High prevalence of antibodies against *Chlamydiaceae* and *Chlamydophila abortus* in wild ungulates using two “in house” blocking-ELISA tests

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1. Introduction

The link between wildlife, environment, pathogens and human activities creates a dynamic where new pathogens or new hosts frequently emerge; changes in population density or host behaviour affect disease prevalence, and disease agents can suddenly boost their virulence and widen their host range (Gortázar et al., 2007). There is growing evidence that chlamydial infections may result in adverse pregnancy outcomes in humans and domestic animals (Pospischil et al., 2002; Baud et al., 2008), and it is well known that chlamydiosis is a multi-host disease affecting a huge variety of vertebrates (Everett et al., 1999).

*Chlamydiaceae*, which includes six species, is a bacterial genus that, together with the genus *Chlamydia*, both belongs to the family *Chlamydiaceae*. *Chlamydiaceae abortus* is the most common cause of reproductive failure in sheep- and goat-breeding countries in Europe, and where it has a substantial economic impact (Aitken, 1993). Infected pregnant ewes and goats abort late in gestation or give...
birth to weak lambs as a result of affected placenta. Bacteria excreted at abortion with the uterine discharge are the main source of infection for susceptible animals through ingestion or inhalation. After the onset of abortion, most animals acquire immunity and reestablish success fully (Rodolakis et al., 1998). Data from the few existing reports on chlamydial seroprevalence in domestic ruminants show geographical differences, ranging from 11 to 51% in sheep and goat flocks (Cuello et al., 1992; Markey et al., 1993; Mainar-Jaime et al., 1998; Borel et al., 2004) and from 24 to 45% in cattle (Cavirani et al., 2001; Petit et al., 2008). Data related to the prevalence and relevance of chlamydial infections in wild mammals are even more scarce (Hotzel et al., 2004).

Early diagnosis of chlamydial infections is important to prevent and limit the spread of the microorganism. For diagnosis purposes, serological assays are more suitable for screening large numbers of samples. The complement fixation test (CFT) is the most widely used test and is still recommended by the Organisation Internationale des Epizooties (OIE) (http://www.oie.int). However, it lacks specificity due to its antigen, which consists mainly of the heat-resistant lipopolysaccharide (LPS), common to all Chlamydiaceae species (Brade et al., 1987), particularly in ruminants, which are widely infected by Chlamydiophila pecorum (Rodolakis et al., 1998). Moreover, CFT requires a prime quality serum which, in wildlife studies, is not always available (Mason and Fleming, 1999). Several ELISA methods have been developed in order to improve chlamydial serology. These include ELISA tests using purified whole elementary bodies (EB), LPS or more specific assays based on the C. abortus major outer membrane protein (MOMP) (Kaltenboeck et al., 1997; Salti-Montesanto et al., 1997; Hoelzl et al., 2004; Livingstone et al., 2005). Comparative serological studies have revealed that a new commercially available ELISA test based on a polymorphic outer membrane protein (POMP) could serve as an improved alternative to CFT or to classical indirect ELISAs for the serological diagnosis of OEA Q1 (Buendia et al., 2001; Vretou et al., 2007).

Although there are numerous indications that Chlamydiaceae are present in a wide range of wildlife host species, few studies have been conducted to determine the prevalence of C. abortus, most probably due to the absence of suitable species-specific serological tools and reagents to test sera from wild animals. While the role of Chlamydiaceae as pathogens in European wild boar (Sus scrofa) has yet to be established, the detection by PCR of a spectrum of Chlamydiaceae species identical to those in domestic pigs (including C. abortus) in a small sample of hunter-harvested wild boar from Germany, revealed a possible wildlife reservoir for these bacteria. The possibility that these animals acquired chlamydiae through contact with domestic pigs appeared very unlikely (Hotzel et al., 2004).

