DR. JOAQUÍN VICENTE (Orcid ID : 0000-0001-8416-3672)

DR. MARIA CRUZ ARNAL (Orcid ID : 0000-0002-6770-3400)

MS. ANA BALSEIRO (Orcid ID : 0000-0002-5121-7264)

DR. FRANCISCO RUIZ-FONS (Orcid ID : 0000-0002-0820-5292)

PROF. CHRISTIAN GORTAZAR (Orcid ID : 0000-0003-0012-4006)

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Distribution of *Pestivirus* exposure in wild ruminants in Spain

Short title: Pestivirus in wild ruminants in Spain

Authors: S. Jiménez-Ruiz[1,2], J. Vicente[1*], I. García-Bocanegra[2], Ó. Cabezón[3,4], M.C. Arnal[5], A. Balseiro[6], F. Ruiz-Fons[1], F. Gómez-Guillamón[7], S. Lázaro[8], F. Escribano[9], P. Acevedo[1], L. Domínguez[10], C. Gortázar[1], D. Fernández de Luco[5], M.A. Risalde[11]

Affiliations:

[1] Grupo de Sanidad y Biotecnología (SaBio). Instituto de Investigación en Recursos Cinegéticos IREC (UCLM-CSIC-JCCM). Universidad de Castilla-la Mancha (UCLM), 13071 Ciudad Real, España.

[2] Departamento de Sanidad Animal. Facultad de Veterinaria. Universidad de Córdoba (UCO), 14014 Córdoba, España.

[3] UAB, Centre de Recerca en Sanitat Animal (IRTA-CReSA), Campus UAB, 08193 Bellaterra, Spain

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[4] Wildlife Conservation Medicine Research Group (WildCoM). Departament de Medicina i Cirurgia. Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, España.

[5] Departamento de Patología Animal. Facultad de Veterinaria. Universidad de Zaragoza (UNIZAR), 50013 Zaragoza, España.

[6] Departamento de Sanidad Animal, Facultad de Veterinaria. Universidad de León (ULE), 24071 León, España.

[7] Programa de Vigilancia Epidemiológica de la Fauna Silvestre. Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible (CAGPDS). Junta de Andalucía, 29010 Málaga, España.

[8] Unidad Analítica Regional de Sanidad Animal. Consejería de Agricultura, Medio Ambiente y Desarrollo Rural de Castilla-la Mancha, 45600 Talavera de la Reina, España.

[9] Programa de Conservación y Recuperación de Fauna Silvestre, Dirección General del Medio Natural de la Región de Murcia, 30008 Murcia, España.

[10] Centro de Vigilancia Sanitaria Veterinaria (VISAVET). Universidad Complutense de Madrid (UCM), 28040 Madrid, España.

[11] Departamento de Anatomía y Anatomía Patológica Comparadas y Toxicología. Facultad de Veterinaria. Universidad de Córdoba (UCO), 14014 Córdoba, España.

*** Corresponding author**: Joaquín Vicente. *E-mail address*: joaquin.vicente@uclm.es

Summary

A large-scale study was carried out to determine the prevalence of antibodies against *Pestivirus* species in wild ruminants and describe their spatial variation in mainland Spain. Serum samples of 1,874 wild ruminants from different regions of this country were collected between the years 2000 and 2017. A total of 6.6% (123/1,874) animals showed antibodies against *Pestivirus* by both blocking ELISA (bELISA) and virus neutralization tests (VNT). The prevalence of antibodies against pestiviruses was different both among species and regions. Seroprevalence by species was 30.0% (75/250) in Southern chamois (*Rupicapra pyrenaica*), 7.0% (25/357) in fallow deer (*Dama dama*), 2.5% (10/401) in red deer (*Cervus elaphus*), 2.4% (8/330) in Iberian wild goat (*Capra pyrenaica*), 1.1% (4/369) in roe deer (*Capreolus capreolus*) and 0.8% (1/130) in mouflon (*Ovis aries musimon*), not detecting seropositivity (0/37) in Barbary sheep (*Ammotragus lervia*). The results confirm that exposure to pestiviruses was detected throughout mainland Spain, with significantly higher seroprevalence in Northern regions associated with presence of Southern chamois. This indicates an endemic circulation of pestiviruses in Southern chamois and a limited circulation of these viruses in the remaining wild ruminant species during the last two decades, thus suggesting that non-chamois species are not true *Pestivirus* reservoirs in Spain. Nonetheless, the high spatial spread of these viruses points out that new epidemic outbreaks in naïve wild ruminant populations or transmission to livestock may occur, evidencing the usefulness of monitoring pestiviruses in wild ruminants, especially at the wildlife-livestock interface.

