Specific IgG antibody responses in *Oestrus ovis* L. (Diptera: Oestridae) infected sheep: Associations with intensity of infection and larval development

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Abstract

Larvae of *Oestrus ovis* (Diptera: Oestridae) are ubiquitous parasites of nasal and sinusal cavities of sheep and goats. According to the chronobiology of *O. ovis* infections in Sardinia and the seasonal pattern of the IgG response, the optimal period to investigate the relationships between *O. ovis* larval populations and intensity of local and systemic IgG antibody responses was mid-July in the summer season. *Sarda × Lacaune* ewes (*n* = 186), divided into three ram-families were used in the study. Systemic and local IgG responses were measured by ELISA tests using second stage larval crude extracts (L2CE) and L2 (L2SGC) and L3 (L3SGC) salivary gland contents as coating antigens. The number of larval instars, larval length of L1, L2 and L3 larvae, and larval weight of L2 and L3 larvae were individually recorded after ewe necropsy. Negative correlations among larval establishment and/or larval development on the one hand and intensity of local or systemic IgG responses on the other hand were found in two out of three studied ram-families.

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Keywords: *Oestrus ovis*; Salivary gland; Sheep; Immunoglobulin G; Larval growth; Genetic resistance; ELISA

1. Introduction

Larvae of *Oestrus ovis* (Diptera: Oestridae) are common parasites of the nasal and sinus cavities of sheep and goats (Dorchies et al., 2000; Scala et al., 2001) causing losses in meat, wool and milk production. Nasal discharges and sneezing frequently seen in summer are the major clinical signs in *O. ovis* infected sheep. Female adults deposit first-instar larvae (L1) into the host nostrils where they quickly crawl into the nasal cavities. First stage larvae (L1) migrate from the nasal septum and turbinates to the ethmoid where the first moult to the second stage larvae (L2) occurs. Then, L2
latter migrate to the sinus where they moult to the third stage larvae (L3). These L3 larvae are later expelled by the host sneezing onto the ground where they pupate. The growth patterns of *O. ovis* larvae were estimated by Cepeda-Palacios et al. (1999). Larval weight increases from 0.23 mg in L1 larvae to 49 mg in late L2 larvae. The highest increase in weight occurs after the L2–L3 moult, especially during the early L3 period, when larvae acquire about 45% of the average mature weight (520 mg). The estimated critical weight for mature larvae is 280 mg; below this value, larvae are expected to produce non-viable adult flies (Cepeda-Palacios et al., 2000).

During larval development, a specific immune reaction is initiated by the host with a local and systemic antibody response and the recruitment of eosinophils and mast cells in the upper respiratory mucosae (Tabouret et al., 2003). The respective roles of these two types of response are not yet known. The survival of *O. ovis* larvae after artificial infections was higher in immunosuppressed animals compared to the controls (Marchenko and Marchenko, 1989; Jacquet et al., 2005). Moreover, an immunization of sheep with excretory-secretory products (ESP) or gut membrane proteins of L3 larvae provided an inhibitory effect on larval growth (Fruge`re et al., 2000; Angulo-Valadez et al., 2007a). These results suggested that at least a partial immune regulation of *O. ovis* larval development could occur in sheep.

In the Mediterranean island of Sardinia, *O. ovis* infections are frequent and severe during the summer in sheep (Scala et al., 2002). In ewes, systemic IgG antibody titers reached their highest values from July to August (summer season) and decreased November to December (autumn and winter seasons), when they were measured by ELISA using crude extracts of second stage larvae as coating antigens (unpublished data). These periods corresponded to the active growth phase (L1, L2 and L3 in similar proportions within the hosts) and the diapausal phase (majority of hypobiotic L1 within the host), respectively (Scala et al., 2001). Previous studies in an experimental flock of 975 Sarda × Lacaune back-cross ewes, divided into 10 ram-families and maintained in the same farm, showed that the intensities of IgG responses were highly correlated from 1 year to another. Likewise, some ram-families had high IgG responses and others had lower responses repeated year by year (Jacquet et al., 2004). Therefore, the objective of this study was to investigate the relationships between *O. ovis* larval burden and development and local and systemic IgG responses in July. To take into account a possible ram-effect, these relationships were measured in three selected ram-families; i.e. a total of 186 adult ewes, one group with usually low IgG responses (Ram-family 1), another group with high responses (Ram-family 3), and the third one showing intermediate values (Ram-family 2).

