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Article type : Original Article

Monitoring of bluetongue virus in zoo animals in Spain, 2007-2019

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Abstract

Bluetongue (BT) is an emerging and re-emerging communicable vector-borne disease of animal health concern. A serosurvey was performed to assess exposure to BT virus (BTV) in zoo animals in Spain and to determine the dynamics of seropositivity in longitudinally-sampled individuals during the study period. Serum samples were collected from 241 zoo animals belonging to 71 different species in five urban zoos (A-E) in Spain between 2007 and 2019. Twenty-four of these animals were longitudinally surveyed at three of the sampled zoos (zoos B, C and E) during the study period. Anti-BTV antibodies were found in 46 (19.1%; 95%CI: 14.1-24.1) of the 241 captive animals analyzed by commercial ELISA. Multiple logistic regression analysis identified zoo B as a risk factor potentially associated with BTV exposure in zoo animals in Spain. A virus neutralization test confirmed specific antibodies against BTV-1 and BTV-4 in 25 (10.7%; 95%CI: 6.7-14.6) and five (3.0%; 95%CI: 0.3-4.0) animals, respectively. Two of the 24 longitudinally- sampled individuals (one African elephant (*Loxodanta africana*) and one aoudad (*Ammotragus lervia*)) showed anti-BTV antibodies at all samplings, whereas seroconversions were detected in one mouflon (*Ovis aries musimon*) in 2016, and one Asian elephant (*Elephas maximus*) in 2019. To the best of the authors' knowledge, this is the first large-scale survey on BTV conducted in both artiodactyl and non-artiodactyl zoo species worldwide. The results confirm BTV exposure in urban zoo parks in Spain, which could be of animal health and conservation concern. Circulation of BTV was detected in yearling animals in years when there were no reports of BTV outbreaks in livestock. Surveillance in artiodactyl and non-artiodactyl zoo species could be a valuable tool for epidemiological monitoring of BTV.

Keywords: *Bluetongue, BTV-1, BTV-4, Vector-borne, Surveillance, Wildlife, Captive, Animal health*.

Introduction

Bluetongue virus (BTV) (genus *Orbivirus*: family *Reoviridae*) is an emerging and re-emerging vector-borne RNA virus mainly transmitted by blood-sucking midges of the genus *Culicoides* (Diptera, family *Ceratopogonidae*) (Rao et al., 2017). This virus has recently been grouped into 24 "classical" (BTV-1 to BTV-24) and eleven atypical serotypes (BTV-25 to BTV-35). Bluetongue (BT) in domestic ruminant species is subject to compulsory eradication in the European Union (Council Directive 2000/75/EC) since it is responsible for significant socioeconomic losses associated with international trade restrictions, loss of production, culling of infected animals and vaccination campaigns. Despite the efforts employed to eradicate the disease, a number of different BTV serotypes, including BTV-1, BTV-2, BTV-3, BTV-4, BTV-8 and BTV-16, are currently circulating in Europe (EC, 2020).

It has previously been suggested that wildlife could play a role in the maintenance of BTV in the Mediterranean Basin (García-Bocanegra et al., 2011; Rossi et al., 2013; Ruiz-Fons, Sánchez-Matamoros, Gortázar, & Sánchez-Vizcaíno, 2014). Both free-ranging and captive wild animals, including carnivores, rodents, perissodactyls and different artiodactyl species, have been shown to be susceptible to BTV infection (EFSA, 2017). Zoo animals are considered useful for surveillance of vector-borne pathogens (Caballero-Gómez et al., 2020, 2021; McNamara, 2007; Robinette, Saffran, Ruple, & Deem, 2017; Sánchez-Romano et al., 2019) due to the large number and wide variety of animal species present in zoological parks and the accessibility of samples during monitoring and management protocols.