Keywords: border disease virus; bovine viral diarrhea virus; ELISA; virus neutralization tests; wild ruminants.

Introduction

The genus *Pestivirus* (family *Flaviviridae*) currently comprises eleven recognized species (*Pestivirus* A to K) (ICTV, 2018; Smith et al., 2017). These viruses can infect a wide range of ungulates including domestic and wild swine as well as both domestic and wild ruminant species (Becher et al., 1997; Vilček & Nettleton, 2006). Pestiviruses are worldwide distributed and have a significant economic impact on livestock productions (Schweizer & Peterhans, 2014). *Pestivirus* A and B [formerly named Bovine Viral Diarrhoea Virus-1 (BVD-1) and BVD-2], and *Pestivirus* D [formerly named Border Disease Virus (BDV)] are among the main causatives of reproductive disorders in cattle and sheep, and antibody prevalence scales up to 85% and 50% in Europe, respectively (Houe, 1999; Nettleton, 2000). In Spain, *Pestivirus* A and D represent the main circulating pestiviruses in livestock and wildlife, being widespread in domestic ruminant species (Berriatua et al., 2006; Diéguez et al., 2017), with seroprevalences ranging from 17.1% in the Northwest to 65.6% in the South of the country (Arnaiz et al., 2012). *Pestivirus* B has been sporadically isolated from livestock, especially in Northwestern and Central Spain (Factor et al., 2016; Elvira-Partida et al., 2017). The susceptibility to infection by *Pestivirus* A and D has also been confirmed in several free-ranging wild ruminant species in this country (Colom-Cadena et al., 2018; Factor et al., 2016; Fernández-Aguilar et al., 2016; Rodríguez-Prieto et al., 2016), where severe outbreaks of a disease associated with infection by a *Pestivirus* D genotype (BDV-4 strain) have been reported in Southern chamois (*Rupicapra pyrenaica*) (Arnal et al., 2004; Luzzago et al., 2016; Marco et al., 2007). However, to date, the dynamics of viruses of the genus *Pestivirus* in the remaining European wild ruminant species and the putative role that these viruses play as disease agents are unknown, mainly because of the limited evidence from experimental infections (Cabezón et al., 2011; Colom-Cadena et al., 2019; Martin et al., 2013).

Populations of wild ruminants have grown and spatially expanded throughout Europe over the last decades, leading to increase the frequency of interactions with sympatric species and causing variable consequences (Acevedo et al., 2011; Apollonio et al., 2011). In mainland Spain, although interaction scenarios are extremely variable due to the wide array of distribution ranges and population densities of wild ruminants, most of wild ruminant populations share pastures and other natural resources with extensively reared livestock (Carrasco-Garcia et al., 2016; Colom-Cadena et al., 2018). In this wildlife-livestock interface, the risk of pestiviruses transmission could be favored by the ability of *Pestivirus* A, B and D to cross-infect different hosts species

(Richomme et al., 2005) and generate immunotolerant animals, conferring them a high capacity to persist in these complex host communities (Vilček & Nettleton, 2006). Thus, though in general the disease related to pestiviruses is uncommon in wildlife, several studies coincided that they should be considered as a threat to the health of wildlife populations and control measures should be considered to reduce their transmission, especially in epidemiological scenarios with wildlife-livestock interface (Colom-Cadena et al., 2018; Marco et al., 2007; Nelson et al., 2016). In this context, the identification of the potential reservoirs for pestiviruses is a key step in the control of such shared pathogens. Hence, the aim of the present study was to determine the prevalence of antibodies against *Pestivirus* and their spatial distribution in the different wild ruminant species in Spain, a country where ruminant livestock is endemically affected.

