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Questing *Dermacentor reticulatus* harbouring *Babesia canis* DNA associated with outbreaks of canine babesiosis in the Swiss Midlands

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ABSTRACT

In 2011 and 2012, outbreaks of clinical canine babesiosis were observed in 2 areas of the Swiss Midlands that had no history of this disease so far. In one area, cases of canine babesiosis occurred over 2 consecutive tick seasons. The outbreaks involved 29 dogs, 4 of which died. All dogs were infected with large *Babesia* sp. as diagnosed in Giemsa-stained blood smears and/or PCR. These were identified as *B. canis* (formerly known as *B. canis canis*) by subsequent partial sequencing of the 18S rRNA gene of *Babesia* sp. Interestingly, the sequence indicated either a genotype with heterogeneity in the ssrRNA gene copies or double infection with different *B. canis* isolates. None of the dogs had a recent travel history, but one had frequently travelled to Hungary and had suffered twice from clinical babesiosis 18 and 24 months prior to the outbreak in autumn 2011. Retrospective sequencing of a stored blood DNA sample of this dog revealed *B. canis*, with an identical sequence to the *Babesia* involved in the outbreaks.

For the first time in Switzerland, the partial 18S rRNA gene of *B. canis* could be amplified from DNA isolated from 19 out of 23 adult *Dermacentor reticulatus* ticks flagged in the same area. The sequence was identical to that found in the dogs. Furthermore, one affected dog carried a female *D. reticulatus* tick harbouring *B. canis* DNA. Our findings illustrate that, under favourable biogeographic and climatic conditions, the life-cycle of *B. canis* can relatively rapidly establish itself in previously non-endemic areas. Canine babesiosis should therefore always be a differential diagnosis when dogs with typical clinical signs are presented, regardless of known endemic areas.

Introduction

Canine babesiosis caused by large *Babesia* spp. occurs in many tropical and subtropical areas worldwide. Formerly, all described large *Babesia* of dogs have been considered as subspecies of *B. canis* (Cacciò et al., 2002), but the different vector tick specificity as well as unambiguous differences in the DNA have led to their reclassification into 3 individual species, namely *B. canis*, *B. rossi*, and *B. vogeli* (Schnittger et al., 2012). *B. canis*, transmitted by the hard tick *Dermacentor reticulatus*, is the most important species in Europe and can cause severe clinical disease in affected animals. The main clinical signs are fever, thrombocytopenia, anaemia, haemoglobinuria, and splenomegaly (Tenter and Deplazes, 2006).

Even though most of Europe has a climate that could support *D. reticulatus* activity (Beugnet et al., 2009), the distribution of this species is highly focal (Gray et al., 2009). In the past decades, a spread of *D. reticulatus* and also of *B. canis* has been observed, and canine babesiosis is now considered as an emerging infectious disease in multiple European areas (Matijatko et al., 2012). In Germany for instance, new foci of *D. reticulatus* have been described (Dautel et al., 2006). Countries as far north as Belgium (Cochez et al., 2012), the Netherlands (Matjila et al., 2005), and Norway (Øines et al., 2010) have reported the occurrence of *D. reticulatus* or autochthonous infections with *B. canis*. The causes of the geographical extension are not completely understood, but most probably climate changes, habitat suitability, dynamics in host populations, and also anthropogenic factors all contributed (Gray et al., 2009; Léger et al., 2012).

In Switzerland, all reported cases of autochthonous canine babesiosis occurred in the biogeographic region 'Swiss Midlands' (Gonseth et al., 2001; Fig. 1). In this region, however, only Geneva represents a stable endemic focus (Jacquier, 1974; Pfister et al.,

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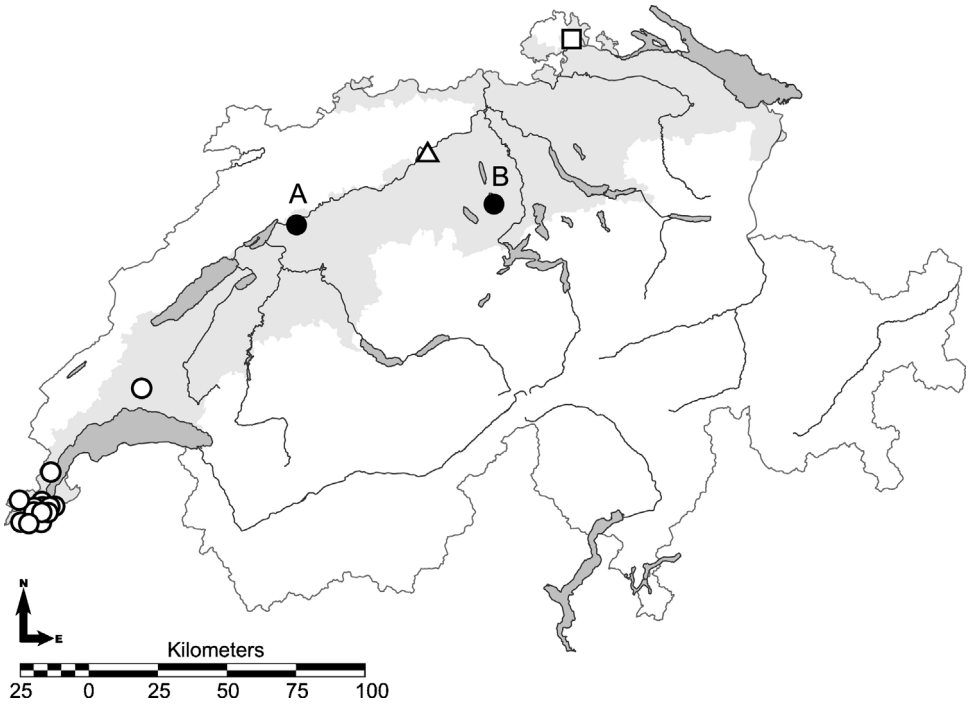