Previous studies have assessed BTV circulation in zoo parks worldwide, providing evidence of BTV exposure in different captive zoo animals (Hourrigan & Klingsporn, 1975; Frölich et al., 2005; Morikawa et al., 2018; Sanchez-Romano et al., 2019; Sanderson et al., 2010). Clinical disease and mortality caused by BTV infection have also been reported in several zoo species, including threatened species (Mauroy et al., 2008; Mazzoni-Baldini et al., 2018). However, the role of zoo animals in the epidemiology of this virus is still poorly understood. In Spain, a country with endemic circulation of two BTV serotypes (BTV-1 and BTV-4) (RASVE, 2020) during the last decade, no studies have been carried out on wild species kept in zoos. The aims of the present study therefore

were: i) to assess BTV circulation in captive zoo animals in Spain and ii) to determine the dynamics of seropositivity in longitudinally-sampled individuals during the study period.

Material and Methods

A total of 241 zoo animals belonging to 71 different species were sampled at five urban zoos (A-E) in Spain between 2007 and 2019 (Table S1). Samples were obtained from serum banks or from individuals subject to surgical interventions, health programs or medical check-ups during the study period. Serum samples were kept frozen at -20 ºC until ready to be taken to the Animal Health Department at the University of Cordoba (Spain) for serological analysis. Epidemiological information, including species, age, health status, zoo provenance and sampling date, was gathered from each animal, whenever possible. In addition, 24 of the 241 analyzed animals were longitudinally sampled (between two and ten samplings per animal) at three of the surveyed zoos (zoos B, C and E). Longitudinally-sampled animals were not translocated during the study period.

The presence of antibodies against the major core protein VP7 of BTV was determined using a commercial double recognition immunoenzymatic assay (ELISA; INGEZIM BTV DR 12.BTV.K0, INGENASA, Spain), INGENASA, Madrid, Spain) according to the manufacturer's instructions. Samples results were expressed as an ELISA percentage (E%), calculated using the following formula: [E% = (sample OD/(0.15 x mean OD of positive controls) × 100]. The positive threshold values were set as suggested by the manufacturers: sera with E% > 100 were considered positive. Whenever possible, ELISA positive sera were tested for the detection of specific antibodies against BTV-1 and BTV-4 by the virus neutralization test (VNT), as previously described (OIE, 2018). Briefly, serum samples were inactivated at 56°C for 30 min and serially diluted (1:2-1:256) in MEM (Eagle's minimum essential medium). Next, sera were mixed with 100 $TCID_{50}$ (50% tissue culture infective doses) of each reference strain of BTV-1 (BTV-1/ALG/2006) and BTV-4 (BTV-4/SPA/2004) and incubated in plates at 37°C for 1 h 30 min. Finally, 100 µL of Vero E6 cell suspension (1.5×10^4 cells/well) were added to cell growth media (MEM supplemented with 15% fetal calf serum, 300 µg L-glutamine/mL, 300 U penicillin/mL and 300 µg streptomycin/mL). The mixture was further incubated at 37ºC for 6-7 days until a cytopathic effect (CPE) developed in control wells containing 100 $TCID_{50}$ of virus and no serum. Samples were considered positive only if

they showed neutralization (absence of CPE) at dilutions ≥1:4 (Lorca-Oró et al., 2012). Controls for cytotoxicity in the absence of virus were included for each sample at a 1:2 dilution. The neutralizing immune response observed was considered specific when VNT titers for a given serotype were ≥ 4-fold higher than titers obtained for the other serotype. Samples showing ≤ 2-fold differences between VNT titers were considered positive but inconclusive for serotype and were, therefore, excluded in order to calculate seroprevalence by serotype.

The overall prevalence of antibodies was estimated by dividing the number of positive animals detected by ELISA by the total number of animals tested, using two-sided exact binomial 95% confidence intervals (95%CI). The distribution of seroprevalence according to sampling period (2007-2013, 2014-2016 and 2017-2019), zoo provenance and Order is shown in Table 1.