Materials and methods

Study design and sampling strategy

The study area, mainland Spain, accounts for almost the 85% of the Iberian Peninsula. Based on landscape structure, major ecosystems, game management practices and socio-political aspects, the Spanish Wildlife Disease Surveillance Scheme splits mainland Spain into five different bioregions (BRs; Figure 1) sharing similar epidemiological features (PNVFS, 2019). The BR1 comprises the Northern areas of temperate Atlantic climate with almost no game management meanwhile the remaining BRs present a Mediterranean climate with an increasing drought gradient from BR2 to BR4. In the Mediterranean BRs, game management is not the norm except for BR3 and the Southwest of BR5, where the highly productive savannah-like or oak forest landscapes are frequently profited for large game production. Mountain habitats are more dominant in BRs 1, 2 and 5 while cereal plains are predominant in BR4. This zoning has been previously exploited to facilitate disease surveillance efforts in wild ungulates in Spain (García-Bocanegra et al., 2016; Lorca-Oró et al., 2014; Muñoz et al., 2010).

The sample size per BR was estimated assuming a Pestivirus seroprevalence of 50% (which provides the highest sample size when prevalence is unknown), with a 95% confidence level and an accepted error of 5% (Thrusfield, 2018). Within each BR, sampling was stratified by species according to their distribution range and population density representativeness, and the number of individuals was chosen to ensure a 95% probability of detecting at least one

seropositive animal for an assumed minimum prevalence of 5%. From each sampling site, that is hunting states or game reserves (n= 93; Figure 1) selected by simple random sampling throughout the study area, the animals (15-20 whenever possible) were also randomly sampled.

A total of 1,874 free-ranging wild ruminants, including 401 red deer (*Cervus elaphus*), 369 roe deer (*Capreolus capreolus*), 357 fallow deer (*Dama dama*), 330 Iberian wild goats (*Capra pyrenaica*), 250 Southern chamois, 130 mouflon (*Ovis aries musimon*) and 37 Barbary sheep (*Ammotragus lervia*), were sampled between 2000 and 2017. The animals were grouped according to the BR of origin (Table 1). Blood samples were obtained by puncture of the heart, the thoracic cavity or the endocranial venous sinuses (Jiménez-Ruiz et al., 2016) at the field necropsies or health veterinary inspections. Upon arrival to the laboratory, sera were obtained after centrifugation, collected into sterile tubes and kept frozen at -20º C until serological analyses.

Laboratorial analyses

All serum samples were tested for the presence of *Pestivirus* specific antibodies against the p80/NS3 antigen using a commercial blocking ELISA (bELISA; INGENASA®, Madrid, Spain). Sensitivity (Se) and specificity (Sp) for this ELISA are 99.5% and 92.0%, respectively, according to manufacturer-specified values (Rodríguez-Prieto et al., 2016). Positive and doubtful sera were then tested in serial using another commercial bELISA (IDEXX®, Liebefeld-Bern, Switzerland). For this bELISA, Se and Sp values provided by manufacturers are 96.3% and 95.0%, respectively (Rodríguez-Prieto et al., 2016).

In addition, doubtful or positive sera to bELISA-INGENASA were also further tested by comparative virus neutralization test (VNT) using isolates belonging to *Pestivirus* A (BVDV-1-NADL strain; GenBank Acc. No. M31182) and *Pestivirus* D (BDV-4-BDV-pig-SP-2007 strain; Genbank Acc. No. HF567456), as was previously described (OIE, 2019). *Pestivirus* A and D isolates were chosen for this analysis since they have been the main circulating *Pestivirus* species in livestock and wildlife in Spain between 2003 and 2018 (Colom-Cadena et al., 2018; Diéguez et al., 2017; Factor et al., 2016; Hurtado et al., 2003, 2004; Luzzago et al., 2016; Marco et al., 2007; Paniagua et al., 2016; Valdazo-González et al., 2006, 2007). Neutralizing antibody titres were expressed as the reciprocal of the highest dilution that neutralized 100 tissue culture infective doses (100 $TCID_{50}$) in all cultures, calculated according to the method described by Reed and Muench (1938). Neutralization was monitored by the immunoperoxidase monolayer assay (IPMA) (OIE, 2019). Sera that showed neutralization at dilutions ≥ 1:10 were designated as positive,

considering antibodies specific of a *Pestivirus* genotype only when titres were at least three times higher than those obtained for the other genotypes (Cabezón et al., 2011; Fernández-Sirera et al., 2012a, 2012b; Fernández-Aguilar et al., 2016).