As regards wild ruminants, the literature on abortions caused by C. abortus includes a case report on a springbok (Antidorcas marsupialis) in Paris zoo (Berri et al., 2004). However, most studies have been made by serological analysis using CFT. In Italy, Giovanini et al. (1988) showed a prevalence of 75% in fallow deer (Dama dama), while Giacometti et al. (1995) showed a prevalence of 31% in alpine ibex (Capra ibex). Similarly, a CFT-based study in populations of wild ruminants including fallow deer, red deer (Cervus elaphus), mouflon (Ovis aries musimon) and Iberian ibex (Capra pyrenaica) in southern Spain indicated that Chlamydiaceae infections could be present in wild ruminant species (Cubero-Pablo et al., 2000). Antibody seroprevalence ranged from 24 to 37% and was higher in those areas where contact with domestic sheep and goats was likely. Salwa et al. (2007) found low titers of a serological reaction to Chlamydiaceae in 45% of European bison (Bison bonasus) selected between 1991 and 2001 for poor body condition, cachexia, lameness and balanoposthitis in Bialowieza (Poland), suggesting that the transmission of pathogens can occur between domestic and wild ruminants. In the present study our objectives were (1) to develop two “in-house” blocking-ELISA tests to detect antibodies against Chlamydiaceae and C. abortus in sera from wild ungulates. These methods would make it possible to analyze simultaneously sera from phylogenetically non-related species, including birds; (2) to establish the relationship between geographical and biological factors such as animal species, age and sex and prevalence of antibodies against Chlamydiaceae and C. abortus in wild ungulates from Spain.

2. Materials and methods

2.1. Wild animal sera and geographical location

The sample included 434 field sera from eight wild ungulate species including one suid species (European wild boar), three cervids (red deer; fallow deer; and roe deer, Capreolus capreolus), and four bovids (mouflon; Barbary sheep, Ammotragus lervia; Southern chamois, Rupicapra pyrenaica; and Iberian ibex). Samples were obtained opportunistically during the hunting seasons between 2005 and 2007 from different geographical regions in Spain. Sampling sites are representative of the most biogeographically relevant landscape types of mainland Spain, with a bias towards the Mediterranean shrub-lands of the central and southern regions, where hunting activities are traditionally important. Thus, sampling sites were divided into four geographic regions, named Cantabrian Mountains, Northern Plateau, South–Central, and South Coast.

The Atlantic climate is represented by the samples from the Cantabrian Mountains, a mountainous coastal region in the north of Spain. Here, no feed is provided to wildlife because of conflict regarding wildlife damage to croplands. Contact between wild ungulates and livestock, mostly cattle, is frequent. Mediterranean shrub-lands are dominant in the South–Central and South Coast sampling sites. These areas are characterized by high densities of wild ungulates due to intensive game management. Fencing and feeding are common and contact with livestock (mostly goats and cattle) is limited within fenced estates. Finally, only Iberian ibex was tested in the Northern Plateau. Most samples came from a fenced estate with ibex, wild boar and roe deer but no domestic ungulates, and two samples were obtained from an open hunting reserve with diverse wild and domestic ungulates.

2.2. Ovine control sera

Seven sera from aborted ewes experimentally infected with the AB7 strain of C. abortus (García de la Fuente et al., 2004), two sera from ewes experimentally infected with the iB1 strain of C. pecorum (Rodolakis and Souriau, 1989) and three sera from SPF lambs were used as ovine control sera. Additionally, 10 field ovine sera from a flock with a known OEA clinical history (Buendía et al., 2001) were used in this work.

2.3. Chlamydial antigen

McCoy cell monolayer in tissue culture flasks of 75 cm² (Sterilin Ltd, Hounslow, UK) were infected with the AB7 strain of C. abortus and elementary bodies (EB) were purified using a renograin method as previously described (Buendía et al., 1997). Infected cells were sonicated for 1 min and then centrifuged at 500 g for 10 min to remove gross cell debris. The resulting supernatant portion was then diluted 1/100 in 0.85% NaCl solution and centrifuged at 10,000 g for 20 min at 4 °C. The pellet was re-suspended in 0.85% NaCl solution and centrifuged at 100,000 g for 60 min at 4 °C. The resulting pellet was resuspended in 20% sucrose (0.1 M) and was used to coat ELISA plates. Protein concentration was determined using the bichinchninic acid protein assay with bovine serum albumin as a standard (Sigma, Madrid, Spain).

