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Babesia divergens-like organisms from free-ranging chamois (*Rupicapra r. rupicapra*) and roe deer (*Capreolus c. capreolus*) are distinct from *B. divergens* of cattle origin – an epidemiological and molecular genetic investigation

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Key words: *Babesia capreoli*; *B. divergens*; chamois; roe deer; cattle; ticks; genotyping.

1. Introduction.

In June 2005 and May 2006, three adult female chamois (*Rupicapra r. rupicapra*) were found dead in the eastern Swiss Alps. Pathological examination revealed pale mucous membranes and musculature, swollen spleen and haemoglobinuria. Blood smears showed small inclusions in the erythrocytes, and PCR/sequencing on DNA extracted from blood or spleen of the infected animals identified the causing organism as *Babesia divergens/B. capreoli* by a 99% -100% identity of a 385-404bp portion of the18S rRNA gene with GenBank entries attributed to these organisms. This was the first confirmed report of fatal *Babesia* infections in chamois, raising the question of an emerging disease in this species (Hoby et al., 2007a).

The presence of a small *Babesia sp.* considered as *B. bovis* was described once in a chamois from Switzerland (Bouvier, 1965), but species identification was based only on microscopical examination of blood smears. In other European countries, *Babesia sp.* were reported in free-ranging roe deer (*Capreolus c. capreolus*), red deer (*Cervus elaphus*) (Duh et al., 2005b) and a farmed reindeer (*Rangifer t. tarandus*) (Langton et al., 2003) which were genetically most similar to *B. divergens*, the known pathogen of cattle. The authors suggested that wild ruminants might act as possible reservoirs for *B. divergens*. In earlier studies, small *Babesia sp*. isolated from roe deer were considered a separate species (*B. capreoli*) based mainly on the fact that this organism was not infective to splenectomized cattle, sheep and goat (Enigk and Friedhoff, 1962). A few subsequent reports on babesial infections of wild ruminants in Europe referred to this species (Dorrestein et al., 1996; Gray et al., 1991). However, *B. capreoli* currently cannot be adequately differentiated by morphological, serological or molecular means from *B. divergens* (Garcia-Sanmartin et al., 2007). The only two sequences of *B. capreoli* (AY726009, AY726010) deposited in GenBank show 99.6% identity (558/560 bp) with part of the 18S rRNA gene of a *B. divergens* isolate from cattle (GenBank accession nr. U07885).

In Switzerland, *B. divergens* has been described sporadically in cattle of southern and western Switzerland based on microscopical diagnosis (Aeschlimann et al., 1975; Galli-Valerio and Stalder, 1918; Gern et al., 1982), and its occurrence was recently confirmed by molecular means (Mathis et al., 2006, Hilpertshauser et al., 2007, and own unpublished data).

The first report of a *B. divergens/capreoli*-like parasite in a new host (chamois) in a new area (eastern Switzerland) tempted us to investigate whether the parasite is

present in cattle and/or ticks from cattle in this Swiss region, and to further genetically characterise and compare it with *B. divergens* originating from Swiss cattle.

2. Material and Methods

2.1 Study area

The region of interest is a rugged, pre-alpine area of about 80 km^2 in eastern Switzerland ('Tössstock', 47°19'N, 8°90'E) comprising grassland and forest. The altitude of the investigated area ranges from 600 to 1300 m above sea level. Several pastures are shared by cattle and free-ranging ruminants such as roe deer, red deer and chamois as observed by the local farmers and game wardens.

2.2 Cattle

Three-hundred and fourteen head of cattle were sampled for this study originating from totally 56 farms. Fourteen farms (F1-14) with 122 animals are located in the study area. The other 42 farms are located outside but their cattle (n=192) grazed on six different pre-alpine pastures (A1-A6) of the study area during summer 2006. Earmark numbers, gender and date of birth were systematically registered. Thirty-four animals were younger than one year, 196 animals were between one and three years old, and 84 were older than three years. Except for five animals, all investigated cattle were females.

2.3 Blood samples

EDTA-blood samples (7 ml blood/cattle) were drawn from the vena jugularis externa. From the cattle (n=122) living year-round in the region of interest, blood was taken once; from the animals of the pre-alpine pastures, blood samples were collected at the beginning (n=140) and at the end (n=162) of the summer grazing season.

