Potential impact of diseases transmissible by sperm on the establishment of Iberian ibex () genome resource banks

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ORIGINAL PAPER

Potential impact of diseases transmissible by sperm on the establishment of Iberian ibex (*Capra pyrenaica*) genome resource banks

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Abstract The long-term cryopreservation of the germplasm (spermatozoa and oocytes) of threatened species offers flexibility in their genetic management and security against disasters or outbreaks of disease that might seriously affect subpopulations. A wide range of infectious diseases can, however, be transmitted via artificial insemination, and the risk of infectious diseases being spread via manipulated germplasm needs to be carefully managed in order to avoid fertility problems in the dams and decreased survival of offspring. Furthermore, accidental introduction of exotic microorganisms into ecosystems remain a major threat. The aims of the present study were to assess the impact of diseases transmissible by sperm on the establishment of germplasm banks for the Iberian ibex, a wild mountain ungulate, and to determine the influence of the presence of these pathogens on sperm functionality. Blood and sperm samples were obtained from 52 mature ibex males legally shot in southern Spain. Sperm motility, morphological abnormalities, acrosome integrity and plasma membrane integrity were assessed for each sample to determine in vitro sperm quality. All serum samples underwent serological analysis for bovine herpes virus type I, bluetongue virus (BTV), bovine leukaemia virus, caprine arthritis–encephalitis virus, pestivirus, *Brucella*, *Coxiella burnetii*, *Chlamydiophila abortus*, *Mycoplasma agalactiae* and *Borrelia burgdorferi*. The highest prevalence (30.7%) was recorded for *B. burgdorferi*, followed by *C. burnetii* (13.4%). A total of 734 sperm doses containing approximately 200×10^6 spermatozoa each were frozen in straws. Forty-five straws (6.1% of the total number) came from animals seropositive for diseases listed in the Terrestrial Animal Code of the OIE for collection and processing of bovine and small ruminant semen (in this case, pestivirus and BTV). A total of 271 frozen straws (36.9% of the total of frozen straws) were provided by animals seropositive for pathogens potentially transmissible by semen not included in the above OIE code (in this case, *B. burgdorferi* and *C. burnetii*). The values of sperm variables were not affected by seropositivity for any of the pathogens transmissible by sperm.

Keywords Diseases · Genome resource banking · Seroprevalence · Spanish ibex · Spermatozoa

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Introduction

Genetic resource banking can play an important role supporting breeding programmes in small populations of

threatened wild species kept in captivity in anticipation of future opportunities for reintroduction to the wild. The long-term cryopreservation of the germplasm (e.g. spermatozoa and oocytes) is an insurance against a catastrophic loss of live animals related to natural disasters or outbreaks of disease (Kirkwood and Colenbrander 2001), and they may also be an aid to maintenance of genetic diversity in small breeding populations (Holt 1994). A wide range of infectious diseases can, however, be transmitted via artificial insemination (Philpott 1993), and the risk of infectious diseases being spread via manipulated germplasm needs to be carefully managed in order to avoid fertility problems in the dams and/or decreased survival of offspring. Furthermore, accidental introduction of exotic microorganisms into ecosystems remains a major threat (Kirkwood and Colenbrander 2001).

Bull semen can contain bovine herpes virus type I (BHV-1; Afshar and Eaglesome 1990) and bluetongue virus (BTV; Foster et al. 1980; Philpott 1993). Although bovine leukaemia virus (BLV) is rarely found in semen (Lucas et al. 1980), it is probably transmissible by artificial insemination (Kirkwood and Colenbrander 2001); seronegative sires are therefore in demand (Philpott 1993). Certainly, caprine arthritis–encephalitis virus (CAEV) has been detected in the semen of bucks (Travasos et al. 1999), and *Brucella abortus* (Robison et al. 1998), *Brucella melitensis* (Amin et al. 2001), *Coxiella burnetii* (Kruszewska and Tylewska-Wierzbanowska 1993) and *Chlamydiophila psittaci* (Lozano 1986) have also been shown to be transmissible by semen. Recently, *Mycoplasma agalactiae* has been detected in semen of goat bucks (de la Fe et al. 2009). Therefore, specific serological testing for this pathogen is also required before sperm collection. Although the transmission of *Borrelia burgdorferi* occurs primarily via the bite of infected ticks of the *Ixodes ricinus* complex, several studies report it might also be sexually transmitted (Burgess et al. 1993; Bach 2001; Harvey and Salvato 2003). Indeed, the existence of *B. burgdorferi* in semen has been confirmed (Bach 2001).