2.4. Serological tests

2.4.1. Reference techniques

Three serological assays were carried out as reference techniques using control and field ovine sera to select the cut-off for the “in house” blocking-ELISAs: (i) the complement fixation test (CFT), (ii) a classical indirect ELISA using purified C. abortus EB as antigen (ELISA-EB), and (iii) the ELISA kit commercialized by Pourquier Laboratories (Institut Pourquier, Montpellier, France) now IDEXX Laboratories that used an C. abortus recombinant fragment of the 80–90 kDa protein not defined by the manufacturer (ELISA-Pourquier).

The CFT and ELISA-EB were performed essentially as described previously by Buendía et al. (2001) and a titre of ≥1/40 was considered positive for CFT. In the ELISA-EB, a cut-off value was calculated using a group of 10 sera tested negative by CFT and ELISA-Pourquier in a previous study (Buendía et al., 2001). Sera with an OD ≥0.207 (mean optical density for the 10 serum specimens ± 3 standard deviations) were considered positive. The ELISA-Pourquier test was performed according to the instructions of the manufacturer. The final values were expressed as % Sample/Positive control (% S/P). Sera with % S/P equal to or lower than 50% were considered negative for C. abortus infection, sera with a % S/P between 50 and 60% were considered doubtful and sera with a % S/P equal to or higher than 60% were considered positive.

2.4.2. Monoclonal antibodies

Two monoclonal antibodies (mAbs) were selected from a panel in preliminary assays (data not shown) using the ELISA-EB or the ELISA-Pourquier tests: the Chlamydiaceae LPS-specific HASC5 mAb (Salinas et al., 1994) and the C. abortus POMP91B-specific JAC67 mAb (Souriau et al., 1993; Q2 Vretou et al., 2003), respectively. These mAbs were produced from ascitic fluid in Balb/c mice. An optimal working dilution of the mAbs was determined in preliminary experiments, using a checker-board design (data not shown). Both mAbs were titrated to determine the saturation point (OD between 1.6 and 1.8), and working dilutions of 1/100 for HA5C5 and 1/500 for JAC67 were selected for use in the blocking-ELISA-LPS and blocking-ELISA-rPOMP, respectively, with an OD below the saturation point.

2.4.3. “In house” blocking-ELISA Chlamydiaceae lipopolysaccharide-specific (b-ELISA-LPS)

The 96-well ELISA plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4 °C with purified EB at a concentration of 0.2 μg of protein/well in 100 μl of 0.05 M sodium carbonate/bicarbonate buffer (pH 9.6). Non-specific binding was blocked with 5% non-fat dried milk in PBS (PBS-M) for 30 min at room temperature. After three washes with PBS containing 0.05% Tween-20 (PBS-T), serum samples, including pooled sera from SPF lambs as negative control, were applied in duplicate to the plate at 1/10 dilution in PBS-M, incubated at room temperature for 1 h and washed three times as before. The LPS-HA5C5 mAb was added to the plates at the above defined 1/100 dilution and incubated at room temperature for 30 min. After another washing step, goat peroxidase-conjugated anti-mouse immunoglobulins (Sigma), diluted at 1/1000 in PBS-M, was added and incubated at room temperature for 1 h. Bound peroxidase was developed by adding a commercially available substrate containing the chromogen 3,3',5,5' tetramethylbenzidine (TMB). The reaction was stopped after 10 min by addition of 1 M H2SO4. Optical densities were read in a microtitre plate reader at 450 nm wavelength. The results were expressed as percentage of inhibition:

\[
\text{% of inhibition} = 100 \times \frac{\text{mean net OD of sample serum}}{\text{mean net OD of control negative serum}}. \]

2.4.4. “In house” blocking-ELISA recombinant C. abortus POMP-specific (b-ELISA-rPOMP)

The b-ELISA-rPOMP was performed using the ELISA kit commercialized by Pourquier Laboratories according to the instructions of the manufacturer with some modifications. The plates were coated with a C. abortus-specific...
Table 1 Serological results for ovine control sera and for field sera from sheep with known clinical OEA history, estimated by all the ELISA tests.

