Short communication

Spatiotemporal monitoring of selected pathogens in Iberian ibex (*Capra pyrenaica*).

Running title: Monitoring of selected pathogens in Iberian ibex

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Summary

An epidemiological surveillance program was carried out to assess exposure and spatio-temporal patterns of selected pathogens (*Brucella* spp., *Mycobacterium avium* subsp. *paratuberculosis* (MAP), *Mycoplasma agalactiae*, *Pestivirus* and bluetongue virus (BTV)) in Iberian ibex (*Capra pyrenaica*) from Andalusia (southern Spain), the region with the largest population of this species. A total of 602 animals in five distribution areas were sampled during 2010-2012 (P1) and 2013-2015 (P2). The Rose Bengal test (BRT) and complement fixation test (CFT) were used in parallel to detect anti-*Brucella* spp. antibodies. Commercial ELISAs were used to test for antibodies against the other selected pathogens. Sera positive for BTV and *Pestivirus* by ELISA were tested by serum neutralization test (SNT) to identify circulating serotypes/genotypes. The overall seroprevalences were: 0.4% for *Brucella* spp. (2/549; CI95%: 0.1-1.3) (14/555 positive by RBT; 2/564 by CFT), 0.5% for MAP (3/564; CI95%: 0.1-1.5), 5.7% for *M. agalactiae* (30/529; CI95%: 3.9-8.0), 11.1% for *Pestivirus* (58/525; CI95%: 8.5-14.1) and 3.3% for BTV (18/538; CI95%: 2.0-5.2). Significantly higher seropositivity to both *M. agalactiae* and BTV was observed in P1 compared to P2. Spatiotemporal clusters of high seroprevalence were also found for *M. agalactiae* in four of the five sampling areas in 2010, and for BTV in one of five areas in 2012. Specific antibodies against BTV-4, BDV-4 and BVDV-1 were confirmed by SNT. Our results indicate that the Iberian ibex may be considered spillover hosts of *Brucella* spp. and MAP rather than true reservoirs. The prevalence of antibodies against *M. agalactiae* and BTV suggests spatiotemporal variation in the circulation of these pathogens, while *Pestivirus* has a moderately endemic circulation in Iberian ibex populations. Our study highlights the importance of long-term surveillance for a better understanding of the spatiotemporal distribution of shared infectious diseases and providing valuable information to improve control measures at the wildlife-livestock interface.

Keywords: *Capra pyrenaica; Brucella* spp.; *Mycobacterium avium; Mycoplasma agalactiae;* Bluetongue; *Pestivirus*; Surveillance

Introduction

The Iberian ibex (*Capra pyrenaica*) is a wild mountain species endemic to the Iberian Peninsula. The populations of this wild caprine, at about 50,000 individuals, are heterogeneously distributed across southern and eastern regions of Spain and were more recently re-introduced to localized areas of northern Portugal and southern France. Currently, the Iberian ibex is listed as "Least concern" in the Red List of Threatened Species of the International Union Conservation of Nature (IUCN). Whereas this wild caprine has recovered and expanded in some regions, in others, the trend in population size has been decreasing in the last few decades. Major factors accounting for the decline in these populations include loss of genetic diversity, imbalances in age structure and sex ratio, the progressive destruction and fragmentation of their natural habitat, uncontrolled hunting and disease (Acevedo and Cassinello, 2009). While it is known that this species can act as reservoir of certain pathogens, knowledge of its role in the epidemiology of shared diseases remains scarce and tends to be limited to cross-sectional studies focused on relatively small geographical areas (González-Candela et al., 2006; Astorga-Márquez et al., 2014).

The Iberian ibex frequently shares habitats and resources with other sympatric wild and domestic species, which may enhance the cross transmission of pathogens. We hypothesize that the Iberian ibex could play a role in the maintenance of shared infectious diseases of animal health and conservation concern at this wildlife-livestock interface. Surveillance could be a useful tool in these epidemiological scenarios for establishing the spatiotemporal distribution of shared diseases and determining their importance for the health status of Iberian ibex populations and for sympatric species. Our aim therefore was to assess exposure and spatiotemporal patterns of selected pathogens (*Brucella* spp., *Mycobacterium avium* subsp. *paratuberculosis* (MAP), *Mycoplasma agalactiae*, *Pestivirus* and bluetongue virus (BTV)) in Iberian ibex populations from Andalusia (southern Spain), the region with the largest populations of this species, at around 30,000 individuals (CAPMA, 2013).