2. Materials and methods

2.1. Sheep population

In 1998, 14 elite Lacaune rams from France were mated to 184 Sarda ewes to produce F1 rams in Italy. Among those, 10 male offspring of 10 different Lacaune sires were mated to 2719 Sarda ewes to produce 975 backcross females born in the winter of 1998–1999 (average 98 ewes per family, range 76–121). From August 1999 to 2004, the ewes were bred in an experimental farm located in South Sardinia under a dry Mediterranean climate (average rainfall 400 mm/year, temperature up to 40 °C). All ewes were grazed daily for 4–5 h on an irrigated pasture of ryegrass, and supplemented with alfalfa hay, maize silage and concentrate, especially during the winter and late spring. The first mating occurred in September 1999 while the 4 following mating seasons occurred in June 2000 and July 2001, 2002, 2003. Thus lambings occurred in winter 2000 (first lactation) and fall 2000, 2001, 2002, 2003. After a suckling period of 3 (1st and 2nd lactation) or 4 weeks (3rd, 4th and 5th lactation), they were milked twice a day. In July ewes were progressively dried off and milked in the morning only. Due to a partial slaughtering in January 2004, only three families were conserved in Monastir until July 2004, i.e. Ram-family 1 (62 ewes), Ram-family 2 (70 ewes) and Ram-family 3 (54 ewes). These ewes were exposed to high rates of natural *O. ovis* infections during six successive warm seasons (from 1999 to 2004).

2.2. *Oestrus ovis* populations

Necropsies were carried out in July (summer season) 2004 at a slaughterhouse in Cagliari (Sardinia). After splitting the heads, larvae were collected from the septum, turbinate, ethmoid, and the sinusal cavities, afterward counted and their stages (L1, L2 and L3) identified according to Zumpt (1965). Larval length of L1, L2 and L3 larvae and weight of L2 and L3 larvae were individually recorded using a micrometer under a dissecting microscope and a 0.01 mg precision balance, respectively (Fruge`re et al., 2000).
2.3. Analysis of systemic and local antibody responses

Blood samples were taken from each individual ewe the day before slaughter and the sera were stored at −20 °C until use. Mucus samples were collected at necropsy by absorption on filter paper (Whatman® Ltd. Madison, UK) strips of 4 cm² each. PBS impregnated strips were deposited on the septal mucosa for 5 min on each side. Strips were then gently agitated in PBS for 2 h at room temperature (Tabouret et al., 2003). Solutions were centrifuged and supernatants stored frozen at −20 °C.

2.4. Parasite proteins

Second stage larvae were homogenized into PBS (pH 7.2) at 0.25 g wet weight per ml of buffer, centrifuged at 5000 × g. Supernatants were filtered through 0.8/0.2 μm Acrodisc sieves (Gelman Laboratory, Ann Arbor, MI, USA) to be used as the L2 crude extracts (L2CE). L2 and L3 larvae were dissected alive in ice-cold PBS medium under a stereoscope (Angulo-Valadez et al., 2007b). The salivary glands were removed and centrifuged at 10,000 × g to expel the contents. Protein concentrations were determined in L2CE and L2 and L3 salivary gland contents (L2SGC and L3SGC) using the bicinchoninic acid assay kit (BCA, Pierce, Rockford, IL). Bovine serum albumin was used as standard.

2.5. Systemic specific IgG

To detect systemic O. ovis specific IgG, an ELISA test was performed according to Jacquiet et al. (2005). L2CE, L2SGC and L3SGC were diluted at 2 μg/ml in carbonate buffer (pH 9.6), distributed (100 μl) in 96 well plates (Nunclon surface, Nunc, Denmark), incubated for 1 h at 37 °C, then overnight at 4 °C. The wells were washed three times with PBST (0.01 M phosphate, 0.15 M sodium chloride, pH 7.2 and 0.1% Tween 20). The antigen-coated wells were then incubated for 30 min with a 10% skimmed milk solution (200 μl) at 37 °C before blotting dry. Triplicate serum samples (100 μl) diluted 1:200 in PBST were incubated for 60 min at 37 °C. The plates were washed three times with PBST before addition (100 μl) of a horseradish peroxidase-conjugated donkey anti-sheep IgG (Sigma A3415, St. Louis, MO) diluted (1:2000) in carbonate buffer (60 min of incubation at 37 °C). Three final washes with PBST were carried out before addition and incubation at 37 °C of 100 μl per well of the chromogen (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium; Sigma A9941, St. Louis, MO, USA). After 1 h, the optical densities (OD) were determined with a spectrophotometer (Dynatech plate reader, Dynatech laboratories Inc., Alexandria, VA) by measuring the absorbance at 405 nm. Results are expressed as OD of samples minus mean OD of three PBS wells. All samples were performed in triplicate. The average of the sample values was used in final calculations.