2.4 Serology

After centrifugation (1500 x g for 15 min) of the blood samples, plasma was examined for specific antibodies against *B. divergens* by the indirect fluorescent antibody test (IFAT) using slides coated with *B. divergens*-infected gerbil erythrocytes (fixed for 10 min in ice-cold acetone and air-dried) and a goat anti-bovine fluorescein-conjugate (Southern Biotech, Reinach BL, Switzerland) diluted 1:200 in PBS (Edelhofer et al., 1998). Serum of a cow originating from a *B. divergens*-free area

was used as a negative control. The positive control serum of a calf with confirmed *B. divergens* infection was used at dilutions ranging from 1:160 to 1:320, negative and field sera were tested using dilutions from 1:40 to 1:80. The positive threshold titer was set at 1:40. Slides were examined at a final magnification of 600x using a standard UV-microscope (Olympus BH2) equipped with a 60x oil-immersion objective.

2.5 Microscopy and DNA extraction

From all blood samples, one Giemsa-stained smear was prepared (Fluka, Biel-Benken, Switzerland). DNA isolation from blood was done with the QIAmp DNA blood mini kit (Qiagen AG, Basel, Switzerland) according to the manufacturer's instruction. DNA extraction of ticks was done as described previously (Hilpertshauser et al., 2006).

2.6 Tick sampling

During spring, summer and autumn 2006, 887 ticks were collected from 138 head of cattle. Of these ticks, 606 originated from the pre-alpine pasture "A6". Only cattle from this location were examined twice for ticks. Ticks were stored in 70% ethanol at 4°C. Species, stage and gender of each tick were determined under the stereo microscope according to the key of the University of Neuchâtel (Cotty, 1985). For DNA isolation, ticks from individual host animals were pooled in groups of maximum 3.

2.7 B. divergens and *B. divergens/capreoli-like isolates*

Specifications of the parasite isolates investigated are compiled in Table 1.

2.8 Polymerase chain reaction (PCR)

PCRs were done in 100-µl assays containing buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4, 2.5 mM $MgCl_2$, 0.5% Tween 20), each deoxynucleoside triphosphate at a concentration of 0.2 mM (with dUTP replacing dTTP) (all reagents from Sigma-Aldrich, Buchs, Switzerland), each primer at a concentration of 1 µM, and 0.5 U uracil DNA glycosylase to control for PCR carryover contamination (Longo et al., 1990) and 5 µl (ticks/blood) or 25 µl (blood) DNA sample. An initial step at 37°C for 10 min was performed in an automatic thermal cycler (DNA engine, MJ Research, Waltham, MA).

After 10 min of heat inactivation of the uracil DNA glycosylase, 2.5 U *Taq* polymerase was added in a hot start.

Primer specifications and cycling conditions are given in Table 2. To check whether amplifiable DNA from ticks was extracted, the tick-specific primers 16S+1 and 16S-1 were employed. Identification of *Babesia* spp. was done by PCR and sequencing of part of the 18S rRNA gene; genotyping was achieved by targeting the rRNA genes locus.

The amplified fragments were visualized by electrophoresis on a 1.5% agarose gel containing ethidium bromide.

2.9 Cloning and sequencing of amplicons

Amplicons of repeated PCRs including dTTP were purified either directly or after excision from agarose gels using commercial kits (qiaquick PCR purification kit; min elute gel extraction kit, Qiagen, Hilden, Germany) following the manufacturer's instruction. Sequencing by a private company (Microsynth, Balgach, Switzerland) was done on the cloned amplicons (topo TA cloning-vector pCR 2.1, Invitrogen, Carlsbad, CA). Sequences determined in this study are deposited in the GenBank database (accession numbers EU182594-EU182603).

2.10 Sequence analyses

Sequences were subjected to BLAST searches in GenBank, and neighbour-joining trees were constructed in combination with the bootstrap method (van de Peer and de Wachter, 1993), using the aligned sequences (MultiAlin) (Corpet, 1988).

3. Results

3.1 Blood samples from cattle

From all the 424 tested blood samples, two samples originating from animals of farm "F7" showed a positive titer (1:160) in the IFAT. However, microscopical and PCR examinations using primers Bab_F/R (Table 2) of all the cattle (n=10) of this farm were negative. Further, all animals of the pre-alpine pasture "A6" (n=30) from which ticks with positive Babesia-PCR were identified (see below) were repeatedly tested by IFAT (to check for seroconversion) with consistently negative results.