Fig. 1. Map of Switzerland showing locations of different canine babesiosis outbreaks in dogs without travel history. Solid circles show the location of the 2 described outbreaks in Dotzigen (A) and in Baldegg (B). Open circles: Porchet et al. (2007); open triangle: Sager et al. (2005); open square: Schaarschmidt et al. (2006). All reported outbreaks originated from the biogeographic region 'Swiss Midlands' (light-shaded area; Gonseth et al., 2001). Questing adult *D. reticulatus* ticks harbouring *B. canis* DNA were found in area B (geodata: Swiss Federal Office for Environment, CH 3003 Bern, Switzerland).

1993), and further dissemination along Lake Geneva was observed (Porchet et al., 2007; Fig. 1). *D. reticulatus* has only been collected in Geneva, but *B. canis* DNA was not demonstrated in those ticks (Pfister et al., 1993). Central, northern, and eastern Switzerland were historically considered free from canine babesiosis, but some years ago, 2 clustered cases of this disease in dogs with no travel history occurred (Sager et al., 2005; Schaarschmidt et al., 2006). These 2 outbreaks were restricted to one tick season, involved only 6 dogs, and the respective transmitting ticks had not been found. Very little is known regarding molecular characterisation of *B. canis* from Switzerland, with only 2 sequences originating from infected dogs deposited in GenBank (Casati et al., 2006).

Here, we report on 2 outbreaks of canine babesiosis in 2011/2012 in seemingly non-endemic areas of the Swiss Midlands and provide the molecular characterisation of the causative agents involved. In one area, *D. reticulatus* harbouring *B. canis* DNA could be collected for the first time in Switzerland. Anamnestic investigations as well as analysis of climate data during the outbreaks provided some insight into the possible mechanisms of the establishment of new foci for *D. reticulatus* and *B. canis*.

Materials and methods

Areas

The recreational areas of Dotzigen (47.186° N, 7.340° E), area A, and Baldegg (47.186° N, 8.277° E), area B, belong to the biogeographic region 'Swiss Midlands' (Gonseth et al., 2001; Fig. 1). Biogeographic regions are defined by the statistical evaluation of the local flora and fauna (Gonseth et al., 2001). The Swiss Midlands are characterized by a moderate relief with an altitude ranging between 260 m and 960 m above sea level (a.s.l.) (Gonseth et al., 2001) and by a fragmented landscape of deciduous and mixed woodland, agricultural areas, and rivers and lakes (Federal Office for Spatial Development, 2012). The climate is continental with an Atlantic influence (mean annual rainfall 1203 mm and mean annual temperature 9.1 °C at the Station Biel; Federal Office of Meteorology and Climatology, 2012). The areas A and B are both characterized by the presence of water, a river in the former and a lake in the latter instance, bordered by environmentally valuable shrubs and woods and surrounded by agricultural land such as prairies and fields. Area A is situated about 430 m and area B about 460 m a.s.l. Both areas are very popular for dog walks.

Climate data

In 2011, Switzerland experienced the highest mean temperature since the beginning of the climate recordings in 1864, with 1.8–2.4 °C above the long-term mean temperatures from 1961 to 1990 (Federal Office of Meteorology and Climatology, 2012). More specifically, March to May and August to September were too warm, with >4 °C and >2 °C above the long-term mean, respectively. Rainfall reached only 70% of the long-term mean in area A and 90% in area B (Federal Office of Meteorology and Climatology, 2012). In contrast, February 2012 was characterized by unusually cold temperatures below 0 °C for more than 2 weeks (Federal Office of Meteorology and Climatology, 2012). Spring 2012 was also a dry and outstandingly warm period (2–2.5 °C above the long-term mean), with March reaching temperatures 3–4.5 °C above the long-term mean temperatures (Federal Office of Meteorology and Climatology, 2012).

