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Redescription of *Babesia capreoli* (Enigk and Friedhoff, 1962) from roe deer (*Capreolus capreolus*): Isolation, cultivation, host specificity, molecular characterisation and differentiation from *Babesia divergens* ☆

Laurence Malandrin *, Maggy Jouglin, Yi Sun, Nadine Brisseau, Alain Chauvin

INRA, UMR1300 Bio-agression, Epidémiologie et Analyse de Risque, F-44307 Nantes, France
ENVN, UMR1300 Bio-agression, Epidémiologie et Analyse de Risque, F-44307 Nantes, France

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ABSTRACT

The recent use of the sole molecular identification of *Babesia* infecting European cervids has led to confusion between the closely related *Babesia divergens* and *Babesia capreoli*, and to their grouping together as "*B. divergens*-like". In order to clarify this taxonomic confusion, *Babesia* from roe deer, cattle and human blood were isolated, cultured and their biological as well as molecular characteristics compared. On this basis, we conclude that: (i) the parasites isolated from roe deer blood are *B. capreoli*; (ii) there are no intraspecific variations in the 18S rDNA within *B. capreoli* and *B. divergens* spp.; (iii) these two species are closely related as demonstrated by their morphology, serological cross-reactions and 99.83% identity in their 18S rDNA; (iv) these two species are distinct as demonstrated by their different abilities to grow in vitro in cattle, human and sheep erythrocytes, by their infectivity for gerbils, and by a conserved three bases difference at positions 631, 663 and 1637 of their 18S rDNA; (v) *B. capreoli* does not pose a threat to either humans or livestock. An integrated description is given of the host range, geographical distribution, biological and molecular characterisation of *B. capreoli*, and reference materials have been deposited at the Museum d'Histoire Naturelle de Paris.

1. Introduction

Babesioses are tick-borne diseases caused by the intra-erythrocytic multiplication of Protozoa of the genus *Babesia* (Apicomplexa, Piroplasmida) (Homer et al., 2000). They are mainly known as diseases of veterinary importance (Zintl et al., 2003), but interest in their zoonotic potential has increased since fatal cases in immunocompromised individuals were observed (Gray, 2006; Hunfeld et al., 2008). In wild animals, clinical manifestations of babesiosis are less documented, however the role of these animals as reservoirs for parasites of their domestic counterparts or humans is often suspected but rarely proven (Penzhorn, 2006). Three species of *Babesia* have been described to date in wild European cervids: *Babesia* EU1, *Babesia divergens* and *Babesia capreoli*.

Babesia EU1 (*Babesia venatorum*) was first described in human babesiosis cases by Herwaldt et al. (2003) in Austria and Italy, and four years later in Germany (Häselbarth et al., 2007). This species has also been identified in blood from roe deer in Slovenia (Duh et al., 2005a), France (Bonnet et al., 2007) and Italy (Tampieri et al., 2008), but not from red deer (Duh et al., 2005a).

The cattle parasite *B. divergens* is the main causal agent of human babesiosis in Europe (Gray, 2006; Hunfeld et al., 2008) and probably has one of the largest host ranges described to date for a *Babesia* species (Zintl et al., 2003). Experimental infection with *B. divergens* has been achieved in splenectomized primates (chimpanzees, rhesus monkeys) (Garnham and Bray, 1959), ungulates (roe deer, fallow deer, red deer, mouflon and sheep) (Enigk and Friedhoff, 1962a; Chauvin et al., 2002) and rodents (rats) (Phillips, 1984) as well as in non-splenectomized reindeer, sheep and gerbils (Nilsson et al., 1965 ; Malandrin et al., 2009; Lewis and Williams, 1979). Fatal naturally acquired babesiosis in captive reindeer in the United Kingdom (UK) was also attributed to *B. divergens* (Langton et al., 2003). The presence of *B. divergens* or *B. divergens*-like parasites in naturally infected roe deer has been recently reported in Slovenia (Duh et al., 2005a), Poland (Sawczuk et al., 2005), Spain (Garcia-Sanmartin et al., 2007), Switzerland (Hoby et al., 2007a) and Italy (Tampieri et al., 2008), in red deer in Slovenia (Duh et al., 2005a) and in chamois in Switzerland (Hoby et al., 2007b). However, the identity of *B. divergens* from wild ungulates based solely on sometimes partial 18S rDNA sequencing is questionable.