Statistical analyses

Differences in the serostatus of *Pestivirus* at individual level (response variable: positive/negative) in relation to host species (seven levels) and the BR (five levels) of origin (fixed independent factors), were analysed by a generalized linear mixed model (GLMM) with a binomial error and a logit link function. The sampling site (hunting state or game reserve) was included as a random effect factor, to account for the samples obtained from the same sampling site. Similar GLMMs were run independently for each species to assess variations among BRs on *Pestivirus* seroprevalence, and Tukey' post-hoc tests were also conducted to assess differences between pairs of BRs. Statistical analyses were carried out using SPSS V24 software (IBM Corp., Armonk, NY, USA).

Results

A total of 149 [8.0%; 95% confidence interval (95%CI): 6.7-9.2] out of the 1,874 sera collected from wild ruminants were positive by bELISA-INGENASA. However, some discrepancies were found between comparative methods regarding the positive bELISA-INGENASA results (see Supplementary file). Of them, seven sera were considered as false positives because they were negative by both bELISA-IDEXX and VNT. Nine samples could not be tested with any of the last two techniques due to limited available volume of sample and 12 could not be examined by VNT because of serum cytotoxicity. Moreover, because of their limited volumes, 16 sera were only used for serological confirmation by VNT (gold-standard method), but not for analysis by bELISA-IDEXX. The two doubtful bELISA-INGENASA sera resulted finally positive by both bELISA-IDEXX and VNT. Thus, seroprevalence to *Pestivirus* was determined from samples positive to both bELISA-INGENASA and VNT.

The overall prevalence of antibodies against *Pestivirus* in wild ruminants in Spain was 6.6% (123/1,874; 95%CI: 5.4-7.7). Seroprevalence results by species and BRs are detailed in Table 1. Antibody titres more than threefold higher against BVDV-1-NADL (*Pestivirus* A) and

BDV-4-BDV-pig-SP-2007 (*Pestivirus* D) strains were shown by VNT in six and 12 animals, respectively. No differences three times higher in titres between the two *Pestivirus* strains used in the VNT were detected in the remaining seropositive individuals (105/123=85.4%), even though 75.2% (79/105) of them showed VNT titres ≥80 for one or both pestiviruses (see Supplementary file). Seropositivity to *Pestivirus* A and D strains was detected in three and four of the five BRs, respectively. The spatial distribution of pestiviruses in wild ruminants in mainland Spain is shown in Figure 1.

Overall, statistically significant differences in pestiviruses exposure were found among species ($F_{6,1863}$= 4.722; P< 0.001) as well as among BRs ($F_{4,1863}$= 2.616; P= 0.034). Independently of BR, a significantly higher seroprevalence was detected in Southern chamois and fallow deer when compared with all the remaining wild ruminants. Independently of species, a lower seroprevalence was obtained in BR3 and BR4 when compared with the other BRs (Figure 2). The GLMM testing the effect of BR independently for each species evidenced significant spatial variations for Iberian wild goat and Southern chamois seroprevalences, but not for the remaining wild ruminant species (Table 2).

Discussion

The role of wild ruminants in the epidemiology of *Pestivirus* has been relatively poorly studied in Europe since most surveys have been focused specifically on few species and/or conducted at regional levels. To the best of our knowledge, this is the first large-scale serosurvey of pestiviruses comprising all the species of free-ranging wild ruminants present in a country taking into account clearly delimitated epidemiological units. The results obtained provide an accurate picture of the prevalence of exposure and spatial variation of these viruses throughout mainland Spain.

The prevalence of antibodies against *Pestivirus* detected in the present study was heterogeneous across species and BRs (Table 1). The significantly higher seropositivity found in Southern chamois (30.0%), especially against the BDV-4 strain used, is consistent with the epizootic of BDV-4 genotype outbreaks that affected this species during the study period (Arnal et al., 2004; Marco et al., 2007). The exposure to pestiviruses in chamois species has been frequently

reported, with evidences of high prevalence of antibodies in both the Southern (Fernández-Sirera et al., 2012a; 2012b; Marco et al., 2011) and the Alpine chamois (*Rupicapra rupicapra*) (up to 73.4% and 38.7%, respectively) if compared to other European wild ruminants (Casaubon et al., 2012; Martin et al., 2015; Olde Riekerink et al., 2005). However, differences in seroprevalence have also been reported among regions for Southern chamois in Spain (Falconi et al., 2010; Fernández-Aguilar et al., 2016), consistently with the lower seropositivity found in Cantabrian chamois (*Rupicapra pyrenaica parva*) from BR1 in comparison to those obtained in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) from BR2 and BR5 in the present study. Our results suggest a widespread circulation of BDV-4 genotype in Southern chamois and suggest a potential risk of infection for sympatric species, especially in the Pyrenees (Colom-Cadena et al., 2018).

