

Relationships of systemic IgG antibody response and lesions caused by *Oestrus ovis* L. larvae (Diptera: Oestridae) in infected goats - Interacciones de la respuesta sistémica de anticuerpos IgG y las lesiones causadas por larvas de *Oestrus ovis* L. (Diptera: Oestridae) en cabras infectadas

Angulo-Valadez, Carlos E.: Centro de Investigaciones Biológicas del Noroeste, La Paz, B.C.S., México | **Cepeda-Palacios, Ramón:** Universidad Autónoma de Baja California Sur, La Paz, B.C.S. México | **Ascencio, Felipe:** Centro de Investigaciones Biológicas del Noroeste, La Paz, B.C.S., México | **Jacquet, Philippe:** UMR INRA/ENVT 1225 Interactions hôtes Agents Pathogènes, Toulouse, France | **Dorchies, Philippe:** UMR INRA/ENVT 1225 Interactions hôtes Agents Pathogènes, Toulouse, France | **Ramírez-Orduña, Juan Manuel:** Universidad Autónoma de Baja California Sur, La Paz, B.C.S. México.

*Contacto: Tel.: +52 612 123 88 00x5412; fax: +52 612 123 88 22.
Correo electrónico: rcepeda@uabcs.mx (R. Cepeda-Palacios).

Abstract

This is the abstract, *Oestrus ovis* (Diptera: Oestridae) is a nasosinusal parasite of sheep and goats that affects the wellbeing and performance of the hosts. Our objectives were (1) to analyze associations of host phenotypic characteristics (age, weight, sex), *O. ovis* larval characteristics, systemic antibody IgG response, and lesions in sinusal and horn cavities in naturally infected goats, and (2) to estimate the serodiagnostic value of salivary gland antigens for oestrosis diagnosis by ELISA test in goats naturally exposed to *O. ovis* infection. *O. ovis* third-instar larvae (L3) were collected, then dissected to remove the salivary gland and to obtain the antigens source (SGC). A total of 251 goats were necropsied. The host's weight, age and sex were individually recorded. The sinusal and horn cavities were examined for the presence of *O. ovis* larvae. Cavitory lesions and lesion intensity in infected goats ($n=38$) were scored according to a severity table. Sera ($n=125$) were analyzed by ELISA to detect specific humoral IgG responses. Annual prevalence of goat oestrosis was 73.9%. A low positive association ($r=0.38$, $P<0.05$) was observed between larval burden and severity of sinus lesions. In general, high sensitivity (90.82%) and low specificity (25.93%) were observed in ELISA. As conclusions, major pathological damages

caused by *O. ovis* were associated with the presence of early L2 and early L3 larvae, probably enhanced by larval molting. SGC antigens were proven valuable antigens for oestrosis diagnosis by ELISA test in goats.

Keywords: *Oestrus ovis* | Salivary gland antigens | IgG response | ELISA test | Goats.

Resumen

Oestrus ovis (Diptera: Oestridae) es un parásito nasosinusal de las cabras y las ovejas que afecta el bienestar y el rendimiento de sus hospedadores. Nuestros objetivos fueron (1) analizar las asociaciones de características fenotípicas del hospedador (peso, sexo, edad), características larvarias de *O. ovis*, la respuesta de IgG, y las lesiones en las cavidades sinusales y cornuales de cabras infectadas, y (2) estimar el valor de los antígenos de la glándula salival para el serodiagnóstico de la estrosis caprina usando la prueba de ELISA. Se capturaron larvas de tercer estadio (L3), se disectaron para remover la glándula salival y obtener la fuente de antígenos (CGS). Se examinaron en necropsias 250 cabras. El peso, sexo y edad fueron registrados. Los senos frontales y cavidades cornuales se examinaron para la presencia de *O. ovis*. La intensidad de las lesiones en cabras infectadas ($n=38$) se registraron de acuerdo con una tabla de severidad. Los sueros ($n=125$) se analizaron por ELISA para detectar la respuesta humoral de IgG. La prevalencia anual de la estrosis caprina fue 73.9%. Una asociación baja positiva ($r=0.38$, $P<0.05$) se observó entre la carga larvaria y la severidad de las lesiones encontradas. En general, se observaron alta sensibilidad (90.82%) y baja especificidad (25.93%) en la prueba de ELISA. Como conclusiones, los daños patológicos mayores se asociaron al número y presencia de larvas tempranas L2 y L3, probablemente inducidos por la muda larvaria. Los antígenos CGS demostraron ser valiosos en la prueba de ELISA para el diagnóstico de la estrosis caprina.