☆ *Note.* Nucleotide sequence data reported in this paper are available in the GenBank database under Accession Nos. FJ944822 to FJ944828.

* Corresponding author. Address: INRA, Ecole Nationale Vétérinaire de Nantes, UMR1300 Bio-agression, Epidémiologie et Analyse de Risque, BP 40706, F-44307 Nantes, France. Tel.: +33 (0) 2 40 68 78 58; fax: +33 (0) 2 40 68 77 51.

E-mail addresses: malandrin@vet-nantes.fr, philippe.maestri@gmail.com (L. Malandrin).

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A closely related species, *B. capreoli*, was first described from roe deer in 1962 in Germany (Enigk and Friedhoff, 1962b), with demonstration of susceptibility of splenectomized red deer and non-splenectomized fallow deer to experimental infections with this organism (Enigk and Friedhoff, 1963). Its presence in naturally infected red and sika deer was also demonstrated (Adam et al., 1976; Hinaidy, 1987; Gray et al., 1990). In these early studies, *B. capreoli* was differentiated from *B. divergens* by the fact that it did not infect splenectomized cattle (Enigk and Friedhoff, 1962b; Adam et al., 1976; Gray et al., 1990) or gerbils (Gray et al., 1990). The two species could not be clearly differentiated on the basis of morphology or serology (Gray et al., 1990, 1991). Subsequently, fatal cases in wild roe deer were attributed to *B. capreoli* throughout Europe: Germany (Müller and Rapp, 1971), Sweden (Christensson and Järplid, 1979), Hungary (Ivanics, 1982), Austria (Hinaidy, 1987) and probably Italy (Cancrini et al., 2008), with parasitemia as high as 20–25%.

Babesia EU1, *B. divergens* and *B. capreoli* share the same vector, *Ixodes ricinus* (respectively, Becker et al., 2009; Joyner et al., 1963; Nikol'skii and Pozov, 1972). DNA of the first two *Babesia* spp. has been successfully amplified by PCR from this tick species in numerous European countries: Slovenia (Duh et al., 2001, 2005b), The Netherlands (Nijhof et al., 2007; Wielinga et al., 2009), France (Bonnet et al., 2007), Switzerland (Casati et al., 2006; Hilpertshauser et al., 2006), Austria (Blaschitz et al., 2008) and Poland (Skotarczak and Cichocka, 2001; Pieniazek et al., 2006).

Whereas *Babesia* EU1 can easily be differentiated from the two other species by molecular means (18S rDNA sequencing), distinction between the closely related *B. divergens* and *B. capreoli* remains uncertain, and their status as different species is sometimes questioned (Gray, 2004; Duh et al., 2005a; Hilpertshauser et al., 2006). There is indeed a gap between the early studies (up until 1991) based on biological characterisation of *B. capreoli* (Enigk and Friedhoff, 1962b; Adam et al., 1976; Gray et al., 1990) and recent studies (from 2005 onwards) where identification has been solely based on molecular comparisons of sometimes only partially sequenced 18S rDNA amplified from ticks or wild ungulates. This led authors to consider the DNA as *B. divergens*, *B. divergens*/*B. capreoli* or *B. divergens*-like, since identities of 99–100% were obtained on partial sequences. This has several important implications. If there is only one species, then roe deer can be a reservoir for the zoonotic *B. divergens*. If the two species are distinct, does the *B. capreoli* host range include humans, thereby adding a new species to the zoonotic *Babesia*? These uncertainties remain to be clarified.

