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ANTIBODY PREVALENCE AND MOLECULAR IDENTIFICATION OF *BABESIA* SPP. IN ROE DEER IN FRANCE

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ABSTRACT: In a region-wide serologic study carried out in 2004 on free-ranging hunted roe deer in various landscapes, we found that 58% of the animals (237 out of 406) were antibody positive for *Babesia divergens* antigen. Serologic and infection status was also analyzed for 327 roe deer live-trapped in two fenced forest areas over 5 yr (2004–08). For two consecutive years during this period, 92 and 94% of the deer in these closed populations were antibody-positive for *B. divergens*. *Babesia* spp. were isolated in autologous red blood cell culture for 131 of the trapped animals (40%). Molecular typing was done on 76 isolates with polymerase chain reaction (PCR)–restriction fragment length polymorphism methods targeted at the *18S* ribosomal subunit gene (18 isolates) and the *Bd37* gene coding for a merozoïte surface antigen implicated in a protective response (60 isolates). Results indicated continuous cocirculation of *B. capreoli* and *B. venatorum* in both forests and possible coinfection of animals with both species. No infection with *B. divergens* was detected. Fifteen isolates were confirmed to be *B. capreoli* by sequencing part of the *18S rRNA* gene. Using PCR detection of the *Bd37* gene, all nine isolates of *B. venatorum* in this study were negative, whereas the 15 confirmed and 50 putative *B. capreoli* isolates showed very variable restriction profiles, distinct from those known for *Bd37* in *B. divergens*. Two isolates showed conflicting results, suggestive of mixed infection.

Key words: *Babesia capreoli*, *Babesia divergens*, *Babesia venatorum*, *Capreolus capreolus*, *Cervus elaphus*, *Ixodes ricinus*, reservoir, zoonosis.

INTRODUCTION

Among tick-transmitted pathogens, protozoan hemoparasites of the genus *Babesia* are a threat to human, livestock, and wildlife health. In wild and domestic ruminants in Europe, three *Babesia* species are of interest: *Babesia divergens*, the main pathogen responsible for cattle piroplasmosis and of sporadic zoonotic cases (Zintl et al., 2003); *B. capreoli*, thought to specifically infect cervids (Enigk and Friedhoff, 1962) but also associated with fatal cases in alpine chamois (*Rupicapra rupicapra rupicapra*; Hoby et al., 2009); *B. venatorum*, first named *Babesia* sp. EU1, detected in severe human cases and subsequently in healthy roe deer (Herwaldt et al., 2003; Duh et al., 2005; Bonnet et al., 2007a). These three species are morphologically and serologically indistinguishable. They are genetically very similar, especially *B. divergens* and *B. capreoli*, which only differ by three nucleotides on the *18S rRNA* gene and were recently confirmed as separate species based on biological criteria (Malandrin et al., 2010). In the absence of concomitant biological data, it is possible that reports of *B. divergens* or *B. divergens*–like organisms in cervids throughout Europe could indeed be *B. capreoli*–type organisms (Langton et al., 2003; Duh *et al.*, 2005; García-Sanmartín et al., 2007; Cancrini et al., 2008, Zintl et al., 2011). Zoonotic issues of *Babesia* infections were recently reviewed (Gray et al., 2010).

All three species share the same vector, *Ixodes ricinus* (Joyner et al., 1963; Nikol'skii and Pozov, 1972; Donnelly and Peirce, 1975; Becker et al., 2009). Other *Babesiae* (e.g., the Missouri-type, *Babesia* MO1, and the *B. microti* complex) are probably also present in Europe in roe deer since related sequences have been identified at least once in roe deer in Italy (Tampieri et al., 2008).

Molecular studies on wild and domestic ruminants and ticks have been made on housekeeping genes (*18S* ribosomal subunit) or internal transcribed spacer sequences (Duh et al., 2001, 2005; Hilpertshauser et al., 2006; García-Sanmartín et al., 2007; Schmid et al., 2008; Blaschitz et al., 2008; Hoby et al., 2009; Zintl et al., 2011). To investigate the extent of *Babesia* infection in healthy roe deer in France, we present results from two studies. First, we carried out a region-wide serologic survey on hunted animals, indicating widespread infection with *Babesia* spp. Second we summarized information on isolation and genetic subtyping of *Babesia* from whole blood samples from two closed populations of roe deer monitored in forest wildlife reserves. We used *18S rRNA* polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) and sequencing, and another genetic marker, *Bd37* gene coding for a merozoite surface antigen, which is known to cluster into six distinct RFLP profiles in *B. divergens* of bovine and human origin (Hadj-Kaddour et al., 2007; Sun et al., 2011). An ortholog of the *Bd37* gene, called *Bcp37/41*, was recently described in *B. capreoli* (Sun et al., 2011).