2.6. Local specific IgG

Flat-bottomed microtiter plates (Nunclon surface, Nunc, Denmark) were coated with O. ovis L2CE, L2SGC or L3SGC (5 μg/ml). The procedure described above was used except that the sera were replaced by mucus preparations, diluted 1:25 in PBS. Optical densities were read with a Dynatech plate reader at 405 nm. Results are expressed as optical densities for local IgG minus the mean OD of three PBS wells. All samples were performed in triplicate. The average of the sample values was used in final calculations.

2.7. ELISA tests parameters

Positive cut-offs were determined using sera from 38 non-infected, 3 month-old lambs, born in winter and reared indoors, i.e. under O. ovis-free conditions. Sensitivity, specificity, positive and negative predictive values were calculated as well as apparent prevalence according to Tabouret et al. (2001).

2.8. Relationships with intensities of infection and larval development

Optical density values of systemic and local IgG responses, number and weight of larvae were transformed (log (x + 1)) to normalize the distributions.

2.9. Statistical analysis

Comparisons of prevalence and larval instar proportions between ram-families were done with Chi-square test. Intensities of infections, weight and length of larvae were compared with ANOVA analysis after log (x + 1) transformation. Correlation coefficients among these variables were calculated. All statistical procedures were carried out using the Statistica software (StatSoft, 1998).
3. Results

3.1. Parasitological parameters

The prevalence and intensity of infection, the lengths of L1, L2 and L3 larvae, the weights of L2 and L3 larvae and the demographic structures of *O. ovis* populations (L1, L2 and L3 relative proportions) are presented in Table 1 for the whole flock (*n* = 186 ewes). All these parameters were similar within the three ram-families (Table 1) except the demographic structures which differ significantly between the three ram-families (*P* = 0.01). A high proportion of L1 larvae was observed in Ram-family 3 burdens, and L2 larvae in Ram-family 1, while L3 larvae accounted 50% of the total larvae recovered from Ram-family 2.

3.2. ELISA analysis (systemic IgG response)

Results of indirect ELISA tests (using L2CE, L2SGC and L3SGC as coating antigens) are summarized in Table 2. High sensitivities were observed using any of the antigen sources. All tests resulted in higher apparent prevalences than the true prevalence (determined after necropsy), i.e. some non-infected sheep at the time of necropsy showed high titers of specific *O. ovis* IgG antibodies. This was observed consistently in the three ram-families (data not shown).

Intensities of local and systemic IgG antibody responses showed high intra-ram-family variation (Fig. 1A and B). Maxima OD values were found using salivary gland proteins as coating antigens in both

![Fig. 1](image.png)

**Fig. 1.** Intensities of *Oestrus ovis* specific, systemic (A) and local (B), Immunoglobulin G antibody response in infected sheep. L2CE: crude extracts of L2 larvae, L2SGC: salivary gland extracts of L2 larvae and L3SGC: salivary gland extracts of L3 larvae.

### Table 1

*Oestrus ovis* populations in three Ram-families necropsied in July (summer season) in Sardinia, Italy

<table>
<thead>
<tr>
<th>O. ovis infection</th>
<th>Ram-family 1 (<em>n</em> = 62)</th>
<th>Ram-family 2 (<em>n</em> = 70)</th>
<th>Ram-family 3 (<em>n</em> = 54)</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence of <em>O. ovis</em> infection</td>
<td>63.0%</td>
<td>64.0%</td>
<td>59.6%</td>
<td>62.2%</td>
</tr>
<tr>
<td>Mean intensity of infection (S.D.)</td>
<td>2.6 (3.2)</td>
<td>2.2 (3.1)</td>
<td>2.1 (3.1)</td>
<td>2.3 (3.1)</td>
</tr>
<tr>
<td>Proportion of L1 (%)</td>
<td>17.6</td>
<td>10.9</td>
<td>25.8</td>
<td>18.1</td>
</tr>
<tr>
<td>Proportion of L2 (%)</td>
<td>47.8</td>
<td>39.1</td>
<td>35.6</td>
<td>40.8</td>
</tr>
<tr>
<td>Proportion of L3 (%)</td>
<td>34.6</td>
<td>50</td>
<td>38.6</td>
<td>41.1</td>
</tr>
<tr>
<td>Mean length (mm) of L1 (S.D.)</td>
<td>1.1 (0.3)</td>
<td>1.0 (0.2)</td>
<td>1.2 (0.2)</td>
<td>1.1 (0.2)</td>
</tr>
<tr>
<td>Mean length (mm) of L2 (S.D.)</td>
<td>9.6 (4.3)</td>
<td>7.8 (3.5)</td>
<td>8.4 (3.1)</td>
<td>8.6 (3.6)</td>
</tr>
<tr>
<td>Mean length (mm) of L3 (S.D.)</td>
<td>18.6 (2.6)</td>
<td>18.4 (2.6)</td>
<td>18.0 (2.2)</td>
<td>18.3 (2.5)</td>
</tr>
<tr>
<td>Mean weight (mg) of L2 (S.D.)</td>
<td>45.3 (38.6)</td>
<td>33.6 (41.9)</td>
<td>45.0 (39.0)</td>
<td>41.3 (39.8)</td>
</tr>
<tr>
<td>Mean weight (mg) of L3 (S.D.)</td>
<td>388.6 (149.7)</td>
<td>386.6 (144.8)</td>
<td>362.5 (134.2)</td>
<td>379.2 (142.9)</td>
</tr>
</tbody>
</table>