In this study, *Babesia* from roe deer physically separated from contact with cattle were isolated, cultivated and cloned. A link between the biological data (host specificity and ability to grow in erythrocytes from different species) that allowed clear distinction between the roe deer *B. capreoli* and *B. divergens* and the molecular data (18S rDNA complete sequencing) was then established which confirmed the roe deer *B. capreoli* as a separate species.

2. Materials and methods

2.1. Origin of Babesia isolates

The 19 *B. divergens* isolates used in this study originated from human (two), from bovine cases of acute babesiosis in different regions of France (15) (L'Hostis et al., 1995) or from asymptomatic cattle carriers (two) (Malandrin et al., 2004) (Table 1). The nine roe deer included in our study issued from live-trapped members of populations housed in the Wild Fauna Reserves of Chizé (Deux Sèvres, France) or Trois Fontaines (Marne, France). For blood sampling, roe deer were physically restrained and jugular blood samples were drawn into Venoject tubes (Terumo Europe, Leuwen, Belgium) containing citrate phosphate dextrose (CPD, Sigma) (1.4 ml CPD/10 ml Venoject tube). Gentamicin (Dutscher, France) and Amphotericin B (Sigma) were added to the CPD to give final concentrations of 50 and 2.5 μg/ml, respectively. Upon arrival in

Table 1
Hosts and geographical origins of *Babesia divergens* isolated from humans or cattle and *Babesia* isolated from roe deer used in this study.

Isolate name	Vertebrate host of origin	Year of isolation	Host status	Geographical origin: County
Rouen 87 F5	Human	1987	Acute babesiosis	Seine-Maritime
CF 2000	Human	2000	Acute babesiosis	Puy-de-Dôme
Bob2A	Cattle	2003	Acute babesiosis	Corrèze
1802A	Cattle	1988	Acute babesiosis	Cher
2210A	Cattle	1988	Acute babesiosis	Côtes d'Armor
2706A	Cattle	1988	Acute babesiosis	Eure
3503B	Cattle	1988	Acute babesiosis	Ille et Vilaine
4411A	Cattle	1988	Acute babesiosis	Loire Atlantique
5311B	Cattle	1988	Acute babesiosis	Mayenne
5604A	Cattle	1988	Acute babesiosis	Morbihan
5606A	Cattle	1988	Acute babesiosis	Morbihan
6116A	Cattle	1988	Acute babesiosis	Orne
64102A	Cattle	1988	Acute babesiosis	Pyrénées Atlantiques
6903C	Cattle	1988	Acute babesiosis	Rhône
7211A	Cattle	1988	Acute babesiosis	Sarthe
7904A	Cattle	1988	Acute babesiosis	Deux Sèvres
8702A	Cattle	1988	Acute babesiosis	Haute Vienne
135[a]	Cattle	2003	Asymptomatic carrier	Ille et Vilaine
C139[a]	Cattle	2004	Asymptomatic carrier	Calvados
2745	Roe deer	2008	Asymptomatic carrier	Trois Fontaines, Marne
2770F6	Roe deer	2008	Asymptomatic carrier	Trois Fontaines, Marne
2787	Roe deer	2008	Asymptomatic carrier	Chizé, Deux Sèvres
2801F10	Roe deer	2008	Asymptomatic carrier	Chizé, Deux Sèvres
2806	Roe deer	2008	Asymptomatic carrier	Chizé, Deux Sèvres
2807	Roe deer	2008	Asymptomatic carrier	Chizé, Deux Sèvres
2820	Roe deer	2008	Asymptomatic carrier	Trois Fontaines, Marne
2824	Roe deer	2008	Asymptomatic carrier	Trois Fontaines, Marne
3085	Roe deer	2008	Asymptomatic carrier	Chizé, Deux Sèvres

[a] We confirmed the pathogenicity of these two isolates cultivated from asymptomatic carriers by experimental infections of two splenectomized calves with development of symptoms typical of acute bovine babesiosis.