Results

A total of 46 (19.1%; 95%CI: 14.1-24.1) of the 241 zoo animals showed seropositivity for BTV by ELISA (Table 1; Supplementary Material, Table S1). Four of the five orders presented at least one seropositive animal, with frequency of seropositivity ranging from 9.8% in the order *Carnivora* to 60.0% in the order *Proboscidea*. Eighteen of the 71 zoo species analyzed showed anti-BTV antibodies (Table S1). Clinical symptoms compatible with BTV infection were not observed at any of the five zoological parks analyzed during the study period. Seropositive animals were detected in four of the five sampled zoos, with within-zoo seropositivity ranging between 6.5% and 38.2% in zoos C and B, respectively (Table 1, Figure 1). Seropositivity was found every year during the study period (2007-2019), including anti-BTV antibodies detected in three yearlings (≤ 12 months old) (two mouflons (*Ovis aries musimon*) and one aoudad (*Ammotragus lervia*)) in zoo B sampled in 2014, 2016 and 2017 (Table 2).

Thirty-nine of the 46 ELISA-positive animals were analyzed by VNT based on the volume of sera available. Neutralizing antibodies against BTV were confirmed in 33 of these individuals (Table 2). Specific antibodies against BTV-1 were detected in 25 animals (25/234; 10.7%; 95%CI: 6.7-14.6), and five animals (5/234; 3.0%; 95%CI: 0.3-4.0) showed anti-BTV-4 antibodies. Potential co-occurrences (neutralizing antibodies against BTV-1 and BTV-4 in the same individual but ≤ 2-fold differences between titers) were found in one banteng (*Bos javanicus*), one Eurasian lynx (*Lynx lynx*)

and one mouflon (Table 2). Specific anti-BTV-1 antibodies were detected at the four seropositive zoos, and in 2008 and every year during the periods 2010-2012 and 2014-2018. By contrast, neutralizing antibodies against BTV-4 were observed only in zoo animals from zoo B, and every year between 2014 and 2018 (Figure 1; Tables 1 and 2).

Twenty of the 24 longitudinally sampled zoo animals tested negative by ELISA at all samplings (Table 3). Two individuals (one African elephant (*Loxodonta africana*) and one aoudad) remained seropositive throughout the study period. Neutralizing antibodies against BTV-1 were confirmed by VNT in the ELISA-positive aoudad, which was sampled in 2017 and 2018 at zoo B. At this zoo park, one Asian elephant (*Elephas maximus*) was seronegative in May 2018 but showed seropositivity in January and February 2019 by ELISA, although the serotype could not be determined. One seronegative mouflon sampled in January 2016 at the same zoo was also shown to be exposed to BTV-4 in a second sampling carried out in December of the same year (Table 3).

Discussion

To the best of the authors' knowledge, this is the first large-scale survey on BTV conducted in both artiodactyl and non-artiodactyl zoo species worldwide. We also report for the first time BTV exposure in the banteng, fossa (*Cryptoprocta ferox*), Iberian wolf (*Canis lupus signatus*), Malayan tapir (*Tapirus indicus*) and South American sea lion (*Otaria byronia*). The seropositivity value detected in animals belonging to the order *Artiodactyla* (20.0%) is within the range of those previously observed in the limited surveys carried out on captive artiodactyls worldwide (3.1-37.2%) (Frölich et al., 2005; Yeşilbağ et al., 2011). The high frequencies of seropositivity obtained in the present study in some of the species, including the aoudad (69.2%), mouflon (62.5%) and red deer (*Cervus elaphus*) (33.3%), suggest that zoo artiodactyls could act as potential reservoirs of BTV in Spain, as has been demonstrated in those that are free-ranging (García et al., 2009; García-Bocanegra et al., 2011; Lorca-Oró et al., 2014; Ruiz-Fons, Reyes-García, Alcaide, & Gortázar, 2008). The seropositivity detected in these species could be of animal health and conservation concern. Even though most artiodactyl species have been shown to be asymptomatic for BTV infection, and clinical symptoms compatible with BT were not observed at any of the five zoos analyzed during the study period, clinical disease and mortality have previously been reported in free-living mouflon and Iberian ibex (*Capra*

pyrenaica) in Spain (Fernández-Pacheco, Fernández-Pinero, Agüero, & Jiménez-Clavero, 2008; Gómez-Guillamón et al., 2020a).