3.2 Ticks

3.2.1 Ticks species

All the collected ticks belonged to the genus *Ixodes*, and 853 were identified as *Ixodes ricinus*. Identification to the genus level only was possible with 34 ticks (all *Ixodes).* The overwhelming majority of the ticks were adult stages (n=880) and 7 were nymphs.

3.2.2 Detection of babesial DNA in ticks by PCR

Among the 887 analysed ticks, DNA of *Babesia sp.* was detected in 6 samples. Sequence analysis of the amplicons obtained with primers Bab_F/R (Table 2) revealed that 5 isolates clustered together with *Babesia sp.* genotype EU1 (99-100% identity). The sixth isolate (Z6) had a sequence identical to GenBank entries attributed to *B. divergens/B. capreoli*. All infected ticks were adult females of *I. ricinus* collected from cattle summering on the pasture “A6”.

3.3 Analysis of the 18S rRNA gene

Full length (1728 bp, isolates B1, B2, C1) or nearly full length (1621 bp, isolate D1) sequences of the 18S rRNA gene revealed identities higher than 99% among the Babesia isolates from cattle (B1, B2), chamois (C1) and roe deer (D1). A total of 19 polymorphic sites were present with 17 being unique to single sequences, and at two positions (positions 631 and 1637) the sequences of the two isolates from cattle were identical but differed from the identical sequences from the isolates of the free-ranging ruminants. A dendrogram using the sequence of the closely related *B. odocoilei* (GenBank AY046577) as outgroup clustered the bovine isolates together apart from the other two isolates. However, the topology of the tree was only weakly supported by bootstrapping (not shown).

3.4 Analysis of rDNA internal transcribed spacers 1 and 2 (ITS1, ITS2), 5.8S and part of the 28S rRNA genes

An approx. 940 bp fragment spanning the ITS1 and 2 and including the full 5.8S and part of the 28S rRNA genes was amplified (using primers ITS_F/R; Table 2), cloned and sequenced of all 6 isolates listed in Table 1. Repeated sequencing of 6 clones of the isolates B2 (of cattle origin) and C1 (chamois) revealed no sequence heterogeneity within one isolate. A robust dendrogram could be derived clearly separating the sequences of the *Babesia* isolates of cattle from those of chamois and

roe deer (Fig. 1). Three sites could be identified which are clearly distinct between these two groups of genotypes (Fig. 2). PCR and sequencing of part of this target (using primers ITS2_F/28S-rev, Table 2) revealed that the *Babesia* isolate from a tick (Z6) with an 18S rRNA gene similar to *B. divergens/B. capreoli* (see above) clusters with the isolates from the free-ranging ruminants.

4. Discussion

The emergence of lethal infections in chamois caused by a *Babesia divergens/capreoli*-like parasite in the eastern Swiss Alps (Hoby et al., 2007a) raised fears that the cattle parasite *B. divergens* had expanded its geographical distribution unnoticed and that it had spilled over to wildlife or, vice versa, that it had been introduced to the area by migrating game and constitutes a threat to local cattle. However, neither the microscopical and the serological nor the molecular examinations gave evidence that cattle in this region were infected with *Babesia sp*. Furthermore, clinical signs of bovine babesiosis have never been observed by the local farmers (n=48), the 4 persons in charge of the 6 pre-alpine pastures, the veterinarians (n=13) or the game wardens (n=2).

Our molecular genetic analyses of these “small” *Babesia* from cattle and free-ranging ruminants (chamois, roe deer) showed that their nearly full-length 18S rRNA genes have very high identities (>99.4%). In contrast, *Babesia* sp. EU1 which has repeatedly been identified in game or in ticks in Europe (Casati et al., 2006; Duh et al., 2005a; Duh et al., 2005b; Hilpertshauser et al., 2006 and this work) and which also has been reported in three splenectomized human patients (Haselbarth et al., 2007; Herwaldt et al., 2003), differs by 3% in this gene sequence. The analyses of part of the 28S rRNA gene and of the more variable locus, the rDNA ITS, revealed again a high similarity, but the isolates from cattle could clearly be differentiated from those of free-ranging ruminants (Fig. 2). Hence, these data indicate that *B. divergens*-like organisms from cattle and from free-ranging ruminants are closely related but distinguishable and have different host specificities. These findings confirm earlier observations of different infectivity of such isolates for Mongolian gerbils (*Meriones unguiculatus*) and cattle which are susceptible to isolates originating from cattle but not to those from game (Gray, 2006; Langton et al., 2003). Hence, these genetic and biological differences support the use of the discrete

species name *B. capreoli* (Enigk and Friedhoff, 1962) for the *B. divergens*-like organisms from chamois and roe deer.