The sanitary control of sperm donors (OIE 2009) should be made routine in order to avoid the use of contaminated sperm in assisted reproduction. Moreover, there is an additional risk of cross-contamination during semen processing prior to freezing when the straws are sealed with polyvinyl alcohol powder (Clarke 1999) and during liquid nitrogen storage (Tedder et al. 1995; Clarke 1999). Apart from causing disease, many pathogens affect sperm functionality, reducing the quality of collected sperm (Revell et al. 1988). Pathogens may affect reproductive function indirectly by causing hyperthermia, which influences spermatogenesis, or directly by causing micro-vascular lesions in the reproductive tract (Osburn 1994), by damaging the epithelium of the seminiferous tubules and excurrent ducts (Hrudka 1984) or inducing necrosis in the epididymes (Kruszewska et al. 1996). Sperm contamination by micro-organisms may also negatively affect the response of sperm to freezing–thawing (Santiago-Moreno et al. 2009), compromising the viability of sperm stored in germplasm banks.

The Iberian ibex (*Capra pyrenaica*) is a wild caprine found only in the mountains of Spain and Portugal. Several ibex populations, mainly from southern Spain, have suffered at the hands of sarcoptic mange; in certain cases, the mortality rate has been over 95% (Fandos 1991). This disease continues to affect most populations of ibexes in southern Spain, although now with less virulence. Given the marked vulnerability of many populations due to sarcoptic mange and the loss of heterozygosity derived from habitat fragmentation, the Andalusian Regional Government together with the Spanish Ministry for Science and Innovation have recently supported the establishment of a germplasm bank for different populations of the species. This would help guarantee their preservation in the face of possible natural disasters or outbreaks of further disease. The long-term cryopreservation of ibex spermatozoa requires special attention be paid to avoiding the risks associated with infectious diseases that can be transmitted via sperm. The aim of the present study was to assess the seroprevalence of pathogens at least theoretically transmissible via sperm in a population of Iberian ibex and to determine their impact (samples discarded due to potential presence of infectious diseases) on the establishment of a genome resource bank. Due to the fact that there is no health code for the official sanitary control of wild animal semen collection, some of the indications of the Terrestrial Animal Code of the World Organisation for Animal Health (OIE) for collection and processing of bovine and small ruminant semen (chapter 4.5) have been followed (OIE 2009). Therefore, the pathogens sought were classified into two groups: (1) those listed by the Terrestrial Animal Health Code of the OIE for collection and processing of bovine and small ruminant semen (IBR/BHV-1, pestivirus, BTV, CAEV, *Brucella* and *M. agalactiae*) and (2) those not listed but still posing a possible risk of semen transmission as reported in the literature (BLV, *Chlamydiophila abortus*, *C. burnetii* and *B. burgdorferi*). The influence of these pathogens on sperm quality was also analysed.

Materials and methods

Semen samples were obtained from 52 mature (8–14 years of age) Iberian ibex males legally hunted in the Tejeda and Almijara Game Reserve in southern Spain (36° N latitude; UTM coordinates 30S 400,785–420,937 4,084,512–4,066,334) during the rutting season (December). All animals showed a good body condition and had no symptoms of disease by external examination. The testes, with their scrotal sac, were transported to the laboratory

immediately after removal. To reduce the death-to-spermatozoa-collection time, a small laboratory was set up in the mountains of the game reserve. The collected testes were kept at ambient temperature (about 11°C) during transport and laboratory processing. All epididymal spermatozoa reached the laboratory within 9 h of death. The testes and epididymes were removed from the scrotal sac and the caudae epididymides isolated from the testes and surrounding connective tissue. The sperm mass of the epididymides was collected by a method previously described for this species (Santiago-Moreno et al. 2007). It was then diluted in 1 ml of a medium (warmed to room temperature in a sterile plastic petri dish [Sterilin®, UK]) composed of 3.8% Tris ($w\ v^{-1}$), 2.2% citric acid ($w\ v^{-1}$), 0.6% glucose ($w\ v^{-1}$) and 6% egg yolk ($v\ v^{-1}$) (all compounds were purchased from Panreac Química S.A. [Barcelona, Spain] and Sigma Chemical Co. [St. Louis, MO, USA]). This solution was adjusted to pH 7.0 with NaOH at room temperature; its osmolality was 345 mOsm/kg.