Exposure to BTV was found in the threatened African and Asian elephants, which is consistent with previous surveys conducted on free-ranging animals of these species (Anderson & Rowe, 1998; Saminathan et al., 2020). However, the individuals that were analyzed by VNT showed negative results for the serotypes assessed, which may indicate the absence of a humoral neutralizing immune response against BTV in these species or exposure to other serotypes of BTV. In this regard, BTV-8 exposure was detected in wild ruminants in Spain between the 2007/2008 and 2010/2011 hunting seasons (García-Bocanegra et al., 2011, Loca-Oró et al., 2014). BTV-8 outbreaks were also reported in livestock between 2008 and 2010 in different Spanish regions, and more recently in northern Spain (RASVE, 2020). However, since information about the origin of some of these elephants could not be obtained, exposure to other serotypes that were not circulating in Spain cannot be ruled out. The seropositivity detected in the Eurasian lynxes sampled could be of conservation concern, since BT cases and mortality have previously been reported in this species after being fed ruminant fetuses and stillborn animals in areas with BTV outbreaks (Jauniaux et al., 2008). It should be noted that this virus is endemic in southwestern Spain, where the endangered Iberian lynx (*Lynx pardinus*) is mainly distributed (MITECO, 2020). Taking into account that BTV circulation has been detected in free-ranging wild ungulates in this area (García-Bocanegra et al., 2011; Gómez-Guillamón et al., 2020b) and that these may be prey species for free-ranging Iberian lynxes (Delibes, 1980), further studies are warranted to assess the susceptibility to BTV exposure of this threatened species.

Animals with anti-BTV antibodies were detected in four of the five sampled zoos. Three of them (zoo A, B and E) had seropositive animals born in the same zoo where they were sampled. In zoo C, two animals showed neutralizing antibodies against BTV-1 and BTV-4, which is consistent with the serotypes reported in livestock and wild ruminants in this area (RASVE, 2020). Even though these individuals were probably exposed to BTV at this zoological park, given information on previous movements of these animals could not be obtained, contact with these serotypes in other zoos cannot be ruled out. The within-zoo seroprevalence ranged between 6.5% and 38.2%, which suggests widespread but not homogeneous circulation of BTV in zoos in Spain. The highest seroprevalence

was observed at zoo B. This zoo is located in Andalusia (southern Spain), the Spanish region reporting the highest number of BT outbreaks (5189/12496; 41.5%) in domestic ruminants (RASVE, 2020). Southern and western areas of Spain also have the highest densities of *Culicoides imicola,* the major competent vector of BTV in this country (Acevedo et al., 2010; Calvete et al., 2008; Cuéllar et al., 2018). Zoo B was also shown to be a risk factor for the circulation of Schmallenberg virus, another re-emerging *Culicoides*-borne virus (Caballero-Gómez et al., 2021). Because the presence of *Culicoides* spp. has been demonstrated in different zoological parks worldwide (Labuschagne, Gerber, Espie, & Carpenter, 2007; Nelder, Swanson, Adler, & Grogan, 2008; Vilar, Guis, Krzywinski, Sanderson, & Baylis, 2011; England et al., 2020), entomological surveillance programs should be implemented in zoos in Spain to assess the role of different vector species on BTV transmission in these epidemiological scenarios.

Since Spain is considered a high-risk country for the introduction or reintroduction of BTV (Rao et al., 2017; MAPA, 2020), the development of surveillance programs in zoo animals could improve the early detection of circulation of these viruses, particularly in areas where livestock species are vaccinated. In this regard, the only BTV outbreak reported during the 2019/2020 vector season in Spain was a BTV-4 outbreak detected by passive surveillance in a captive black wildebeest (*Connochaetes gnou*) at a zoo park located in a restricted area for this serotype (RASVE, 2020). The seropositivity detected in one forest buffalo (*Syncerus caffer nanus*), born in August 2009 in zoo E (eastern Spain) and sampled in 2013, indicates BTV circulation after the last BTV outbreak was reported in livestock in this region in January 2009 (RASVE, 2020). This hypothesis is also supported by the detection of specific antibodies against BTV-1 in another three forest buffalos sampled in 2014 and 2015, and in one lion from the same zoo in 2013.