### Table 2

ELISA test used for predictive diagnosis of oestrosis in sheep, using L2CE, L2SGC and L3SGC as coating antigens at 2 μg/ml

<table>
<thead>
<tr>
<th>Assay parameters</th>
<th>ELISA test coating antigen source</th>
<th>L2CE</th>
<th>L2SGC</th>
<th>L3SGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>95.0</td>
<td>99.0</td>
<td>97.1</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>48.2</td>
<td>22.4</td>
<td>52.6</td>
<td></td>
</tr>
<tr>
<td>+Predictive value</td>
<td>74.0</td>
<td>66.6</td>
<td>78.5</td>
<td></td>
</tr>
<tr>
<td>−Predictive value</td>
<td>85.9</td>
<td>95.2</td>
<td>90.7</td>
<td></td>
</tr>
<tr>
<td>Apparent prevalence</td>
<td>78.4</td>
<td>90.6</td>
<td>79.6</td>
<td></td>
</tr>
</tbody>
</table>


b Apparent prevalence was estimated on systemic IgG response. True prevalence determined by ewe necropsy was 62.2%.
systemic and local responses. No significant differences in intensity of IgG responses were observed among the three ram-families.

3.3. Relationships between IgG antibody responses and *O. ovis* larval burden or larval development

Systemic and local IgG responses were highly correlated ($P = 0.001$) in Ram-families 1 ($r = 0.57$), 2 ($r = 0.58$) and 3 ($r = 0.53$). No statistically significant relationships between IgG responses and larval burden or larval development were shown in Ram-family 1. Nevertheless, some significant negative correlations were observed in Ram-family 2 and Ram-family 3 (Table 3).

3.4. Larval burden

L2 larval burden was negatively correlated to the systemic L3SGC specific IgG response in Ram-family 2 only (Table 3). Likewise, L3 larval burden was negatively correlated to the intensity of local IgG response in Ram-family 2 (L3SGC as antigen coating) and Ram-family 3 (L2CE antigen as antigen coating).

3.5. Larval development

L2 and L3 larval weights were negatively correlated to both systemic and local IgG responses in Ram-family 2 and only to local IgG response in Ram-family 3 (Table 3).

4. Discussion

L2 and L3 salivary gland proteins are known to be the most antigenic fractions of *O. ovis* larval proteins (Angulo-Valadez et al., 2007b; Innocenti et al., 1995). Indirect ELISA using L2CE, L2SGC and L3SGC as coating antigens showed high sensitivity but low specificity in the summer period. Data reported in this study confirmed previous reports from France (Tabouret et al., 2001) and Sardinia (Suárez et al., 2005). False positives observed were either due to non-specific reactions or, more likely, to animals which did not host larvae at necropsy but which could have been previously infected. High levels of specific IgG antibodies could persist after the expulsion of any remaining mature larvae or after larvicidal treatment (Jacquet et al., 2005).