<table>
<thead>
<tr>
<th>Control sera from ewes</th>
<th>ELISA-EB (OD)</th>
<th>b-ELISA-LPS (% Inhibition)</th>
<th>ELISA-Pourquier (% S/P)</th>
<th>b-ELISA-rPOMP (% Inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum number 1</td>
<td>0.610 (+)</td>
<td>41</td>
<td>70 (+)</td>
<td>39</td>
</tr>
<tr>
<td>Serum number 2</td>
<td>1.275 (+)</td>
<td>72</td>
<td>82 (+)</td>
<td>52</td>
</tr>
<tr>
<td>Serum number 3</td>
<td>1.032 (+)</td>
<td>38</td>
<td>76 (+)</td>
<td>46</td>
</tr>
<tr>
<td>Serum number 4</td>
<td>0.512 (+)</td>
<td>44</td>
<td>61 (+)</td>
<td>32</td>
</tr>
<tr>
<td>Serum number 5</td>
<td>1.719 (+)</td>
<td>69</td>
<td>113 (+)</td>
<td>71</td>
</tr>
<tr>
<td>Serum number 6</td>
<td>0.826 (+)</td>
<td>36</td>
<td>92 (+)</td>
<td>48</td>
</tr>
<tr>
<td>Serum number 7</td>
<td>0.410 (+)</td>
<td>43</td>
<td>48 (−)</td>
<td>29</td>
</tr>
<tr>
<td>Serum number 8</td>
<td>0.221 (+)</td>
<td>38</td>
<td>33 (−)</td>
<td>21</td>
</tr>
<tr>
<td>Serum number 9</td>
<td>0.276 (+)</td>
<td>35</td>
<td>27 (−)</td>
<td>16</td>
</tr>
<tr>
<td>Serum number 10</td>
<td>0.097 (−)</td>
<td>21</td>
<td>24 (−)</td>
<td>0</td>
</tr>
<tr>
<td>Serum number 11</td>
<td>0.139 (−)</td>
<td>18</td>
<td>12 (−)</td>
<td>11</td>
</tr>
<tr>
<td>Serum number 12</td>
<td>0.116 (−)</td>
<td>12</td>
<td>31 (−)</td>
<td>7</td>
</tr>
<tr>
<td>Field sera from ewes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum number 1</td>
<td>0.196 (−)</td>
<td>34</td>
<td>21 (−)</td>
<td>25</td>
</tr>
<tr>
<td>Serum number 2</td>
<td>0.143 (−)</td>
<td>25</td>
<td>32 (−)</td>
<td>22</td>
</tr>
<tr>
<td>Serum number 3</td>
<td>0.118 (−)</td>
<td>16</td>
<td>17 (−)</td>
<td>9</td>
</tr>
<tr>
<td>Serum number 4</td>
<td>0.465 (+)</td>
<td>25</td>
<td>66 (+)</td>
<td>27</td>
</tr>
<tr>
<td>Serum number 5</td>
<td>1.541 (+)</td>
<td>49</td>
<td>103 (+)</td>
<td>61</td>
</tr>
<tr>
<td>Serum number 6</td>
<td>0.620 (+)</td>
<td>39</td>
<td>72 (+)</td>
<td>30</td>
</tr>
<tr>
<td>Serum number 7</td>
<td>0.963 (+)</td>
<td>57</td>
<td>66 (+)</td>
<td>39</td>
</tr>
<tr>
<td>Serum number 8</td>
<td>1.640 (+)</td>
<td>72</td>
<td>112 (−)</td>
<td>84</td>
</tr>
<tr>
<td>Serum number 9</td>
<td>0.306 (+)</td>
<td>33</td>
<td>11 (−)</td>
<td>18</td>
</tr>
<tr>
<td>Serum number 10</td>
<td>0.820 (+)</td>
<td>49</td>
<td>63 (+)</td>
<td>33</td>
</tr>
</tbody>
</table>

Sera with % S/P <50: negative result; sera with % S/P 50–60: doubtful result; sera with % S/P >60: positive result.

Chi square tests or, where appropriate, Fisher’s exact tests were used to compare prevalence data. 95% confidence intervals for prevalence were estimated by the expression

\[
\text{S.E. 95\%C.I.} = 1.96\sqrt{(\frac{p(1-p)}{n})/1/2}
\]

\(p\) was known in 127 hosts. For statistical purposes, animals were classified into age classes following Vicente et al. (2006).