Materials and methods

Study area and data collection

An active epidemiological surveillance program, coordinated by the Regional Government of Andalusia, was carried out in Andalusia in the five distribution areas of Iberian ibex with more than one individual per km^2 (CAPMA, 2013) (Figure 1). In each of these areas, the most important population nuclei of this species in terms of population abundance (n=11) were selected (Supplementary material. Table S1). All the sampled ibex herds were located in areas where livestock was also present. Domestic ruminants in the study areas are subjected to national brucellosis and bluetongue eradication and control programs (RASVE, 2020a). In addition, *M. agalactiae*, MAP and *Pestivirus* have also been shown to be widespread in livestock in this region (Astorga-Márquez et al., 2014; Paniagua et al., 2016), although compulsory control programs were not implemented during the study period.

Sample collection was divided into two consecutive periods: 2009-2012 (P1) and 2013-2015 (P2). The number of Iberian ibexes sampled per area in each period was chosen to ensure a 95% probability of detecting at least one seropositive animal, assuming a minimum within-sampling-area prevalence of 7.5%. Whenever possible, a minimum of 39 individuals were sampled in each sampling area and study period. Blood samples were collected from 602 ibexes in all: 284 in P1 and 318 in P2. Samples were centrifuged at 400 *g* for 10 min for serum extraction and stored at –20 °C until analysis. Data on age (juveniles: < 2-year-old; sub-adult: 2-6-years-old; adult: > 6-years-old), sex, body condition and presence of clinical signs and lesions were recorded for each animal, whenever possible.

Laboratory analysis

The Rose Bengal test (BRT) and complement fixation test (CFT) were used in parallel to detect anti-*Brucella* spp. antibodies, as previously described (OIE, 2016). Seroprevalence of *Brucella* spp. was established from sera positive by RBT and CFT. Commercial ELISAs were used

to test for antibodies against MAP (*Mycobacterium paratuberculosis* Antibody Test Kit, IDEXX, Spain), *M. agalactiae* (CIVTest Ovis M. agalactiae, Hipra, Spain), BTV (INGEZIM BTV DR 12.BTV.K0, INGENASA, Spain) and *Pestivirus* (INGEZIM Pestivirus Compac 12BVD, INGENASA, Spain), in accordance with the manufacturers' recommendations.

Serum samples positive for BTV and *Pestivirus* by ELISA were subsequently analyzed by serum neutralization test (SNT) to detect specific neutralizing antibodies against the three BTV serotypes that have circulated in Spain in the past decade (RASVE, 2020b): BTV-1 (BTV-1/ALG/2006), BTV-4 (BTV-4/SPA/2004) and BTV-8 (BTV-8/BEL/2006) (for further information see García-Bocanegra et al., 2011). Only samples that showed neutralization (absence of CPE) of 100 $TCID_{50\%}$ of virus at ≥ 1:4 dilutions were considered positive. For *Pestivirus*, SNT was also performed to detect neutralizing antibodies against BDV-4 and BVDV-1 NADL, the main ruminant *Pestivirus* strains circulating in Spain (for further information, Paniagua et al., 2016). Samples were considered positive when neutralization of 100 $TCID_{50\%}$ of virus at ≥ 1:10 dilutions was observed. Given the possibility of cross reactions among BTV serotypes or *Pestivirus* genotypes, the neutralizing immune response observed was considered specific when SNT titers against one virus increased ≥ 2-fold relative to titers against the other viruses. Samples showing ≤ 2-fold differences between SNT titers for BTV or *Pestivirus* were considered positive but inconclusive for serotype or genotype, respectively.

Statistical analysis

Differences in seroprevalence between study periods were analyzed by the Chi-square or Fisher's test, as appropriate. A spatiotemporal statistical scan was performed using the Bernouilli model to detect significant clusters with high seroprevalence at sampling area and year level, for each pathogen. The number of Monte Carlo simulations was set to 1,000 for the cluster scan statistic. The analysis was performed using the "SpatialEpiApp" package in R software. Clusters were considered significant at $p < 0.05$.