Several reports suggest that at least a partial immune regulation of *O. ovis* larval populations might occur in sheep. For instance, retardation in larval development and high systemic IgG response were observed in immunized sheep with excretory-secretory products (containing salivary gland antigens) or digestive membrane proteins of *O. ovis* (Frugère et al., 2000; Angulo-Valadez et al., 2007a). However, larval establishment was not affected in these studies. Furthermore, adult sheep seem to be less susceptible to *O. ovis* infection than young lambs (Dorchies and Yilma, 1994; Scala et al., 2001) suggesting that continuously exposed sheep develop immune competency. Protective responses to the blowfly *Lucilia cuprina* are dependent on the frequency and the amount of larval exposures as well as the genetic background of the sheep (Eisemann et al., 1990; Sanderman et al., 1992). In vitro, larval growth was significantly delayed by sera (containing high levels of IgG levels) from repeatedly infected or vaccinated sheep (Bowles et al., 1987; Eisemann et al., 1990; Tellam and Eisemann, 1998; Colditz et al., 2002). However, *in vivo* evaluation using larval challenge models failed to demonstrate any significant protection.

<table>
<thead>
<tr>
<th>Larval characteristic</th>
<th>Antibody response</th>
<th>Ram-family</th>
<th>n*</th>
<th>Larval instar</th>
<th>Correlation coefficient ($r$)</th>
<th>$P$</th>
<th>Antigen coatingb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burden</td>
<td>Systemic IgG</td>
<td>2</td>
<td>38</td>
<td>L2</td>
<td>−0.38</td>
<td>0.01</td>
<td>L3SGC</td>
</tr>
<tr>
<td></td>
<td>Local IgG</td>
<td>2</td>
<td>42</td>
<td>L3</td>
<td>−0.35</td>
<td>0.025</td>
<td>L3SGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>25</td>
<td>L3</td>
<td>−0.39</td>
<td>0.025</td>
<td>L2CE</td>
</tr>
<tr>
<td>Weight</td>
<td>Systemic IgG</td>
<td>2</td>
<td>35</td>
<td>L2 + L3</td>
<td>−0.32</td>
<td>0.05</td>
<td>L2CE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>38</td>
<td>L3</td>
<td>−0.30</td>
<td>0.05</td>
<td>L3SGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>17</td>
<td>L3</td>
<td>−0.52</td>
<td>0.025</td>
<td>L2CE</td>
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<td></td>
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<td></td>
<td></td>
<td>L2 + L3</td>
<td>−0.43</td>
<td>0.05</td>
<td>L2CE</td>
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<tr>
<td></td>
<td>Local IgG</td>
<td>2</td>
<td>38</td>
<td>L3</td>
<td>−0.30</td>
<td>0.05</td>
<td>L3SGC</td>
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<td>0.025</td>
<td>L2CE</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L2 + L3</td>
<td>−0.43</td>
<td>0.05</td>
<td>L2CE</td>
</tr>
</tbody>
</table>

* n: Number of positively infected sheep with this larval instar.

using crude larval, excretory-secretory preparations or purified serine-proteases (Elkington and Mahony, 2007). By comparison, the roles of antibodies have been clearly demonstrated in the regulation of development of Teladorsagia circumcincta (Stear et al., 1999) and Haemonchus contortus (Lacroux et al., 2006) in sheep. In these two latter parasite infections, antibodies should act directly by neutralizing parasite enzymes and also in cooperation with eosinophils in an antibody dependent release of toxic granules in the contact with the cuticle of worm (Meeusen et al., 2005; Terefe et al., 2007).

In our study, necropsies and antibody level determinations were done in animals coming from the same flock, located in South Sardinia where O. ovis infections are frequent and severe, especially in July (summer season). These ewes were divided into three ram-families (half-sib ewes within a family). Due to common management, we may consider that all 186 ewes were exposed to identical O. ovis infection rates regardless of the ram-family to which they belonged. Moreover, at the time of the study, all ewes had experienced natural repetitive O. ovis infections during six periods of fly activity and should be considered as highly immune sheep. Regarding prevalence and intensity of infections, larval lengths and weights showed high variability in each ram-family without significant differences among ram-families. However, the proportions of the three larval instars were significantly different among ram-families suggesting that larval development differed from one ram-family to another. In addition, significant correlations between local or systemic IgG responses and larval establishment and development were observed in some ram-families but not in others. This is the first report in which larval development differed from one ram-family to another. In addition, significant correlations between local or systemic IgG responses and larval establishment and development were observed in some ram-families but not in others. This is the first report in which larval development differed from one ram-family to another.

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5. Conclusion

Naturally O. ovis infected adult ewes showed high local and systemic IgG antibody responses to salivary glands proteins of L2 and L3 larvae. IgG levels appeared to be negatively correlated to larval survival and development inside the host. This phenomenon was likely under genetic control as some but not all ram-families showed these negative associations.

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