2.5. Statistical analysis

The serology analyses revealed that 41.7 ± 4 of the sera were positive for the b-ELISA-LPS and 18.9 ± 3% for the b-ELISA-rPOMP (Table 2). Antibodies to Chlamydiaceae LPS were detected in sera from all eight ungulate species with a prevalence ranging from 23 to 60%. Iberian ibex was the only wild ungulate without seropositivity to the C. abortus specific POMP. The prevalence of anti-POMP antibodies in the other seven wild ungulate species ranged from 7 to 40%. The inter-specific differences found in antibody seroprevalence (Fig. 1) were significant both for anti-LPS antibodies (Chi-square statistic = 22.3, 7 d.f., \(p < 0.001\)) and for anti-POMP antibodies (Chi-square statistic = 29.9, 7 d.f., \(p < 0.001\)).
Sixty-two (14.3%) and 234 (53.9%) hosts tested positive and negative, respectively, to both b-ELISA tests. One hundred and nineteen (27.4%) hosts (26 wild boar, 41 red deer, 24 fallow deer, 9 roe deer, 3 mouflon, 4 Barbary sheep, 5 Southern chamois and 7 Iberian ibex) were positive for b-ELISA-LPS but negative for b-ELISA-rPOMP. Surprisingly, 19 (4.3%) hosts (2 wild boar, 2 red deer, 6 fallow deer, 4 roe deer, 1 mouflon, and 4 Southern chamois) yielded a positive b-ELISA-rPOMP result while testing negative for the b-ELISA-LPS.

Due to the significant inter-species differences, the influence of sampling region on antibody prevalence was analyzed for wild boar and deer species separately. Data on bovids were excluded since sampling was limited to one region per species. As regards the European wild boar, no significant difference in anti-LPS antibody prevalence was observed (Chi-square statistic = 2.6, 2 d.f., p > 0.05). However, the differences in anti-POMP antibody prevalence were significant ( Fisher’s exact test, p = 0.002), with high antibody levels observed in wild boar from the Cantabrian Mountains, in northern Spain. For red deer no significant difference in anti-LPS antibody prevalence was observed among regions (Chi-square statistic = 0.8, 2 d.f., p > 0.05) but again, the differences in anti-POMP antibody prevalence were significant (Chi-square statistic = 36, 2 d.f., p < 0.001). The prevalence in red deer from the South Coast was high compared with the absence of anti-POMP antibody in this species from Central and Northern Spain. In fallow deer, no significant difference was observed in anti-LPS or in anti-POMP antibody prevalence among regions (Chi-square statistic < 2.5, 2 d.f., p > 0.05). In contrast, roe deer showed significant regional variation in anti-LPS antibody prevalence, with the highest levels recorded in the South Coast samples ( Fisher’s exact test, p = 0.018), but no variation was found for anti-POMP antibody prevalence ( Fisher’s exact test, p > 0.05).

Sex was known for 282 hosts (Table 3). The between-sex differences for anti-LPS (Chi-square statistic = 0.5, 1 d.f., p > 0.05) and for anti-POMP antibody prevalence (Chi-square statistic = 0.6, 1 d.f., p > 0.05) were not significant. Age class was known for 127 hosts (Table 4). No age-related differences were found for anti-LPS (Chi-square statistic = 1.4, 3 d.f., p > 0.05) or for anti-POMP antibodies prevalence (Chi-square statistic = 5.5, 3 d.f., p > 0.05) considering the whole mixed-species sample. When tested independently, red deer (n = 60 known age of individuals) and wild boar (n = 73) yielded no significant age effect on antibody prevalence (Chi-square statistic < 2.5, 3 d.f., p > 0.05, for both species).

4. Discussion

The use of two “in house” blocking ELISAs provided a useful tool for the serological diagnosis of contact with Chlamydiaceae and C. abortus in wild ungulates, as well as showing a marked prevalence of antibodies against Chlamydiaceae in Spanish wild ungulates. To the best of our knowledge, this is the first report on the detection of C. abortus antibodies in red deer, fallow deer, roe deer, mouflon, Barbary sheep, Southern chamois, Iberian ibex and wild boar. Lack of reports on C. abortus antibody
prevalence in wild ungulates is related to the absence of species-specific serological assays for this microorganism, unlike the cases for domestic ruminant species (Salti-Montesanto et al., 1997; Kaltenboeck et al., 1997; Buendia et al., 2001; Hoelzle et al., 2004; Livingstone et al., 2005; Vretou et al., 2007). However, using a commercial kit that offers good sensitivity and specificity (Buendia et al., 2001; Vretou et al., 2007), we developed a C. abortus-specific “in house” blocking-ELISA test. Similarly, we developed another “in house” blocking-ELISA test to detect anti-Chlamydiaceae LPS antibodies, adapted from a previously evaluated indirect ELISA (Buendia et al., 2001).