The seroprevalence for BTV-1 (10.7%) found in zoo animals in the present study was higher than that detected for BTV-4 (3.0%). This result is in accordance with the higher number of BTV-1 outbreaks (11,492) compared to BTV-4 (518) reported in livestock in Spain during the study period (RASVE, 2020). Anti-BTV-1 antibodies were detected at four of the five sampled zoos, whereas specific seropositivity to BTV-4 was only observed in zoo B. Related to this, BTV-1 outbreaks were reported in livestock in several regions of Spain during the study period, whereas BTV-4 outbreaks were

mainly reported in southern and western regions of the country, 70% of which were observed in Andalusia (RASVE, 2020).

Two of the 24 longitudinally-sampled animals remained seropositive at all samplings, which could be associated with the persistence of antibodies during those years, although repeated exposure to BTV cannot be ruled out. One mouflon housed in zoo B seroconverted against BTV-4 between January and December 2016, and two yearling animals of the same species sampled in January the same year and in November 2014 in the same zoo showed neutralizing antibodies against this serotype. Although the presence of maternal antibodies in yearling mammals cannot be ruled out, these results indicate BTV-4 circulation in 2014 and during the 2015/2016 vector season in southern Spain. It should be noted that between 2015 and 2016, only one BTV-4 outbreak (recorded in January 2016) was reported in livestock located in the same province as zoo B (RASVE, 2020). Antibodies against BTV-1 were also detected in one yearling aoudad sampled in July 2014 at zoo B, which suggests that this animal may have been exposed to this serotype during that year. Interestingly, no BTV-1 outbreaks in livestock were reported in the same year and region as the animals sampled (RASVE, 2020).

In conclusion, the results obtained in the present study confirm BTV circulation, particularly BTV-1 and BTV-4, in urban zoo parks in Spain, which could be of animal health and conservation concern. The high frequency of seropositive animals and the dynamics of seropositivity observed indicate that certain artiodactyl and non-artiodactyl zoo species could be useful sentinel species to monitor BTV activity, particularly in areas where domestic ruminants are vaccinated. Further molecular and serological studies are warranted in these candidate species to assess the role of zoo animals in the epidemiology of BTV.

Acknowledgements

We are grateful to Cordoba Zoo Conservation Center, Bioparc Valencia, Bioparc Fuengirola, Zoo-Aquarium Madrid and Río Safari Elche for providing the valuable samples. J. Caballero-Gómez is supported by an FPU grant of the Spanish Ministry of Science, Innovation and Universities (FPU17/01319).

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.

Ethical statement

The collection of samples was performed by personal of the zoos. All samples were collected from serum banks or from animals subjected to health programs, medical check-ups or surgical interventions during the study period. No ethical approval was necessary.

Data availability statement

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

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Table Legends

Table 1. Seropositivity to bluetongue virus in zoo animals in Spain by ELISA.

Table 2. Results of virus neutralization test (VNT) for the detection of antibodies against bluetongue virus in ELISA-positive zoo animals in Spain.

Table 3. Antibodies against BTV in longitudinally sampled zoo animals. Colored dots indicate antibodies to BTV (pink: anti-BTV-1 antibodies; blue: anti-BTV-4 antibodies; yellow: anti-BTV antibodies; green: negative). In seropositive animals, titers against the BTV serotype are shown in brackets. When two samplings were carried out in the same year, the number of interval months is indicated in superscript. Blank spaces indicate absence of sampling.

Table S1. Antibodies against bluetongue virus detected by ELISA in zoo species sampled in zoo parks in Spain.

Figure captions

Figure 1. Distribution of the zoos (A-E) sampled in Spain. The number of positive (red) and negative (green) animals analyzed by ELISA at each zoo park is represented in a pie chart. Coloured dots indicate antibodies to BTV detected at each zoo by VNT (pink: anti-BTV-1 antibodies; blue: anti-BTV-4 antibodies; brown: anti-BTV-1 and/or anti-BTV-4 antibodies; yellow: undetermined BTV serotype; black: absence). Years when seropositive yearling animals (≤ 12-months-old) were detected are listed above each species.