Whether the few human infections which were attributed to *B. divergens* in the past (reviewed by Gray, 2006; Zintl et al., 2003) were caused by *Babesia* sp. EU1, *B. divergens* of cattle or *B. capreoli* of wild ruminant origin remains to be determined, and the rDNA locus with the genetic differences reported here seems a useful marker to differentiate the latter two.

Roe deer in Europe seems to host at least two different *Babesia* sp. namely *B. capreoli* and *Babesia* sp. EU1, and first studies revealed prevalences of up to 30% (Bonnet et al., 2007; Duh et al., 2005b; Hoby et al., 2007b). Interestingly, the parasites seem to have a low virulence in roe deer, and it may be speculated that roe deer acts as a reservoir for the parasite affecting chamois. The *Babesia* isolate from a fatal case in chamois is closely related to three isolates from roe deer of different geographic areas in Switzerland (Fig. 1). Further investigations are required to assess whether these differences are of biological importance or whether chamois is more susceptible to this parasite.

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Figure 1. Dendrogram deduced from sequences comprising internal transcribed spacer 1 (ITS1), 5.8S rRNA gene, ITS2 and part of 28S rRNA gene of *Babesia* isolates from Switzerland (isolates B1, B2 from cattle, C1 from chamois, D1-D3 from roe deer). Neighbour-joining analysis with bootstrap values of 1000 replicates given at the nodes. No corresponding sequence is known from a closely related organism.

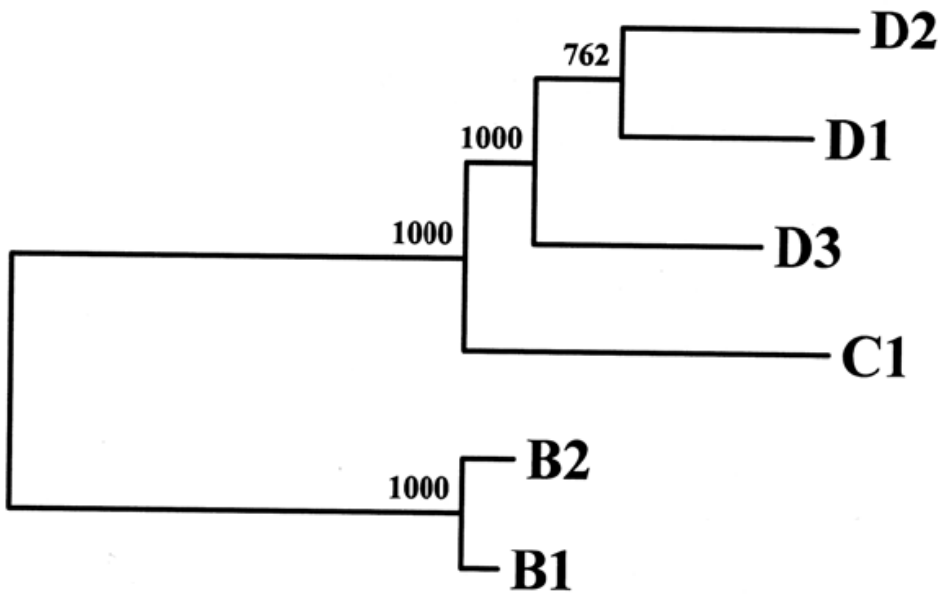

Figure 2. Characteristic sequence differences of rDNA internal transcribed spacers 1 and 2 (ITS1, 2) and 28S rRNA gene of *Babesia* isolates from cattle (B1, B2), chamois (C1), roe deer (D1, D2, D3) and a tick (Z6). Sequence positions indicated refer to the sequence B1 (GenBank accession nr. EU182598). Dots indicate gaps, differing nucleotides are underlined.

	ITS1	ITS2	28S rRNA gene
Position	230	630	790
Isolate	----------	----------	----------
B1	TGTAGTTGGCGTACACTTGG	CTTTTACT-------CCTGGTAA	ATTACTATTGCAGTATTTCTA
B2	TGTAGTTGGCGTACACTTGG	CTTTTGCT-------CCTGGTAA	ATTACTGTTGCAGTATTTCTA
C1	TGTAGTTGATATACACTTGG	CTTTTACTGGTTTACTCTGGTAT	ATTACTATTG---TATTTCTA
D1	TGTAGTTGATATACACTTGG	CTTTTACTGGTTTACTCTGGTAT	ATTACTATTG---TATTTCTA
D2	TGTAGTTGATATACACTTGG	CTTTTACTGGTTTACTCTGGTAT	ATTACTGTTG---TATTTCTA
D3	TGTAGTTGATATACACCTGG	CTTTTACTGGTTTACTCTGGTAT	ATTACTATTG---TATTTCTA
Z6	(not determined)	CTTTTACTGGTTTACTCTGGTAT	ATTACTATTG---TATTTCTA

Table 1. Designation and origin of the investigated *Babesia* isolates.