The seroprevalence detected in fallow deer (7.0%) was also significantly higher than the one obtained for the remaining non-chamois wild ruminant species (Figure 2), although lower than those observed in previous studies conducted in fallow deer in the Italian Tuscany region, in red deer in Central Spain and in mouflon in the French south Alps against *Pestivirus* A (58.0% in fallow deer and 19.5% in red deer) (Giovannini et al., 1988; Rodríguez-Prieto et al., 2016) and *Pestivirus* D (61.1% in mouflon) (Martin et al., 2011). The low exposure to pestiviruses in the other species surveyed in the present study is in line with the absence or very low circulation of these viruses reported in red deer (Paniagua et al., 2016), Barbary sheep (Candela et al., 2009), mouflon (López-Olvera et al., 2009), Iberian wild goat (Astorga-Márquez et al., 2014) and roe deer (Morrondo et al., 2017) in other Spanish regions and in other European countries (Besi et al., 2018; Casaubon et al., 2012; Fernández-Sirera et al., 2012b). These findings suggest that wild ruminants other than Southern chamois do not constitute a significant potential source for pestiviruses interspecies transmission. Nonetheless, these results cannot be underestimated because the introduction of pestiviruses into naïve wild ruminant populations may lead to unexpected consequences (Vilček & Nettleton, 2006).

A widespread exposure to pestiviruses was found for mainland Spain since seropositivity was detected across BRs. However, the spatial distribution of pestiviruses was not homogenous, with a significantly higher seroprevalence in both Atlantic Spain (BRs 1 and 2) and the Mediterranean basin (BR5). These regions perfectly match with those showing statistical differences for particular species in the post-hoc tests (Table 2). This situation, joined to the high exposure to pestiviruses of livestock species in Spain (Arnaiz et al., 2012; Diéguez et al., 2017),

suggest that non-chamois wild ruminants act as spillover hosts rather than true reservoirs of *Pestivirus*. In this sense, most studies carried out in domestic species were focused on dairy husbandry, so that the distribution patterns and the infection status by pestiviruses in other livestock production systems are still little known in several regions of mainland Spain. Further research is necessary in order to elucidate the mechanisms responsible for the maintenance and transmission of pestiviruses, especially at the wildlife-domestic interface on extensive production systems.

As suggested by the higher reactivity observed against the BVDV-1-NADL (*Pestivirus* A) and the BDV-4 (*Pestivirus* D) strains, it seems likely that the BVDV-1 genotype in BRs 1, 2 and 4, and the BDV-4 genotype in all BRs except BR4 are the causative agents. Both genotypes seemed to be circulating in the three species populations with the highest seroprevalence to pestiviruses: Southern chamois, fallow deer and red deer. However, exposure to other *Pestivirus* genotypes in wildlife in Spain, although unlikely, cannot be ruled out. Our results strongly suggest a widespread distribution of *Pestivirus* A and D genotypes in mainland Spain, concurring with previously reported studies (Colom-Cadena et al., 2018; Diéguez et al., 2017; Factor et al., 2016; Hurtado et al., 2003, 2004; Luzzago et al., 2016; Marco et al., 2007; Paniagua et al., 2016; Valdazo-González et al., 2006, 2007). However, although the main *Pestivirus* A and D genotypes detected in Spain were analysed in this study, other potential circulating pestiviruses, like the recently detected *Pestivirus* B in domestic ruminants in Spain (Aduriz et al., 2015; Eiras et al., 2019; Elvira-Partida et al., 2017; Factor et al., 2016), the Tunisian-like *Pestivirus* detected in small domestic ruminants in Tunisia, Italy and France (Ciulli et al., 2017; Martin et al., 2015; Thabti et al., 2005), or the novel ovine Italian *Pestivirus* found in ovine and wild boar in Italy (Casciari et al., 2020; Sozzi et al., 2019), cannot be ruled out.