Sperm motility, morphological abnormalities, acrosome integrity and plasma membrane integrity were assessed for each sample. The percentage of motile spermatozoa and the quality of sperm motility were evaluated subjectively using a phase-contrast microscope (Zeiss, Germany) at ×400 in samples previously incubated for 20 min at 37°C. The vigour with which the sperm cells moved was scored on a scale from 0 (lowest) to 5 (highest). Sperm viability was assessed by staining an aliquot of sperm suspension with nigrosin–eosin (Campbell et al. 1956). Simultaneously, plasma membrane integrity was assessed using the hypo-osmotic swelling test (Jeyendran et al. 1984). Morphological abnormalities were assessed by phase-contrast microscopic examination of glutaraldehyde-fixed samples (counting 200 cells). Spermatozoa with cytoplasmic droplets were considered morphologically normal since these are commonly seen in epididymal sperm cells. The percentage of spermatozoa with intact acrosomes was assessed by observing 200 spermatozoa in samples fixed in buffered 2% glutaraldehyde solution at 37°C, using phase-contrast microscopy (magnification ×1,000). Individual spermatozoa that showed a smooth, crescent-shaped apical ridge were classified as having an intact acrosome. Spermatozoa classified as not showing acrosome integrity were those with an irregularly shaped apical ridge, no apical ridge or a loose, vesiculated acrosomal cap (Pursel and Johnson 1974).

Blood samples of about 5 ml were obtained postmortem via intracardiac puncture and placed in sterile tubes without anticoagulant. These samples were then centrifuged at 400×*g* for 15 min and the serum obtained frozen at −15°C until required for serological analysis. All serum samples underwent serological analysis for the specific detection of antibodies against pathogens transmissible by sperm, including BHV-1, BTV, BLV, CAEV, pestivirus (bovine viral diarrhoea virus and border disease virus), *Brucella*, *C. burnetii*, *C. abortus*, *M. agalactiae* and *B. burgdorferi*. The serological techniques used are listed in Table 1.

The values of the sperm variables showed a skewed distribution (Shapiro–Wilks W test: $P<0.001$; Lilliefors test for normality, $P<0.01$) and were, therefore, subjected to arcsine transformation before statistical analysis. The impact

Table 1 Seroprevalence of different diseases potentially transmissible by sperm in the studied Iberian ibex specimens ($n=52$)

Pathogens	Diagnostic test used	Seroprevalence level
Pestivirus (BVDV and BDV)[a]	Commercial ELISA (Institut Pourquier, France)[c]	3.8% (2/52)
BTV[a]	Commercial competitive ELISA (ID VET, France)[d]	5.7% (3/52)
CAEV[a]	Commercial ELISA (Idexx, Netherlands)	0% (0/52)
Brucella[a]	Complement fixation test (as prescribed by OIE)[e]	0% (0/52)
IBR/BHV-1[a]	Commercial ELISA (Idexx, The Netherlands)	0% (0/52)
M. agalactiae[a]	Commercial ELISA (Institut Pourquier, France)[f]	0% (0/52)
C. burnetii[b]	Commercial indirect immunofluorescence test (Biomerieux, France)	13.4% (7/52)
C. abortus[b]	Commercial ELISA (Idexx, Netherlands)	0% (0/52)
B. burgdorferi[b]	Commercial indirect immunofluorescence test (Biomerieux, France)	30.7% (16/52)
BLV[b]	Blocking ELISA (as prescribed by OIE)[g]	0% (0/52)

[a] Diseases requiring sanitary control in semen collection and processing included in the Terrestrial Animal Health Code from the OIE (OIE 2009) as applied to ruminants

[b] Diseases not included in the Terrestrial Animal Health Code but still posing a possible risk of transmission via semen

[c] The ELISA detects specific antibodies against the conserved P80 protein of bovine viral diarrhoea virus (BVDV) and border disease virus (BDV)

[d] The ELISA detects specific antibodies against the VP-7 protein of blue tongue virus (BTV)

[e] OIE Terrestrial Manual 2008 (chapter 2.7.2)

[f] The ELISA detects specific antibodies against P48 major surface lipoprotein of *M. agalactiae*

[g] OIE Terrestrial Manual 2008 (chapter 2.4.11).

of the presence of microorganisms on sperm variables was analysed by one-way ANOVA. All calculations were performed using the STATISTICA v.5.0 software package (StatSoft 1995).