Palabras clave: *Oestrus ovis* | Antígenos de la glándula salival | respuesta de IgG | Prueba de ELISA | Cabras.

Introduction

Oestrus ovis (Diptera: Oestridae) is a worldwide spread insect, which larvae are obligate parasite of the upper respiratory tract of sheep and goats. *O. ovis* flies deposits first instar (L1) into the host nostrils and they develop toward second (L2) and third (L3) instars inside of the host. During the infection, sneezing and nasal mucus discharges are the main evident clinical signs in infected animals.

Host humoral and cellular immune responses are provoked by *O. ovis* infective larvae during their development (i.e. mainly L2 and L3 instars, Tabouret et al., 2003). Among them, the systemic IgG antibody response has been the best immune reaction for oestrosis diagnosis purposes and seroepidemiological studies using both somatic extracts or excreted – secreted products (ESP) as coating antigens in ELISA (Suárez et al., 2005; Alcaide et al., 2005a).

Previously, Tabouret et al. (2001) demonstrated that salivary gland antigens are the most antigenic proteins to be potentially used for the disease diagnosis in sheep. However, to date there are no available reports on the value of salivary gland antigens for diagnosing oestrosis in goats. In addition, despite *O. ovis* infection has been reported to be associated with host's phenotypic characteristics (Abo-Shehada et al., 2003; Yilma and Genet, 2000; Cepeda et al., 1998), the relationships among immune responses and nasal-sinusal lesions caused by *O. ovis* larvae have not been investigated in infected goats so far. Our objectives were (1) to analyze associations of host phenotypic characteristics (age, weight, sex), *O. ovis* larval burden with larval development characteristics, systemic antibody IgG response, and the severity of lesions in sinusal and horn cavities in naturally infected goats, and (2) to estimate the serodiagnostic value of salivary gland antigens for oestrosis diagnosis by ELISA test in naturally exposed goats to *O. ovis* infection.

Material and methods

Oestrus ovis collection and larval dissections

O. ovis third-instar larvae (L3) were collected from the head of goats slaughtered in La Paz, Baja California Sur, Mexico. Larvae were washed with a PBS (pH 7.4) solution containing penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹) prior to dissection (Angulo-Valadez et al., 2007). Briefly, larvae were checked for viability (absence of integumental fungal attacks, damages to cuticle, diminished vigor).

Each live larva was fixed to a paraffined Petri dish with entomological pins and dissected under a stereomicroscope using ophthalmic

surgical equipment. Dissection was carried out on an ice bath using cold (4–10 °C) PBS-antibiotics as liquid medium. After removing ventral coelomic wall, as well as the surrounding fat body, tracheal and nervous tissues, the entire salivary gland ($n=25$) was collected and placed in a tube with PBS-antibiotics.

Salivary gland antigens

Salivary glands were centrifuged (10,000 g x 20 min at 4 °C) to expel the contents and the supernatant was recovered (SGC) and stored (–20 °C) until use as coating antigens in ELISA tests. Protein concentration of SGC was determined using the bicinchoninic acid assay kit (BCA, Pierce, Rockford, Illinois). To ensure that SGC antigens were not degraded prior using in ELISA, protein integrity was achieved visually by polyacrylamide gel electrophoresis (Laemmli, 1970) and proteolytic activity was determined by Azocoll test (Angulo-Valadez et al., 2007).