MATERIALS AND METHODS

Region-wide antibody prevalence against *B. divergens* antigen

Intracardial coagulum was taken from 406 free-ranging, adult roe deer (180 females and 226 males) that were shot in woodlands and hedgerow landscapes of six counties in western France (Ille et Vilaine, Loire-Atlantique, Maine et Loire, Mayenne, Sarthe, Vendée) during the 2003 hunting season (November 2003–March 2004). Serum was extracted by centrifugation at 1,200 × G.

Sera were submitted to an indirect immunofluorescent assay as described in Chauvin et al. (1995) on slides coated with the Rouen 1987 *B. divergens* strain, a human isolate passaged in gerbils. The assay used anti–goat IgG conjugate labeled with fluorescein isothiocyanate (Sigma, Saint Quentin Fallavier, France). The sample was considered positive if a 1:80 dilution gave distinct fluorescence on the slides.

Tests on blood from roe deer from fauna reserves

To obtain *Babesia* isolates from roe deer, we took part in a monitoring program in which whole blood samples for cultivation and molecular characterization were taken regularly from healthy adult roe deer captured by drive-nets in the fenced, protected woodland areas of Chizé (Deux Sèvres, western France 46°07′48″N, 0°25′03″W, 2,370 ha) and Trois Fontaines (Marne, eastern France 48°43′11″N, 4°56′22″E, 1,360 ha). We collected 327 samples from 2004 to 2008, mainly during the winter months, in tubes supplemented with heparin or citrate phosphate dextrose (Sigma) and stored in refrigerators. The samples were taken from 325 animals, which were marked before being released. This sampling was not designed to be a longitudinal survey, therefore, only subsets of the samples were submitted to different tests (summary of tests presented in Table 1).

An indirect immunofluorescent assay (IFA) with *B. divergens* antigen was performed on plasma, as in the 2004 serologic survey. The IFA with *B. capreoli* and *B. venatorum* antigens was conducted on slides prepared respectively with cloned merozoites 2801F10 and rd201 (Bonnet et al., 2007a; Malandrin et al., 2010) as described above. Direct examination was performed under a microscope after Giemsa-based staining of whole blood smears (100×; Diff-Quick, Dade Behring, Deerfield, Illinois, USA). For autologous cultivation (i.e., with red blood cells [RBCs] from the sample), the RBC pellet was washed twice in Roswell Park Memorial Institute (RPMI) 1640 (Lonza, Levallois-Perret, France) (with centrifugation at 800 × G), and 150 µl was cultivated in 2-ml wells in RPMI supplemented with 20% decomplemented fetal calf serum (Lonza; Malandrin et al., 2004). When parasitemia reached 10 to 20% (after 2–3 wk in autologous cell culture), blood cells were taken for molecular characterization of isolates.

A subset of cultures was submitted to molecular identification, 62 from Chizé forest and 14 from Trois Fontaines forest (Table 1). DNA was extracted with Promega Wizard genomic DNA purification kit (Promega, Charbonnieres, France) from pelleted blood cells of the positive roe deer cell cultures, and stored at −20 C until use. The primers used for PCR amplification, the RFLP enzymes (New England Biolabs, Ipswich, Massachusetts, USA; Promega, Madison, Wisconsin, USA), and the expected fragment lengths are summarized in Table 2. A subset of *18S rRNA* PCR products from each forest has been sequenced (Biogenouest, Nantes, France), as described in Table 3. Details on the complete sequencing of the

TABLE 1. Number of blood samples (*n*) collected in two wild fauna reserves, by sampling year and test. All samples were submitted to autologous red blood cell (RBC) culture. Subsets of samples were tested by immunofluorescent assay (IFA) with antigens of *Babesia divergens* (Bd), *B. capreoli* (Bc), or *B. venatorum* (Bv). Subsets of isolates were submitted to molecular identification of species.

	Chizé						Trois Fontaines		
Year	*n*	RBC culture +	IFA Bd +/tested	IFA Bc +/tested	IFA Bv +/tested	Molecular identification	*n*	RBC culture +	Molecular identification
2004	30	20				13	16	8	2
2005	96	42	47/57			31	0		
2006	10	5				5	0		
2007	50	17	46/50	50/50	50/50	0	15	5	2
2008	56	21	53/56	54/56	56/56	13	54	13	10
Total	242	105	146/163	104/106	106/106	62	85	26	14

18S rRNA gene on nine isolates that had a *B. divergens/B. capreoli* profile in PCR-RFLP are provided by Malandrin et al. (2010).