Results

Table 1 shows the prevalence of the infectious diseases examined. No serum sample returned positive results for IBR/BHV-1, BLV, CAEV, *Brucella*, *C. abortus* or *M. agalactiae*. Thirty-two of the 52 ibexes were negative for all the diseases studied. Twenty males were seropositive for at least one disease; eight of these were simultaneously positive for two diseases. The highest seroprevalence (16/52, 30.7%) was recorded for *B. burgdorferi*, followed by *C. burnetii* (7/52, 13.4%).

One ibex (1/52; 1.9%) showed unilateral cryptorchidism; spermatozoa were therefore collected from descended testicle only. The sperm variable values of this animal were normal. A total of 734 sperm doses containing approximately 200×10^6 spermatozoa each were frozen in straws. A total of 45 frozen straws (45/734, 6.1%) belonged to animals seropositive for an OIE-listed pathogen (pestivirus and BTV); a further 271 (271/734, 36.9%) belonged to animals seropositive for a non-OIE-listed disease (*C. burnetii* and *B. burgdorferi*).

The values of sperm variables were not affected by seropositivity for any of the pathogens transmissible by sperm (Table 2).

Discussion

Attempts to assess the disease risks posed to wild species by germplasm banks and to investigate the transmission of disease via germplasm in wild animals have been scarce (Robison et al. 1998; Kirkwood and Colenbrander 2001). In the present work, the seroprevalence of pathogens at least theoretically transmissible via sperm in a population of Iberian ibex has been evaluated. It is important to keep in mind that the sperm was directly collected from the epididymis, and thus pathogens transmitted in the seminal fluid fraction of semen are unlikely to be transmitted through this sperm bank. Moreover, we have made a serological study, but the presence of these pathogens in the sperm samples was not tested, which should be taken into account in data interpretation.

About 6% of the frozen straws would have required elimination, according the conditions of the Terrestrial Animal Code of the OIE for collection and processing of bovine and small ruminant semen (OIE 2009), since they came from animals seropositive for pestivirus and BTV (OIE-listed pathogens). The removal of these sperm doses would reduce the dissemination of these diseases when the sperm was used in artificial insemination and the possible contamination of other samples by contact during storage (Tedder et al. 1995; Russell et al. 1997).

Bovine viral diarrhoea virus (BVDV) can be isolated from bull semen (Paton et al. 1989). The infection of heifers has been reported after insemination using semen taken from acutely infected bulls (Meyling and Jensen 1988). Serological surveys performed in different countries indicate the prevalence of antibodies to BVDV or border disease virus in domestic goats in the range of 3–16% (Smith and Sherman 2009). This is similar to the 3.8% observed in the ibex of the present study. Pestiviruses can be the cause of miscarriages or the birth of shaker kids in domestic goats and might cause the same in wild ibexes. The sperm variables were not affected in the seropositive animals, unlike reports documenting poor semen quality in infected bulls (Revell et al. 1988; Paton et al. 1989).

The seroprevalence of BTV (5.8%) was lower than that observed in another ibex population from southern Spain (10.8%; Garcia et al. 2009). These differences could be due to variations in the distribution of *Culicoides* vectors from one area to another (Mellor and Wittmann 2002; Calvete et al. 2006). The influence of the sex and age of sampled animals should not be disregarded; in contrast with these earlier studies (Garcia et al. 2009), all the present were males of fully adult age (8–14 years). The dissemination of competent *Culicoides* vectors is the first step in the dissemination of BTV. Although it is now understood that the trading of infected livestock plays a comparatively minor role in the dissemination of the disease (MacLachlan and Osburn 2006), BTV continues to have a significant impact on international trade in live animals, semen and embryos (OIE 2009). In rams and bulls, BTV infection is known to induce infertility, and the virus can be found in

Table 2 Sperm quality parameters in seronegative (controls) and seropositive ibexes