Necropsy analysis and sera

A total of 251 slaughtered goats were slaughtered and necropsied. Before goat slaughtering, host live body weight, age (determined by dental examination) and sex were individually recorded. Each goat's head was carefully examined (Yilma y Dorchies, 1993) for L2 and L3 *O. ovis* larvae at the sinus and horn cavities. Larval numbers were counted and developmental instars were identified according to Zumpt (1965).

Blood samples from randomly selected goats ($n=125$) collected and sera were obtained by centrifugation (1500 g for 5 min at 4 °C), and then frozen at -20 °C until analysis of IgG antibody response.

Oestrosis lesions in infected goats

A table for classifying the lesions and lesion intensity caused by *O. ovis* infective larvae was constructed, based on findings of oestrosis at necropsy in infected animals (Table 1). During six months ($n=9$ sampling dates), the lesions found in sinus and horn cavities of infected goats ($n=38$) were recorded. Average age and body live weight of the sampled goats were 2.39 ± 1.40 and 36.83 ± 13.21 , respectively. The normal mucosa of kids never infected ($n=4$) was used as reference. The number of *O. ovis* larvae was recorded and its larval development (from early L2 to L3D5 larvae, i.e. 3 to 11 interstadia) classified according to Cepeda-Palacios et al. (1999). All analyses were done by the same trained technician.

Table 1. Scale used for evaluation of severity of lesions caused by *O. ovis* larvae in the sinusal and horn cavities of necropsied goats^a.

Severity of lesions	Lesion score
Absent	0
Low	1
Moderate	2
High	3

^aThe score of lesions were additive and the lesion severity in a particular host was the sum of each lesion finding as: the presence of blood or pus, the quantity of secreted mucus, and the level of inflammation according to the relative thickness of the mucosa. The normal mucosa of kids never infected ($n=4$) was used as reference

ELISA tests

A standardized ELISA using SGC as coating antigens was used (Angulo-Valadez et al., 2009). Briefly, SGC antigens were diluted at 2.5 µg/ml in carbonate buffer (pH 9.6), distributed (100 µl) in 96 well plates (Nunclon surface, Nunc, Denmark). The antigen-coated wells were then incubated with a 10% skimmed milk solution (200 µl). Triplicate serum samples (100 µl) diluted 1:50 in PBST were added. The plates were incubated (100 µl) with horseradish peroxidase-conjugated anti-goat IgG whole molecule (Sigma, A5420 St. Louis, MO) diluted (1:2000) in carbonate buffer. The wells were washed three times in all steps with PBST (0.01 M phosphate, 0.15 M sodium chloride, pH 7.4 and 0.1% Tween 20) except after skimmed milk step. Then, 100 µl per well of the chromogen (0.4 mg/ml OPD; Sigma, St. Louis, MO, USA) was added. All incubations were done by 30 minutes at 25°C. Optical densities (OD) were determined with a spectrophotometer (BioRad, Microplate reader 3350-UV) by measuring the absorbance at 454 nm. All samples were performed in triplicate. The average of the each sample values was used in final calculations. Pools of negative ($n=19$ uninfected kids) and positive ($n=5$ naturally *O. ovis* infected goats) sera were used as controls and PBS wells as blank. The cut off value was established to be the mean of negative control + 3SD.

Statistical analysis

Necropsy and sera data were grouped into two periods (spring-summer and autumn-winter) according to dates of sampling. ELISA data were analyzed using epidemiological software of tabulated data (EPIDAT 3.1, 2006). Descriptive statistics of host and larval variables were calculated and simple linear correlation analyses were carried out. The effects of larval development stadia and anatomic side on

the severity of lesions were evaluated by one-way ANOVA. All analyses were done by Statistica software (Statsoft, 1998).

Results

O. ovis infection and host phenotypic characteristics

The necropsy data of naturally exposed goats to *O. ovis* infection are summarized in Table 2. Annual overall prevalence of goat oestrosis was 73.9%. For the spring-summer and autumn-winter, oestrosis prevalences were 72.9% and 75.3%, respectively. The total number of larvae recovered from the sinus and horn cavities in infected goats during spring-summer period was 648 larvae (L3, $n=423$; L2, $n=228$), while in the autumn-winter period, 424 larvae were obtained (L3 $n=267$; L2 $n=158$).