RESULTS

Antibody prevalence in free-ranging roe deer

From the free-ranging, hunted roe deer, 111 females and 126 males (237 adult animals out of 406) were antibody positive for *B. divergens* at a 1:80 dilution. This antibody prevalence of 58.4% suggests that 53.6–63.2% of the local population of roe deer is antibody positive, at a risk of 5% (normal approximation of a binomial distribution), assuming that the sampling was randomly distributed in space and among categories of habitats.

Test results on blood from roe deer in fenced forests

The number of samples, sampling years, and tests performed are summarized in Table 1.

Asymptomatic circulation of Babesia in roe deer: Of the samples collected in Chizé forest in 2007 and 2008, 92–100% were IFA-positive with the three antigens tested (*B. divergens*, *B. venatorum*, and *B. capreoli*). Only four were negative for *B. divergens* in 2007, while in 2008, three samples were negative for *B. divergens* and two for *B. capreoli* (Table 1). No *Babesia* were detected by direct examination of Giemsa-stained smears in any of the 96 samples collected in Chizé in 2005; however, 42 samples showed *Babesia* multiplication after a few days of autologous cell culture. Of the 327 whole blood samples, *Babesia* was isolated from 131 in autologous cell culture (40%). In a comparison of the IFA and cell culture results, agreement between the tests (either positive or negative for both) on 158 samples was 39.5%. *Babesia* was isolated in vitro from only 37% of antibody-positive samples, whereas nine of 21 antibody-negative samples were culture positive.

Molecular identification of Babesia isolates: The results of molecular typing are shown in Table 3 (see Table 1 for the number of isolates typed by year and by forest). The RFLP profiles of the *18S* rRNA gene were as predicted (Table 2), allowing the discrimination between *B. venatorum* and the two other species. The identification of species was confirmed by complete or partial sequencing of the *18S rRNA* gene for 15 putative *B. capreoli* isolates, two putative *B. venatorum*, and two mixed cultures (Table 3). Among these, only four sequences were deposited in GenBank, one for each species *B. capreoli* and *B. venatorum*, and from each forest, Chizé and Trois Fontaines (accession numbers in Table 3). The *B. capreoli* sequences from both locations showed 100% identity with *B. capreoli* reference sequence AY726009, which was also obtained from a roe deer isolate from Chizé in 2003 (Malandrin et al., 2010). The *B. venatorum* sequences were 100% identical to the reference

TABLE 2. Methods of molecular typing of *Babesia* isolates in order to discriminate between the species *B. capreoli* (Bc), *B. divergens* (Bd), and *B. venatorum* (Bv).

Targeted gene	PCR primers		Sizes of PCR amplicon (bp)	RFLP enzymes	Species	Expected RFLP profile(s)	References
18S rRNA	BAB-GF2	5′-GYYTTGTAATTGGAATGATGG-3′	Approx. 560	*Cla*I	Bc/Bd	560 bp	Bonnet et al., 2007b; this study
	BAB-GR2	5′-CCAAAGACTTTGATTTCT-CTC-3′			Bv	185 and 375 bp	
				*Hin*fI	Bc/Bd	50 and 510 bp	
					Bv	28, 170, and 362 bp	
18S rRNA	CRYPTOF	5′-AACCTGGTTGATCCTGCCAGT-3′	Approx. 1,700				Herwaldt et al., 2003
	CRYPTOR	5′-GCTTGATCCTTCTGCAGGTT-CACCTAC-3′					
Bd37, *Bcp37/41*	Bd37-9	5′- ATGAAAACCAGTAAGATTC-TCAACAC-3′	Approx. 900–1,100	*Bgl*II	Bd	6 specific profiles	Hadj-Kaddour et al., 2007; Sun et al., 2011
	Bd37-10	5′- GCCGTATAGCAAATCCATCAT-3′			Bc	Very diverse profiles, different from the 6 known in Bd	
				*Rsa*I	Bd	6 specific profiles	
					Bc	Very diverse profiles, different from the 6 known in Bd	

TABLE 3. Molecular typing of *Babesia* isolates obtained from roe deer in two fenced forests (2004–2008): Typing criteria and available results.

