag.

Sera from sheep infected with other parasites such as *C. cerebralis*, *D. filaria* and *H. contortus* without *O. ovis* infection; none of these sera were reacted with AC ag of *O. ovis* in indirect-ELISA and Dot-ELISA. Thus, no cross-reactions were recorded with this antigen (AC ag).

The results of Dot-ELISA could detect anti- *O. ovis* IgG antibodies at 200 ng/mL with 99.5 % sensitivity and

100 % specificity when postmortem examination of the skull and horn was fixed as the test standards.



Figure 4. Dot-ELISA plate showing the wells of strongly positive sera represented by arrows, while the well with no arrows, shows the negative sera

The test standards revealed that two hundred sheep were positive for *O. ovis* larvae while in indirect ELISA and Dot-ELISA two and one sera were respectively false negative. No sera in the two tests give false positive results. The two tests confirm the test standards.

4. Discussion

Infection with *O. ovis* is very difficult to diagnose and could be misdiagnosed with other diseases which had a similar manifestation. Also, *O. ovis* larvae can't be diagnosed using blood smears or even fecal examination. So, the serological or molecular tests are the only tests which could diagnose oestrosis in sheep and goats.

Indirect ELISA was used for the diagnosis of anti- *O. ovis* antibodies in the collected sera versus anterior cone antigen of the third- stage larvae, in order to detect the systematic IgG which reacts with polypeptides of the larval cuticle of 56 KDa which prove to be the strongest antigenic materials. However, a salivary gland protein 28 KDa is also the most immunogenic fractions; this hypothesis was recorded by (Innocenti *et al.* 1995; Tabouret *et al.* 2001). So, this study used the anterior cone antigen of L3 which combines the salivary glands and cuticle antigen based on the results of the two previous authors.

In this study, the AC ag of L3 was used as an antigen for indirect ELISA and Dot- ELISA. The assay confirmed the results of the macroscopic survey (test standard) since positive results (O.D. varying from 0.5- 2) were recorded for all the infected sheep. Low optical density of 0.5- 0.7 was reported when L2 was only present, but when L2 and L3 infect sheep (O.D. 0.9- 2). According to the authors, the low O.D values associated with the second larval stage infection may be related to a short duration of the infection period of L2, the immunogenic nature of this larval stage, and/or the antigen used from L3; this gives good results in determining the stage present in the animals. Similar results were reported by Innocenti *et al.* (1995) who used the salivary glands' proteins of the *O. ovis* larvae to detect the antibodies' levels of these botfly larvae and stated that the salivary glands were the most immunogenic proteins in the infected sheep. However, this study confirms that the AC ag gives similarly high values to the results of the study using the salivary gland from L3 by Angulo-Valadez *et al.* (2009) in their study of the systemic IgG response using the salivary glands of L3.

Angulo- Valadez *et al.*, (2011) studied the immunogenic reaction against oestrosis in sheep and detected a rise in Th2 immune response. Other studies on the immunogenic relationship between *O. ovis* and other concurrent helminths infection revealed that there is no cross-reaction between *O. ovis* larvae and other parasitic gastro-enteritis helminthes such as *Strongylus* spp., *Trichostrongylus colubriformis* and *H. contortus* (Yacob *et al.*, 2002; Terefe *et al.*, 2005). Otherwise, the infection of the gastro-intestinal tract with nematodes did not interact with the life cycle of *O. ovis* larvae infection, but oppositely, the infection with *O. ovis* larvae was reduce in the nematode egg excretion and worm burdens (Yacob *et al.*, 2002; Terefe *et al.*, 2005); these ideas prove that there was no cross-reaction in this study.

Tabouret *et al.* (2003) evaluated the mucosal IgG and IgA responses using ELISA in sheep infected experimentally with *O. ovis* larvae with a low sensitivity and specificity recorded. Some studies investigated the seroprevalence studies using ELISA on *O. ovis*-specific IgG in sheep (Papadopoulos *et al.*, 2001; Scala *et al.*, 2002, Silva *et al.* 2012) with low sensitivity and specificity in comparison with the present study. This may be attributed to using more sensitive and specific antigens (AC ag).

In the present study, the Dot ELISA gave high sensitivity and specificity results nearly like those by the indirect ELISA. However, the Dot-ELISA has many advantages in comparison with indirect ELISA. The reaction of Dot- ELISA can be read only with a color development which can be evaluated by the eye with no need for a special tool as ELISA reader. Also, several studies examined the validity of antigens dotted onto (NC) which may be stored for over three years at 4°C, or may be valid either for up to ten days or for three months at room temperature or 37°C, so this antigen dotted onto NC membrane could be moved to any country to be used in diagnosis tests, Yamaura *et al.* (2003) and Gupta *et al.* (2008).

Because of the many values of Dot- ELISA, this assay could be used in several diagnoses of many helminthes. It was used in this study on botfly (*O. ovis*) and required only few hours. It is rapid compared to the indirect ELISA which needs two days to be performed.

Gupta *et al.* (2008) explained the advantages of Dot-ELISA confirming that it is a rapid and simple assay and could be performed only using some chemicals and a NC membrane, which means that it could be used in the field. In general, the assay does not require any complicated instruments and the results can be read by the eye. Moreover, Dot- ELISA does not need any special training.

Many parasites were diagnosed using Dot- ELISA including Fascioliasis by Dixit *et al.* (2002), Zimmerman *et al.* (1985); Hydatid cysts by Siavashi *et al.* (2005); Sangaran *et al.* (2017). Toxocariasis by Paller *et al.* (2017); Schistosomiasis by Lakshmanan *et al.* (2016) in addition to many protozoan parasites such as Leishmaniasis, Babesiosis, Amebiasis by Yamaura *et al.* (2003), Toxoplasmosis by Teimouri *et al.* (2018).

5. Conclusion

This study differentiates between two assays to detect the sensitivity and specificity of each test. While the two

tests gave nearly similar sensitivity and specificity results, the Dot- ELISA has more advantages as it can be used for diagnosing oestrosis right in the field or in laboratories because of its simple and rapid procedures. On the other hand, ELISA requires special devices with specific laboratory procedures. In addition, its results' development requires two days. To the authors' best knowledge, the Dot- ELISA is the newest serological technique in the diagnosis of oestrosis. Therefore, this study was conducted to evaluate this rapid technique in comparison to the old assay which is frequently used by different scientists. In this study, the two tests confirm the test standards (post-mortem examination), so, the Dot- ELISA (rapid field test) can be easily used in the diagnosis of oestrosis in live animals.

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