Table 1. Seropositivity to bluetongue virus in zoo animals in Spain by ELISA

Variable	Categories	No. Positives/No. analyzed (%)
Sampling period	2007-2013	11/82 (13.4%)
	2014-2016	19/94 (20.2%)
	2017-2019	16/65 (24.6%)
Zoos	A	4/32 (12.5%)
	B	26/68 (38.2%)
	C	3/46 (6.5%)
	D	0/11 (0.0%)
	E	13/84 (15.5%)
Order	*Artiodactyla*	29/145 (20.0%)
	Carnivora	6/61 (9.8%)
	Diprodontia	0/3 (0.0%)
	Perissodactyla	5/22 (22.7%)

Table 2. Results of virus neutralization test (VNT) for the detection of antibodies against bluetongue virus in ELISA-positive zoo animals in Spain.

Species	Age	Zoo	Sampling year	E%	VNT titers		Interpretation
					BTV-1	BTV-4	
African elephant	Adult	E	2009	730.1	NA	NA	Undetermined BTV serotype
	Adult	E	2011	238.5	Negative	Negative	Undetermined BTV serotype
	Adult	E	2014*	357.3	Negative	Negative	Undetermined BTV serotype
	Adult	E	2016	335.2	NA	NA	Undetermined BTV serotype
	Adult	E	2016	159.1	Negative	Negative	Undetermined BTV serotype
Aoudad	Unknown	B	2011	949.4	512	Negative	BTV-1
	Adult	B	2012	656.1	4	Negative	BTV-1
	Young†	B	2014	719.6	32	4	BTV-1
	Adult	B	2015	902.7	Negative	32	BTV-4
	Adult	B	2016	834.4	64	Negative	BTV-1
	Young‡	B	2017	900.3	256	Negative	BTV-1
	Adult	B	2017	851.7	128	8	BTV-1
	Adult	B	2017*	780.6	32	Negative	BTV-1
	Adult	B	2018	982.1	512	Negative	BTV-1
Asian elephant	Adult	B	2019	394.6	Negative	Negative	Undetermined BTV serotype
Banteng	Unknown	C	2008	979.3	512	512	BTV-1 and/or BTV-4
	Unknown	C	2008	929.0	256	64	BTV-1

Eurasian lynx	Adult	B	2018	912.6	NA	NA	Undetermined BTV serotype
	Unknown	B	2018	892.6	8	16	BTV-1 and/or BTV-4
Fallow deer	Unknown	B	2018	748.9	256	8	BTV-1
	Unknown	B	2018	994.2	256	4	BTV-1
Forest buffalo	Young[§]	E	2013	889.9	NA	NA	Undetermined BTV serotype
	Adult	E	2014	899.8	512	8	BTV-1
	Adult	E	2015	948.0	128	Negative	BTV-1
	Adult	E	2015	838.9	512	Negative	BTV-1
Fossa	Adult	E	2015	761.2	NA	NA	Undetermined BTV serotype
Iberian ibex	Unknown	B	2017	125.1	Negative	Negative	Undetermined BTV serotype
Iberian wolf	Adult	B	2017	711.0	512	Negative	BTV-1
Llama	Adult	B	2016	792.9	Negative	32	BTV-4
Lion	Adult	E	2010	575.9	64	Negative	BTV-1
Malayan tapir	Adult	A	2014	610.3	16	Negative	BTV-1
	Adult	A	2017	144.0	32	Negative	BTV-1
	Unknown	C	2007	723.4	NA	NA	Undetermined BTV serotype
Mouflon	Young[††]	B	2014	650.4	256	512	BTV-1 and/or BTV-4
	Unknown	B	2016	525.9	512	4	BTV-1
	Young[‡‡]	B	2016	951.7	4	512	BTV-4
	Unknown	B	2017	805.3	4	32	BTV-4
	Young[††]	B	2016	950.5	Negative	512	BTV-4
Red deer	Adult	B	2014	104.0	64	16	BTV-1