We acknowledge that this study has some limitations. The first bELISA screening test gave as positives some sera classified as negative or non-conclusive by the gold-standard confirmatory test (VNT), which may have led to a light underestimation of the overall seroprevalence of pestiviruses in wild ruminants in Spain. The high cross-reactivity in the VNT response of wild ruminants to *Pestivirus* A and D, the non-dilution of sera to lower concentrations and the impossibility to include homologous circulating *Pestivirus* strains from the wildlife populations studied, could have contributed to the failure of VNT elucidating the genotypes to what wild ruminants had been exposed to.

Conclusions

Except for the Pyrenean chamois, the results obtained suggest a limited implication of wild ruminants as natural reservoirs of pestiviruses in the Iberian Peninsula. Nevertheless, the widespread distribution of pestiviruses across BRs affecting most of the wild ruminant species present in Spain along with the high interspecific infection capacity of *Pestivirus*, evidence the need of monitoring pestiviruses in wild ruminants in order to detect early circulation in unexposed populations and establish specific control strategies in sympatric livestock species. This study provides a good basis on which further studies can be designed targeting particular species, regions or management situations that are necessary to better understand the epidemiological role of each wild ruminant species.

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Conflict of interest

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Data Availability Statement

Data available on request from the authors.

Ethical approval

All the animals were legally hunted under Spanish and EU legislation by hunters with appropriate permits during the hunting season (October to February) or culled within population control programs of game reserves. This study did not involve purposeful killing of animals and the blood samples were not collected specifically for this study. Protocols, amendments and other resources were done according to the guidelines approved by each Autonomous government following the R.D.1201/2005 of the Ministry of Presidency of Spain. No ethical approval was deemed necessary. The collection of blood samples was being performed for routine procedures before the design of this study in compliance with the Ethical Principles in Animal Research.

Orcid

Saúl Jiménez-Ruiz: https://orcid.org/0000-0003-2090-9353
Joaquín Vicente: http://orcid.org/0000-0001-8416-3672
Ignacio García-Bocanegra: https://orcid.org/0000-0003-3388-2604
Óscar Cabezón: https://orcid.org/0000-0001-7543-8371
María Cruz Arnal: https://orcid.org/0000-0002-6770-3400
Ana Balseiro: https://orcid.org/0000-0002-5121-7264
Francisco Ruiz-Fons: https://orcid.org/0000-0002-0820-5292
Pelayo Acevedo: https://orcid.org/0000-0002-3509-7696
Lucas Domínguez: https://orcid.org/0000-0002-4697-7832
Christian Gortázar: https://orcid.org/0000-0003-0012-4006
María A. Risalde: https://orcid.org/0000-0001-5105-4824

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Figure legends

Figure 1. Spatial distribution of pestiviruses exposure in wild ruminants in Spain (bioregions, BR1-5). Symbols represent the sampling sites included (n= 93). VNT: virus neutralization tests.

Figure 2. Results of the generalized linear mixed models (GLMM) showing the differences in pestiviruses seroprevalence in Spain according to species (A) and bioregions (B). Y-axes are represented in a logit scale and therefore they are only informative for comparative purposes. BS: Barbary sheep; FD: fallow deer; IWG: Iberian wild goat; M: mouflon; RD: red deer; RoD: roe deer; SCh: Southern chamois; BR: bioregion.

Table legends

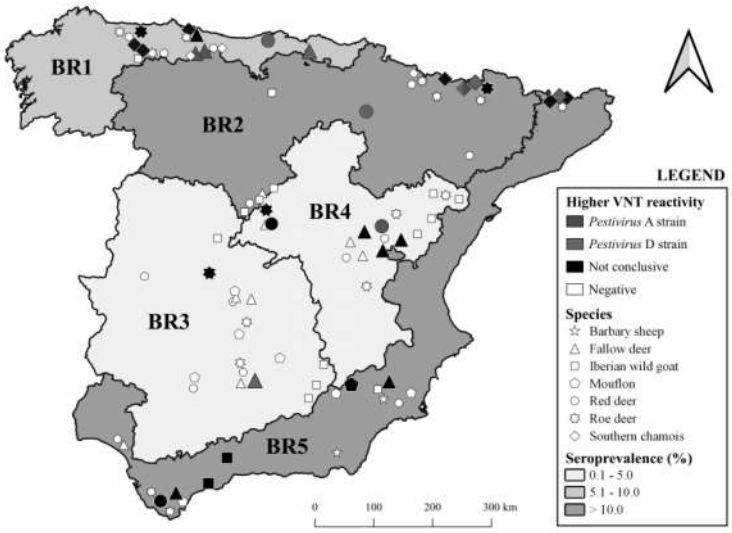
Table 1. Sample size and serological results of pestiviruses circulation in wild ruminant species in Spain (bioregions 1-5).