Isolate designation	Host species	Geographical origin	Reference
B1[a]	Cattle	North western Switzerland (Jura, Clos-du-Doubs)	Mathis et al., 2006
B2[b]	Cattle	Southern Swiss Alps (Grisons, Puschlav)	Hilpertshauser et al., unpublished data
C1[c]	Chamois	Eastern Swiss Alps (Tössstock)	Hoby et al., 2007a
D1[d]	Roe deer	Eastern Swiss Alps (Tössstock)	Hoby et al., 2007b
D2[d]	Roe deer	Eastern Swiss Alps (Tössstock)	Hoby et al., 2007b
D3[d]	Roe deer	Western Swiss Alps (Simmental)	Hoby et al., 2007b

[a]Obtained from a cow living in a known endemic region of *B. divergens*.

[b]Obtained from a splenectomized calf which was infected with the blood of a cow infected with *B. divergens*.

[c]Obtained from a chamois found dead with clinical signs of acute babesiosis.

[d]Obtained from roe deer collected by game wardens in regions where fatal babesiosis in chamois has been reported

Table 2: Features of PCR primers used in this study and reaction conditions

Primer designations	Specificity	Locus	Sequence (5'-3')	Fragment size (bp)	Annealing temp (ºC)	Extension time (s)	No. cycles	Reference
16S+1/-1	Ticks	Part of mitochondrial 16S rRNA gene	CTGCTCAATGATTTTTTAAATTGCTGTGG TTACGCTGTTATCCCTAGAG	~ 300	62	45	40	(Black and Piesman, 1994), modified
BAB_F/R	*Babesia* spp.[a]	Part of 18S rRNA gene	GTTTCTGMCCCATCAGCTTGAC CAAGACAAAAGTCTGCTTGAAAC	422-440	62	45	40	(Hilpertshauser et al., 2006)
CRYPTO_F/R	Generic apicomplexa	Complete 18S rRNA gene	AACCTGGTTGATCCTGCCAGT GCTTGATCCTTCTGCAGGTTCACCTAC	~ 1700	65	90	45	(Herwaldt et al., 2003)
DON_F/R	*Babesia* spp.[b]	Nearly complete 18S rRNA gene	TGTCTAAGTACAAACTTTTTAC TTAAGTGATAAGGTTCACAAG	~ 1660	56	90	45	(Duh et al., 2005a), modified
ITS_F/R	*Babesia* spp.[c]	Part of rRNA locus [d]	GTGAACCTTATCACTTAAAGG TTC(A,G)CTCGCCG(C,T)TACT	~ 940	51	90	40	(Zahler et al., 1998), modified

ITS2_F/28S-rev	[e]	Part of rRNA locus [f]	CCCGTTTCAGTGAGCCCCCTTTCC CTTAAATTCAGCGGATAGCCTCAC	314	66	45	40	This study

[a] Bovine Babesia spp. (*B. divergens, B. bigemina, B. major*), *Babesia* sp. genotype EU1, *B. odocoilei, B. canis*, *B. ovata*, *B. motasi*, and *B. crassa*

[b] Bovine *Babesia* spp (*B. divergens*, *B. bigemina*, *B. bovis*), *Babesia* sp. genotype EU1 , *B. odocoilei*, *B. caballi*, *B. gibsoni*

[c] forward primer targets conserved sequence of *Babesia* spp. (*B. canis*, *B. bigemina*, *B. bovis*, *B. caballi*, *B. divergens*, *B. equi*, *B. microti*, *B. odocoilei*, *B. rodhaini*); reverse primer targets 28S RNA gene region highly conserved among eukaryotes.

[d] internal transcribed spacer 1 (ITS1), 5.8S rRNA gene, ITS2, part of 28S rRNA gene.

[e] internal primers to fragments obtained with primers ITS_F/R with isolates from this study

[f] ITS2, part of 28S rRNA gene.

Lebenslauf

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