Results and discussion

Table 1 shows the distribution of seroprevalences in Iberian ibex in southern Spain according to sampling area, study period, age class and sex. Differences between seroprevalences, sampling areas and sampling periods (Table 2) indicate different epidemiological scenarios for the tested pathogens in this wild caprine in the study area. Wild ruminant species are considered to be spillover hosts of *Brucella* spp. in Spain (Muñoz et al., 2010) and our results support this hypothesis, since only two (0.4%; confidence intervals of 95% (CI 95%): 0.1-1.3) of the 549 analyzed ibexes had antibodies against these zoonotic bacteria (Tables 1 and 2). The seroprevalence obtained is consistent with previous observations in this species (Muñoz et al., 2010; Astorga Márquez et al., 2014) and other wild ruminants in the Iberian Peninsula (Boadella et al., 2010; Muñoz et al., 2010). The study area is officially brucellosis-free in cattle and no cases were reported in this species between 2010 and 2015. Nevertheless, even though the prevalence of brucellosis has decreased sharply in small ruminants in Spain over the past decade, the disease continues to be endemic in the study area (RASVE, 2020a). *Brucella* spp. exposure in naïve ibex populations may have important implications since the Iberian ibex has been shown to be susceptible to infection (Muñoz et al., 2010) and clinical signs and mortality involving *Brucella melitensis* have also been reported in other wild Caprinae, such as the Alpine ibex (*Capra ibex*) (Garin-Bastuji et al., 2014).

The low seroprevalence of MAP (3/564; 0.5%; CI 95%: 0.1-1.5) found in Iberian ibex in our study (Tables 1 and 2) coincides with previous reports in Spain, both in this species (Astorga Márquez et al., 2014) and in red deer (*Cervus elaphus*) (Carta et al., 2012), but contrasts with the higher seropositivity observed in other wild ruminant species in this country (Balseiro et al., 2008; Boadella et al., 2010). A markedly high seroprevalence of MAP has been reported in the study area in livestock species, including the domestic goat (Barrero-Domínguez et al., 2019). Our results suggest that it is unlikely that the Iberian ibex makes a significant contribution to the maintenance of *Brucella* spp. and MAP in Mediterranean ecosystems.

149 *Mycoplasma agalactiae* exposure has previously been detected in Iberian ibex, with
150 prevalence values ranging from 0.9% to 14.3% (González-Candela et al., 2007; Astorga Márquez et
151 al., 2014). The seroprevalence obtained in our study (30/529; 5.7% CI 95%: 3.9-8.0) (Tables 1 and
152 2) falls within these values, although the temporal distribution over the two study periods was not
153 homogeneous. The seroprevalence detected in P1 (10.7%) was significantly higher than in P2
154 (1.1%) ($p < 0.001$) (Table 2). A significant cluster ($p < 0.001$) involving four of the five sampling
155 areas (areas 1, 2, 3 and 5; $p < 0.001$) was also detected in 2010, with seroprevalences ranging from
156 20.0% to 41.2% (Figure 1). The spatiotemporal aggregation observed suggests active circulation of
157 this bacterium in P1, particularly during 2010 (Figure 1). In P2, however, seroprevalence was low
158 (1.1%) and similar to the 0.9% found in captive ibexes sampled in area 1 by Astorga-Márquez et al.
159 (2014) between 2008 and 2009. Temporal variations may be related to *M. agalactiae* exposure at a
160 particular point in time and the short-lived IgG response in this species (Fernández-Aguilar et al.,
161 2017). Environmental changes or interactions with other reservoirs have been shown to be factors
162 involved in spatiotemporal fluctuations of *Mycoplasma* spp. in wild Caprinae species (Fernández-
163 Aguilar et al., 2017).

164 While clinical infections and mortality due to ruminant pestiviruses have been reported in
165 the Pyrenean chamois (Colom-Cadena et al., 2018), there is little information about the impact of
166 these viruses in the other wild caprinae species from the Iberian Peninsule, the Iberian ibex. The
167 overall seroprevalence obtained in the present study (58/525; 11.1% CI 95%: 8.5-14.1) (Tables 1
168 and 2) indicates that *Pestivirus* exposure in this species is higher than previously reported (ranging
169 from 0.0 to 2.3%) (Astorga Márquez et al., 2014; Paniagua et al., 2016). A higher seropositivity
170 was found in those sampling areas with highest population densities (Tables 1 and S1). In this
171 regard, the host density has been previously associated with the pestivirus transmission (Schweizer
172 and Peterhans, 2014). Detection of seropositive animals in all sampling areas (Table 1, Figure 1),
173 similar seropositivity rates in both study periods (12.6% in P1 and 9.6% in P2) (Table 2) and the
174 presence of anti-*Pestivirus* antibodies in 14 juvenile ibexes in both study periods (Supplementary

material. Table S2) provide evidence of a moderately endemic circulation of these viruses in the study area. Since intraspecies and interspecies transmission of *Pestivirus* has been confirmed previously (Ricci et al., 2019), differentiation of *Pestivirus* species by SNT is essential to describe the epidemiology of these viruses. In our study, 21 of the 58 *Pestivirus*-positive samples by ELISA were tested by SNT, resulting in two BVDV-1-positive samples collected in 2011 and 2012, and one BDV-4-positive ibex sample in 2012 (Supplementary material. Table S2). These findings indicate exposure to both BDV-4 and BVDV-1 in the Iberian ibex, which could be of ecological and animal health concern. Further studies are warranted to clarify the impact of *Pestivirus* on the health of Iberian ibex populations.