Table 2. Host phenotypic characteristics and larvae recovered from the sinusal and horn cavities at necropsy examinations of goats naturally exposed to *O. ovis* infection^a.

Host/ <i>O. ovis</i>	<i>n</i>	Mean	S.D.
Goat			
Live body weight	193	35.3	11.9
Age	191	3.5	2.5
Male	71		
Female	126		
Intensity of infection			
L2 larvae	105	3.7	4.4
L3 larvae	121	5.7	5.1
Total larvae	139	7.7	7.8

^a n =Valid number of necropsied goats.

Live weight (range 8-95 kg) and age (range <1 year to 10 years, based on 0-4 dental pair replacement and wear of permanent teeth) of necropsied goats were found significantly correlated ($r=0.43$, $P<0.05$). However, the total number of larvae and/or the larval development were not correlated with body live weight, age, or sex of the infected hosts.

Systemic IgG antibody response

The systemic IgG antibody response against SGC antigens was significantly correlated with total larval count ($r=0.27$, $P<0.05$) and with number of L3 larvae ($r=0.31$, $P<0.05$). There were not associations among systemic IgG level and age, body live weight, or sex of the goats.

Severity of lesions caused by *O. ovis*

Relationship of host characteristics with larval burden, larval development, and lesions

None correlation ($P>0.05$) was found among phenotypic characteristics (body live weight, age, or sex) with total count of larvae as well as with *O. ovis* larval development (Table 3). Moreover, no association ($P>0.05$) was observed between weight or age of the host and severity of lesions found in sinusal and horn cavities.

Table 3. Descriptive statistics of *O. ovis* infection and severity of oestrosis lesions in sinusal and horn cavities of goats.

Variable	<i>n</i>	Mean	S.D.
<i>O. ovis</i>			
No. Larvae			
right lateral	38	2.61	3.41
left lateral	38	2.39	3.49
^a Larval development			
right lateral	80	5.69	3.46
left lateral	75	5.07	3.77
Total no. of larvae	38	3.50	3.94
Severity of oestrosis lesions in sinus and horn cavities ^b			
Right lateral	37	5.19	3.56
Left lateral	37	4.97	3.53
Lateral average	37	5.08	3.45

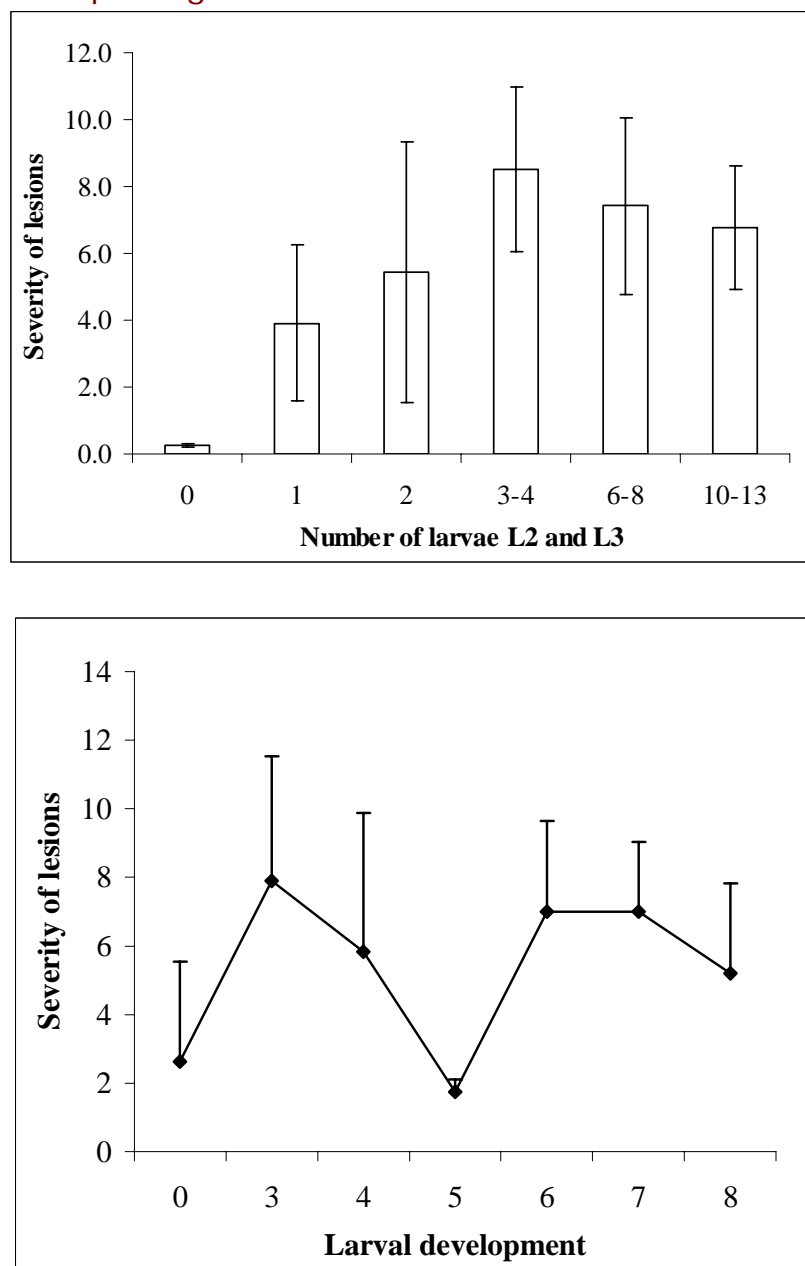
^aAverage larval developmental of *O. ovis* in the host was recorded from early L2 to L3D5 larvae (3 to 11 interstadia) classified according to Cepeda-Palacios et al. (1999).