Species diagnosis of isolates	No. of isolates			Typing criteria				
	Origin			*18S rRNA* gene[a]			*Bd37* gene	
	Chize	Trois-Fontaines	Total	PCR amplification	RFLP profile	Sequencing	PCR amplification	RFLP profile
B. capreoli	5	4	9	+	Bc/Bd	FJ944827; FJ944828[b]	+	Diverse[c]
	3	3	6	+	Bc/Bd	Partial sequence 560 bp[b]	+	
	0	1	1	+	Bc/Bd	nd	+	Diverse[c]
	47	2	49	+	nd	nd	+	Diverse[c]
B. venatorum	1	0	1	+	Bv	EF185818	−	
	0	7	7	+	Bv	nd	−	
	0	1	1	+	nd	HQ830266 (partial sequence)	−	
Mixed culture	1	0	1	+	nd	Partial sequence 512 bp[d]	+	Diverse[c]
	0	1	1	+	Bv and Bc/Bd	Partial sequence 560 bp[b]	−	
Total no.	62	14	76					

[a] RFLP = restriction fragment length polymorphism; PCR = polymerase chain reaction; Bd = *Babesia divergens*; Bc = *Babesia capreoli*; Bv = *Babesia venatorum*; nd = not done.

[b] 100% identity to Bc sequence AY726009 (Malandrin et al., 2010).

[c] Different from six profiles known in Bd (Hadj-Kaddour et al., 2007; Sun et al., 2011).

[d] 100% identity to Bv sequence EF185818 (Bonnet et al., 2007).

sequence AY046575 (Herwaldt et al., 2003). The six partial sequences of the fragment spanning from positions 472 to 1,100 of *B. capreoli* were also 100% identical, including at positions 631 and 663 of the complete sequence, where G and T, respectively, are considered typical for *B. capreoli* (Malandrin et al., 2010).

The 15 isolates confirmed to be *B. capreoli* by *18S rRNA* gene sequencing were positive for *Bd37* gene amplification. All restriction profiles of the *Bd37* PCR products were different from the six profiles described to date in *B. divergens* isolates of bovine or human origin (Hadj-Kaddour et al., 2007; Sun et al., 2011). For 50 more *18S rRNA* PCR-positive isolates (without confirmation of species), chiefly from Chizé forest, a high diversity of *Bd37* PCR-RFLP profiles was observed, even among samples collected in the same forest on the same day (see also the first figure of Sun et al. [2011], which shows restriction profiles from the same set of isolates, compared to restriction profiles of a *Bd37* amplicon in *B. divergens*). For nine isolates assigned to *B. venatorum* either by PCR-RFLP or PCR sequencing of the *18S rRNA* gene, none allowed amplification of the gene *Bd37*.

Cocirculation of different *Babesia* species was observed in both forests. Two samples (from Trois Fontaines in 2008 and Chizé in 2006) yielded either a confusing RFLP profile or conflicting results, suggestive of a mixed infection.

DISCUSSION

Using an isolate from a human patient in Europe as a test antigen, we found high

prevalence for antibody against *B. divergens* in free-ranging roe deer. These results are consistent with previous findings in Trois Fontaines forest, where 40 of 75 tested animals were antibody positive in 1979 (Blancou, 1983), and in the South of Belgium, where four of 13 hunted free-ranging roe deer and 13 out of 26 hunted red deer were antibody positive (Lonneux et al., 1991). Serologic cross-reactivity among the species *B. divergens*, *B. capreoli*, and *B. venatorum* has been long recognized (Enigk and Friedhoff, 1962; Herwaldt et al., 2003) and was recently confirmed with clones of *B. capreoli* characterized by molecular tools (Malandrin et al., 2010). Therefore serology cannot be used to identify the *Babesia* species that circulate in cervid populations. In the second part of our study, in a fenced forest where both *B. capreoli* and *B. venatorum* have been shown to cocirculate and no *B. divergens* have been detected (see molecular results), almost 100% of the plasma samples were reactive to all three species' antigens for two consecutive years (2007 and 2008).

Detection by RBC culture indicated a high prevalence of infection in both forests (40% of samples yielded *Babesia* in cell culture). However, there was poor correlation between antibody positivity and infection, probably due to the difficulty of achieving propagation in autologous cell cultures for *B. capreoli*, as well as low circulating parasitemia.