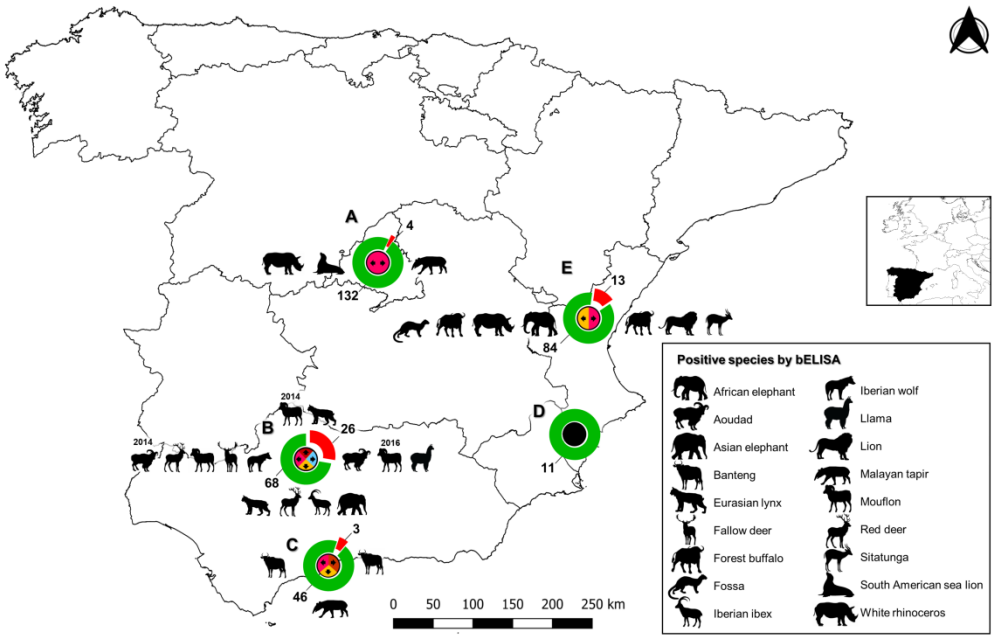
	Unknown	B	2015	690.5	NA	NA	Undetermined BTV serotype
	Unknown	B	2017	870.8	32	4	BTV-1
	Unknown	B	2018	776.5	512	8	BTV-1
Sitatunga	Adult	E	2010	122.0	4	Negative	BTV-1
South American sea lion	Adult	A	2017	531.5	512	64	BTV-1
White rhinoceros	Adult	E	2012	442.2	Negative	Negative	Undetermined BTV serotype
	Adult	A	2016	555.0	4	Negative	BTV-1

*Sampling year of the first positive sample by bELISA in longitudinally surveyed animals; NA: Not analyzed by VNT due to low volume; [†]≤6-months-old; [‡]6-18-months-old; [§]4-years-old; [††]≤12-months-old; [‡‡]1-2-years-old.

Table 3. Antibodies against BTV in longitudinally sampled zoo animals. Colored dots indicate antibodies to BTV (pink: anti-BTV-1 antibodies; blue: anti-BTV-4 antibodies; yellow: anti-BTV antibodies; green: negative). In seropositive animals, titers against the BTV serotype are shown in brackets. When two samplings were carried out in the same year, the number of interval months is indicated in superscript. Blank spaces indicate absence of sampling.

Species	Zoo	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Asian elephant	B											●	●;●[1]
Aoudad	B										●(32)	●(64)	
Jaguar	B					●					●		
Mouflon	B									●; ●(512)[11]			
Malayan tapir	C	●	●	●	●	●	●	●	●	●	●		
Malayan tapir	C		●	●	●	●							
Malayan tapir	C						●		●				
Malayan tapir	C							●	●	●			
Malayan tapir	C			●	●	●							
African elephant	E							●	●	●	●		
African elephant	E							●		●			
Blesbok	E				●	●							
Blesbok	E	●		●									
Bongo	E						●		●				
Common eland	E	●		●	●								
Impala	E		●					●		●; ●[4]			

Impala	E								●		●	
Leopard	E	●; ●[3]										
Thomson's gazelle	E			●	●							
Thomson's gazelle	E								●			●
Thomson's gazelle	E			●				●				
Thomson's gazelle	E		●	●								
Thomson's gazelle	E		●	●								
White rhinoceros	E					●; ●[4]					●	



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