^bA scale for scoring severity of lesions caused by *O. ovis* infective larvae was constructed (see Table 1).

Relationship of larval burden and development with severity of oestrosis lesions

The number of *O. ovis* larvae and the severity of the lesions in frontal sinus of infected goats are shown in Table 3. Maximum values of severity were observed when 6 larvae or more were found in a particular host sinus (mean severity=10). Severity of lesions was the highest when larvae reached the early L2 larvae or early L3 developmental stadia (Figure 1).

Figure 1. Relationships of *O. ovis* larval number (A) and development (B) on the severity of lesions of sinusal and horn cavities in necropsied goats^a.



^aSeverity of lesions was measured based on an additive scale from 0 to 4 (Mean±S.D.)

ELISA analysis

Results of ELISA are shown in Table 4 and Table 5. In general, a high sensitivity and low specificity were observed using SGC as coating antigens. Sensitivity of the ELISA was higher during the autumn-winter period. In contrast, specificity was higher during the spring-summer period (data not shown in tables).

Table 4. Diagnosis of oestrosis in goats by ELISA using SGC as coating antigens and by necropsy examination in naturally infected goats.

ELISA	Necropsy ^a	
	Positive	Negative
Seropositive	89	20
Seronegative	9	7
Total	98	27

^aNecropsy examinations of the sinus and horn cavities of slaughtered goats ($n=125$); goats were considered infected if at least one L2 or L3 larva was present at necropsy.

Table 5. Predictive diagnosis of oestrosis by ELISA in goats naturally exposed to *O. ovis* infection^a.

Parameter	Total (%)
Sensitivity	90.82
Specificity	25.93
Positive predictive value	89.38
Negative predictive value	71.18
Apparent prevalence	87.20
Actual prevalence ^b	78.40
<i>n</i>	125

^aNecropsy examinations of the sinus and horn cavities of slaughtered goats; the goats were considered infected if the presence of at least one L2 or L3 instar larvae was detected.

^bActual *O. ovis* prevalence in goats from which serum samples were collected.