Parasitemia in healthy carriers is very low for *Babesia*, which contrasts with clinically affected animals in which, for example, a parasitemia of 18% was found in smears from intracardiac blood at necropsy from a diseased roe deer (Cancrini et al., 2008). Here in 2005, even when almost half of the animals were found to be infected by using cell culture (42/96), *Babesia* could not be detected by direct examination of Giemsa-stained smears. In domestic ruminants, RBC culture has a detection threshold for *B. divergens* of about 10^{-7}% of parasitemia, corresponding to about 10 parasites/ml of blood (Malandrin et al., 2004). There may be persistence of infection even at this low level, as demonstrated in sheep experimentally infected with *B. divergens* and experiencing a persistent IgG response, despite no currently detectable parasites by RBC culture (Moreau et al., 2009). In the present enzootic context, it is probable that multiple infections occur and that most animals have encountered one or both species *B. capreoli* and *B. venatorum*.

Although it is of scientific interest because of its sensitivity, RBC culture cannot be used routinely because it is labor intensive and vulnerable to saprophyte contamination. It is a useful technique for obtaining single isolates and subsequent in vitro or in vivo testing of the vertebrate host spectrum (as described in Malandrin et al. [2010] for *B. capreoli*). However, it is difficult to obtain the whole blood samples needed for cell culture. Live-capture protocols are cumbersome, and they are only made on a regular basis in a few long-term observational settings. In our study, we took advantage of ongoing monitoring studies in two fenced forests (nature reserves), where there had been no contact with domestic livestock or introduction of cervids for approximately 20 yr. The results show that two species (*B. capreoli* and *B. venatorum*) have cocirculated in these closed populations for many years. The only other ungulate species in contact are red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*).

Surveillance of *Babesia* infections in wildlife should aim for identification at the species level. Differentiating *B. venatorum* from *B. divergens*–related species can be achieved with a simple PCR-RFLP protocol of the *18S rRNA* gene described here. We showed that two species were cocirculating in the two settings that we studied. Clinically affected animals demonstrate high parasitemia, detectable on blood smears performed on cardiac blood (Hoby et al., 2007; Cancrini et al., 2008). In healthy carriers, the possibility of

coinfection of individual animals in addition to low parasitemia must be considered. If direct molecular detection is to be used in healthy carriers, nested PCR protocols should be designed with an internal fragment such as BAB-GF2/BAB-GR2 framing the V4 hyper-variable region. This primer pair is highly conserved among *Babesia* and *Theileria* species and may also detect less closely related genotypes, such as the *Babesia microti* complex or sequences related to *Babesia odocoilei* that have also been detected in roe deer or red deer in Europe (Tampieri et al., 2008; Zintl et al., 2011). To distinguish between the closely related *B. divergens* and *B. capreoli*, it is necessary to at least sequence the *18S rRNA* PCR fragment of the V4 region, with two characteristic nucleotides at positions 631 and 663 of the complete sequence. All currently published sequences of wildlife isolates initially thought to be *B. divergens* (and referenced as such in GenBank) show these differential nucleotides, in addition to another in position 1,637, when the complete sequence is available (Langton et al., 2003; Duh et al., 2005; García-Sanmartín et al., 2007; Hoby et al., 2007; Cancrini et al., 2008; Schmid et al., 2008). Only one recent paper reports true *B. divergens* sequences in healthy free-ranging cervids: six red deer in Ireland (Zintl et al., 2011). However, these sequences are partial and further work is required. Experimental cross-infections and biological and genetic analyses all delineate a host species barrier (Adam et al., 1976; Gray et al., 1990; Malandrin et al., 2010). To deal with the difficulty of species identification, investigators in Switzerland investigating fatal cases of babesiosis in chamois used a more discriminating tool: the sequencing of internal transcribed spacers that clearly showed differences between *B. capreoli* found in roe deer, red deer, and chamois, and *B. divergens* found in bovines from the same areas (Schmid et al., 2008). Subsequently, investigators studying the ecologic factors favoring fatal cases in chamois concluded that there was probably transfer of *B. capreoli* to chamois from healthy roe deer carriers because of increased habitat overlap (Hoby et al., 2009).

We hoped the *Bd37-Bcp37/41* gene and its restriction profile would also be a useful molecular marker. This gene has not been detected in *B. venatorum* with the primer pair used. Further work is required to determine whether an ortholog to *Bd37* gene exists in this species. In contrast to the few typical restriction profiles that exist for *Bd37* from *B. divergens* (Hadj-Kaddour et al., 2007; Sun et al., 2011), *Bcp37/41* from *B. capreoli* shows a wide diversity of RFLP profiles, all different from those known in cattle (Sun et al., 2011; this study). The biological significance of the high variability of *Bcp37/41* in animals sampled in the same location and sometimes on the same day requires further investigation.

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