		Bioregion (BR)†						Seroprevalence (95% CI)	Virus neutralization	
		BR1	BR2	BR3	BR4	BR5	TOTAL (n)		*Pest.* A strain	*Pest.* D strain
Species	Barbary sheep	0	0	0	0	37|0	37	0	0	0
	Fallow deer	80|18.8 (10.2-27.3)	20|0	80|2.5 (0-5.9)	122|4.1 (0.6-7.6)	55|5.5 (0-11.5)	357	7.0 (4.4-9.7)	4/25	3/25
	Iberian wild goat	21|0	80|0	77|0	100|0	52|15.4 (5.6-25.2)	330	2.4 (0.8-4.1)	0/8	0/8
	Mouflon	0	0	80|0	0	50|2.0 (0-5.9)	130	0.8 (0-2.3)	0/1	0/1
	Red deer	80|3.8 (0-7.9)	80|1.3 (0-3.7)	80|0	100|3.0 (0-6.3)	61|4.9 (0-10.3)	401	2.5 (1.0-4.0)	1/10	4/10
	Roe deer	80|1.3 (0-3.7)	83|1.2 (0-3.6)	85|1.2 (0-3.5)	66|1.5 (0-4.5)	55|0	369	1.1 (0-2.1)	0/4	0/4
	Southern chamois	107|7.5 (2.5-12.5)	81|43.2 (32.4-54.0)	0	0	62|51.6 (39.2-64.1)	250	30.0 (24.3-35.7)	1/75	5/75
	TOTAL (n)	368	344	402	388	372	1874	6.6 (5.4-7.7)	6/123	12/123
Seroprevalence (95%CI)		7.3 (4.7-10.0)	10.8 (7.5-14.0)	0.7 (0-1.6)	2.3 (0.8-3.8)	12.6 (9.3-16.0)	6.6 (5.4-7.7)			
***Pest.* A strain positive (n)**		4/27	1/37	0/3	1/9	0/47	6/123			
***Pest.* D strain positive (n)**		4/27	4/37	2/3	0/9	2/47	12/123			

† Sample size (n) | seroprevalence (95%CI).

Table 2. Results of the generalized linear mixed model (GLMM) Tukey' post-hoc tests assessing the differences in pestiviruses seroprevalence among bioregions (BRs) for each studied species in Spain. Different capital letters represent statistical differences among BRs by species in pestiviruses seroprevalence.

	BR1	BR2	BR3	BR4	BR5
Barbary sheep					A
Fallow deer	A	A	A	A	A
Iberian wild goat	AB	A	A	A	B
Mouflon			A		A
Red deer	A	A	A	A	A
Roe deer	A	A	A	A	A
Southern chamois	A	AB			B



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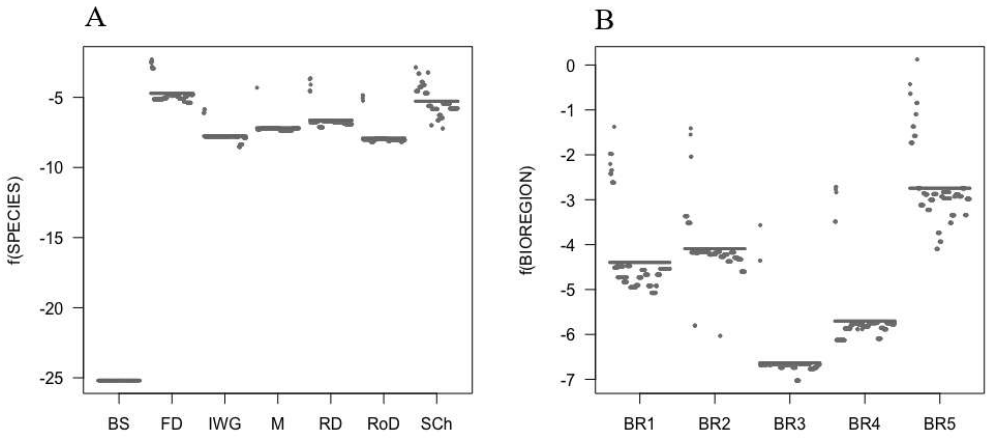

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