Discussion

Cepeda et al. (1998) reported both, a permanent *O. ovis* fly infective activity and high oestrosis prevalence in goats (85.4%) throughout the year in Baja California Sur, Mexico. In our study, the presence of L2 and L3 mature larvae found in sinusal and horn cavities during the study confirmed such results. These variables were especially favorable to investigate host-*O. ovis* relationships under field conditions. Interestingly, severities of lesions were mainly related to the presence of early L2 and early L3 larval instars. This suggests that the strongest pathological damage and immune stimulation were provoked during or immediately after larval molt. According to Innocenti et al. (1997) proteins derived from the larval cuticle are capable to stimulate the host immune system, therefore shedding of old cuticle and the new cuticle should be considered because this pathological effect may be associated to the *O. ovis* larval molt phenomena. In addition, Tabouret et al. (2003) found that pathological damages in infected sheep were more severe in sinus cavities and associated to the presence of L2 and L3 larvae.

Interestingly, host live body weight and age were not negatively associated with the number of *O. ovis* larvae or larval development. Accordingly, older animals usually had always higher opportunities to be repetitively infected during their lifetime and to develop posterior immunity. Therefore, it seems that exist a failure in natural acquired immunity or immune resistance of goats to *O. ovis* infections. In addition, reports on acquired immunity in infected sheep are also conflicting. Despite that Manchenko and Manchenko (1989) demonstrated the important role of the sheep immune system on survival of *O. ovis* larvae, Jacquet et al. (2005) found that after experimental repetitive infections with *O. ovis*, lambs did not develop immune resistance, which lead to think that probably a down immune regulation inside the sheep host was provoked by the parasite (Jacquet et al., 2005; Dorchies et al., 2006).

On the other hand, our ELISA tests using SGC as coating antigens showed high sensitivity and low specificity. However, the necropsy analysis was limited to examine the sinus and horn cavities only. This result, however, seems similar to previously reported ELISA tests in which excretory-secretory products (a mixture of salivary gland secretions and larval excretions) of *O. ovis* has been used in both sheep (Suárez et al., 2005; Alcaide et al., 2005a) and goats (Alcaide et al., 2005b; Sánchez-Andrade et al., 2005) species. Therefore, specificity value of ELISA could be improved by examination of full nasal cavities. On this regard, it was reported that ELISA test using SGC as a coating antigen improved sheep oestrosis diagnosis (i.e. compared with L2 crude extracts), but a low relative specificity was

obtained (Angulo-Valadez et al., 2008). In our study, the ELISA tests used resulted in higher apparent prevalence than the actual prevalence (determined after necropsy in sinus and horn cavities of goats). This means that some non-sinus infected goats showed high titers of specific IgG antibodies to *O. ovis*, resulting in false positive serodiagnoses. False positive cases observed might be due to non-specific reactions, or some goats did not host any larvae in the sinuses but they might have hosted undetected larvae in nasal cavities. Other possible explanation is that false positive cases were previously infected but larvae were already expelled by the sampling time. It has been reported that high levels of specific IgG antibodies can persist after the expulsion of any remaining mature larvae or after larvicidal treatments (Jacquiet et al., 2005).

It is a promising fact that the major *O. ovis* salivary gland antigens are recognized by the immune system of both ovine and caprine species (Tabouret et al., 2001; Angulo-Valadez et al., 2007). These preliminary findings may be useful in the future for development of oestrosis diagnosis. Therefore, the following goal should be to purify specific salivary gland antigens for evaluation in ELISA tests, and to identify the most appropriate for oestrosis diagnosis in sheep and goats. As conclusions, age and weight of the host not influenced the *O. ovis* larval populations in naturally infected goats. Major pathological damages caused by *O. ovis* were associated with the presence of early L2 and early L3 larvae, probably enhanced by larval molting. SGC antigens were proven valuable antigens for oestrosis diagnosis by ELISA test in goats.

Acknowledgments

Authors are grateful with technicians Ing. Zoot. Ana Valverde Onofre and Diana Tapiz Victoria for technical assistance. This research was partially supported by ECOS-ANUIES CONACYT 2009 program.

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REDVET: 2009 Vol. 10, Nº 11

Recibido 26.03.09 - Ref. prov. M030914B – Revisado 28.09.09 - Aceptado 30.10.09
Ref. def. 110907_REDVET - Publicado 15.11.09

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