	Control	Pestivirus	BTV	*C. burnetii*	*B. burgdorferi*	SEM
%MOT	78.8	85.2	87.3	81.0	86.6	1.6
SCORE	3.6	3.5	3.8	3.7	3.9	0.1
%NAR	89.2	87.6	91.3	90.5	91.4	0.7
%HOST	81.4	80.6	85.1	83.3	83.1	1.0
%VIAB	86.8	93.3	84.3	85.6	83.8	1.1
%MORPH	6.3	3.8	4.5	6.2	4.8	0.5

%MOT percentage of motile spermatozoa, *SCORE* quality of sperm movement, *%NAR* percentage of spermatozoa with acrosome integrity, *%HOST* percentage of spermatozoa positive in the endosmosis test (membrane integrity), *%VIAB* percentage of viable spermatozoa according to vital staining (eosin–nigrosin), *%MORPH* percentage of spermatozoa with morphological abnormalities, *SEM* standard error of the mean

domestic goat semen (Smith and Sherman 2009). In bulls, a positive relationship has been found between the infectivity of semen samples from animals latently infected with BTV and abnormalities in the heads of spermatozoa affected by virus-like particles. In rams, BTV infection has a significant impact on a number of sperm variables such as sperm concentration, motility, viability and sperm abnormalities (Kirschvink et al. 2009), which could seriously affect fertility. The present results, however, suggest that seropositivity to BTV has no effect on ibex sperm abnormalities or on other sperm variables. Interspecific differences in pathogenesis might explain these observations. Cattle and sheep are the main species affected, and signs of the disease in goats are less severe (usually subclinical; Komarov and Goldsmit 1951). The time passed since infection might also have an influence on the values of sperm variables; the quality of the semen of affected rams reaches normal reference values around 85 days after the onset of clinical disease (Kirschvink et al. 2009).

Since the accidental introduction of infectious diseases from one ecosystem to another poses a threat to the viability of wild animal populations (Kirkwood and Colenbrander 2001), monitoring for infectious diseases transmissible via the spermatozoa should be undertaken, even if these are not OIE-listed (OIE 2009). These non-OIE-listed diseases may have consequences other than introduction into a different geographic area, such as decreased reproductive success in the case of *C. burnetii* and *C. abortus* and musculoskeletal affections caused by *B. burgdorferi.*

The present data show that about 37% of the frozen straws came from ibexes seropositive for *C. burnetii* and *B. burgdorferi*. Certainly, this is a very high rate, and the impact on the establishment of a genetic resource bank would be very significant if these doses were not used. Although Lyme disease is usually considered an exclusively tick-borne illness, the possibility of venereal transmission in humans (Harvey and Salvato 2003) and animals (Burgess et al. 1993; Leibstein et al. 1998; Gustafson 1993; Gustafson et al. 1993) has been suggested. Indeed, *B. burgdorferi* has been isolated from testes of experimentally infected hamsters (Johnson et al. 1984) and from semen samples of men diagnosed with Lyme disease (Bach 2001). The fact that the sperm variables were not affected in seropositive animals agrees with previous reports in other species (e.g. dogs), suggesting that *B. burgdorferi* and/or its metabolites do not significantly harm the functional and morphological characteristics of spermatozoa (Kumi-Diaka and Harris 1994). Although there are no data regarding the presence of *C. burnetii* in sperm of domestic goats, it has been isolated from bull sperm and cultured from the testes, epididymes, prostate and semen of male mice (Kruszewska and Tylewska-Wierzbanowska 1993). Moreover, sexual transmission via semen of *C. burnetii* has been shown in animals (Kruszewska and Tylewska-Wierzbanowska 1993) and even among humans (Kruszewska et al. 1996). The frozen straws coming from ibexes seropositive for *C. burnetii* and *B. burgdorferi* should not be eliminated a priori. Rather, a careful use of them should be suggested. Microbiological analysis or PCR analysis of sperm samples should be undertaken to determine whether these pathogens are in fact present or not in the sperm. Regarding this, it is important to consider that it has been demonstrated that *B. burgdorferi* is able to survive freezing–thawing in experimentally infected and stored semen (Kumi-diaka and Harris 1995).

In conclusion, this is the first report regarding the impact of pathogens potentially transmissible by semen on the establishment of genome resource banks for a mountain ungulate. The relatively high rates of seropositivity for some of the tested pathogens emphasise the importance of testing of donor animals in order to avoid the use of contaminated sperm in assisted reproduction.

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