Since BTV re-emerged in Spain in 2004, 12,455 BTV-1, BTV-4 and BTV-8 outbreaks have been reported in this country, 41.6% of which were detected in the study area (RASVE, 2020b). While most of Andalusia is considered a restriction area for BTV-1 and BTV-4 and vaccination programs against both serotypes have been implemented in domestic ruminants, a total of 24 BTV-1 and 335 BTV-4 outbreaks were reported in livestock in this region during the study period. The Iberian ibex is susceptible and asymptomatic to BTV-1 and BTV-8 infection in experimental conditions (Lorca-Oró et al., 2012) and it has been suggested that wild ruminant species act as natural reservoirs of BTV in Mediterranean ecosystems in Spain, especially in regions where livestock and wild ruminants share the same habitat (García-Bocanegra et al., 2011; Lorca-Oró et al., 2014). The overall seroprevalence obtained in our study (18/538; 3.3%; CI95%: 2.0-5.2) (Tables 1 and 2) is consistent with the 4.0% found in a previous survey carried out between 2006 and 2009 (Lorca-Oró et al., 2011). These findings indicate a limited but endemic circulation of BTV in Iberian ibex populations in Spain in the last two decades. Interestingly, antibodies against BTV-4 in two ibexes sampled in 2012 were confirmed by SNT (Supplementary material. Table S2). The presence of anti-BTV antibodies in juvenile animals in P1 (7.7%; 2/26), as well as the detection of animals seropositive for BTV-4 in area 5 in 2012 (Table 2), which was not a restricted area for this serotype in livestock at the time (MAAMA, 2012), suggest that the Iberian ibex may play a role

in the sylvatic cycle of BTV in the study area. The spatiotemporal distribution of BTV was also observed to be heterogeneous, with significantly higher seropositivity in P1 (6.7%) compared to P2 (0.4%) ($p < 0.001$) and a significant cluster ($p < 0.001$) in area 5 in 2012 (Figure 1). Temporal variations in BTV seroprevalence have also been reported in wild ruminants and may correlate with BT outbreaks in livestock (Lorca-Oró et al., 2014).

Conclusions

In the present long-term study, we show that the main Iberian ibex populations in terms of abundance were exposed to MAP, *Brucella* spp., *M. agalactiae, Pestivirus* and BTV. The low seroprevalences against MAP and *Brucella* spp. suggest that the Iberian ibex acts as a spillover host, rather than a true reservoir of these two pathogens. The spatiotemporal aggregations of *M. agalactiae* and BTV found reveal epidemiological changes in time and space, identifying regions and periods with a higher risk of exposure to these circulating pathogens, which could be useful for risk-based intervention strategies. The seroprevalences against *Pestivirus* found in both study periods suggest that the circulation of these viruses in Iberian ibex populations in southern Spain is moderately endemic. We also show evidence of the circulation of BTV-4, BVDV-1 and BDV-4 in this wild ruminant species during the study period. The results highlight the importance of long-term surveillance efforts in obtaining a better understanding of the spatiotemporal distribution of shared diseases and providing valuable information to improve control measures. Surveillance in Iberian ibex populations could be a complementary way of monitoring the activity of pathogens, particularly *M. agalactiae*, *Pestivirus* and BTV, at the wildlife-livestock interface.

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231 Laboratory (Algete, Madrid) (Ministry of Agriculture, Fisheries and Food, Government of Spain)
232 with the laboratory analyses.

233 **Conflict of Interest Statement**

234 The authors declare that they have no conflict of interest.

235 **Ethical Statement**

236 This study did not involve purposeful killing of animals. The collection of blood samples
237 from animals captured alive was part of the official Management Program for the Iberian ibex in
238 Andalusia, Spain. Samples from dead ibexes were collected from legally hunted animals, by
239 authorised hunters with the correct permits and licenses and with the permission of landowners. All
240 animals were sampled during the hunting season under Spanish and Andalusian legislation.
241 Therefore, no ethical approval was necessary.

242 **Data Availability Statement**

243 The data that support the findings of this study are available from the authors upon
244 reasonable request.

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338 **Figure legend**

339 **Figure 1.** (a) Sampling areas (1-5) and spatiotemporal clusters identified in two (*M. agalactiae* and
340 bluetongue virus) of the selected pathogens in the Iberian ibex. (b) Cluster characteristics and
341 temporal trends of seropositivity of the two pathogens with spatiotemporal clustering.