The high LPS/POMP antibody ratios detected in wild boar, red deer and Barbary sheep suggest frequent contact of these hosts with Chlamydiaceae other than C. abortus, such as C. pecorum, Chlamydia suis and Chlamydophila psittaci, the last, found by PCR as the predominant species in wild boar from Germany (Hotzel et al., 2004). Alternatively, we might suggest that the particular POMP-fragment used as antigen varies in the C. abortus subtypes present in the field, as occurs with the MOMP in atypical C. abortus strains (Vretou et al., 2001); these variants would raise less-reactive sera from the infected wild ungulates analyzed.

The surprising finding of 4.3% positive sera in wild ungulates to r-POMP but negative to LPS may be explained by the much earlier presence of anti-POMP antibodies detected in naturally (Buendia et al., 2001) and experimentally (Livingstone et al., 2005) C. abortus-infected ewes. Thus, these last authors showed that r-POMP ELISA is considerably more sensitive than other serological tests as CFT, where the LPS is used as antigen. In view of these findings, the use of r-POMP would allow to adopt appropriate control measures to limit contamination and the spread of infection, since it enables a much earlier detection of C. abortus antibodies.

In contrast, there was a high degree of agreement between both b-ELISAs and the prevalence of anti-C. abortus antibodies in Southern chamois and mouflon. In chamois, this may reflect contact with domestic livestock in the open pastures of the Cantabrian Mountains. The high prevalence found in mouflon is more striking, since these animals were sampled exclusively in fenced estates with no direct contact with domestic ruminants. In fact, the detection of anti-C. abortus antibodies in wildlife hosts from fenced estates where there is no contact with livestock suggests that wild animals alone are capable of maintaining the disease cycle, constituting a reservoir of infection, as shown for other shared diseases such as bovine tuberculosis (Gortazar et al., 2005).

This observational study did not allow conclusions to be drawn on the possible effect of Chlamydiaceae on Spanish wild ungulates. However, the high anti-POMP antibody prevalence recorded in some hosts and sampling regions,
Nonetheless, the high prevalence reported herein should be taken into account as regards livestock and human health. Several studies have shown that strains of *C. abortus* and other *Chlamydiaeae* found in wildlife are similar to those found in domestic animals and in humans (Herrmann et al., 2000; Berri et al., 2004; Hotzel et al., 2004). Hence, our data suggest that wildlife hosts, particularly wild ungulates, need to be considered in the control of diseases such as OEA. Moreover, hunters are thought to be at risk (Deutz et al., 2003), and so could other personnel handling carcasses and raw game meat (Hotzel et al., 2004).

In conclusion, contacts with *Chlamydiaeae*, including *C. abortus* and possibly *C. pecorum*, *C. psittaci* and *C. suis* are frequent among wild ungulates. While possible effects on the wildlife hosts remain unknown, a role of wildlife as a reservoir for *Chlamydiaeae* is clear and its possible importance for animal and public health warrants further studies, including the use of direct assays as PCR, immunohistochemistry or isolation of the pathogen. Also, in which host species, including birds and other wildlife animals, and in which conditions *C. abortus* acts just as a commensal or as a pathogen, should be clarified. In view of this, there is an urgent requirement for standardized serological tests (with greater sensitivity and specificity) for the diagnosis of OEA and other chlamydial infections in wildlife hosts in order to ensure comparability and harmonization among international veterinary laboratories. For that reason, the authors suggest the possibility to include new standardized serological assays, as *C. abortus*-specific ELISAs, in substitution of CFT (reference serological test listed in the OIE Manual) that would contribute to the improvement of OEA serological diagnosis.

Conflict of interest statement

None declared.

Uncited reference

Souriau et al. (1994).

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