Dogs

Nine diseased dogs of area A (spring 2011) and 19 diseased dogs of area B (autumn 2011 and spring 2012) could be included in the study. Clinical assessments of the dogs and haematological analyses were carried out. Indirect immunofluorescence antibody

test (IFAT) for *B. canis* was performed using a commercially available kit according to the manufacturer's instructions (MegaScreen® Fluobabesia canis, Megacor, Austria). EDTA-blood samples or DNA samples of dogs that yielded positive test findings for canine babesiosis were submitted to sequencing. Retrospectively, DNA isolated from the blood of a diseased dog in spring 2010 from area B and stored at −20 °C since then was included in the study. In total, blood from 29 dogs was analysed for the presence of *Babesia* spp. (Table 1).

Ticks

Tick flagging was performed on May 8 and on June 21, 2011, in the recreational area of Dotzigen (area A). For this, white cotton blankets were slowly dragged over the vegetation adjacent to the walking paths covering a distance of about 5 km on a route reported as most frequently used by dog owners. Every 10–20 m, the blankets were turned, and collected ticks were transferred with forceps from the blankets to tubes containing 70% ethanol. The same procedure was repeated on March 1, 2012, along Lake Baldegg (area B) covering a distance of about 4 km. Furthermore, we asked the veterinarians in the 2 respective areas to collect ticks on dogs. All ticks were identified using a stereomicroscope and a key for the classification of ticks (Estrada-Peña et al., 2004).

DNA preparation

Genomic DNA from canine blood was isolated using the DNeasy® Blood and Tissue Kit (Qiagen, Switzerland) according to the manufacturer's protocol for non-nucleated blood. Individual ticks were put into single tubes, shock-frozen in liquid nitrogen, and immediately squashed using individual plastic pistils. Subsequently, genomic DNA was isolated from the ticks using the tissue protocol of the DNeasy® Blood and Tissue Kit (Qiagen, Switzerland). DNA of dog blood samples and ticks was eluted in 200 μl elution buffer and stored at −20 °C until further use.

PCR and sequencing

Babesia DNA was detected by PCR using the primers described by Casati et al. (2006), i.e. forward primer 5′-GTCTTGTAATTGGAATGATGG-3′ and reverse primer 5′-TAGTTTATGGTTAGGACTACG-3′. The primers bind to the 18S rRNA gene of different *Babesia* species and yield amplification products ranging from 411 to 452 bp (Casati et al., 2006). PCR conditions were exactly as described by Sager et al. (2005). In brief, 25-μl reactions with 0.5 μM of each primer, 1 Unit Taq Polymerase (Qiagen AG, Basel, Switzerland), 2.5 μl 10× PCR buffer (Qiagen), 2.5 μl 10× dNTP Mix (Qiagen), and 2 μl of DNA template were subjected to an initial denaturation step of 10 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 2 min. Amplification was completed by a further 5-min step at 72 °C. In each run, a positive and a negative control were included. For the positive control, 2 μl of *B. canis* DNA isolated from the blood of an infected dog was substituted for the test DNA, and for the negative control, the test DNA was replaced by 2 μl of water.

Purification of PCR products was performed employing the High Pure® PCR Product Purification Kit (Roche, Switzerland). The purified PCR products were eluted in 50 μl elution buffer and stored at −20 °C. Purified amplicons were sequenced bi-directionally with BigDye v.3.1 (Applied Biosystems) using the amplification primers and were run on a Genetic Analyzer 3130xl (Applied Biosystems). Obtained sequences were blasted against GenBank (http://blast.ncbi.nlm.nih.gov/).

Results

Dogs

Signalment, clinical signs, and haematological changes

A total of 29 dogs were affected by canine babesiosis (Table 1). No sex, age, or breed predisposition could be observed (data not shown). The most prevalent clinical abnormalities were lethargy ($n = 27$; 93%), anorexia ($n = 27$; 93%), fever ($n = 23$; 79%), and dark urine ($n = 19$; 66%) (data not shown). Nine dogs (31%) experienced gastrointestinal disorders such as vomiting and/or diarrhoea, in 2 dogs (7%) icterus could be observed, and one dog (3%) suffered from pain in the neck region and lameness (data not shown). Haematological changes included thrombocytopenia ($n = 27$; 93%), anaemia ($n = 24$; 83%), and leucopoenia ($n = 19$; 66%). The ranges of the abnormal haematological values were $8–103 \times 10^9$ thrombocytes/l (normal limits: $150–500 \times 10^9$/l), $3.13–5.59 \times 10^{12}$ erythrocytes/l (normal limits: $6–9 \times 10^{12}$/l), and $2.36–5.79 \times 10^9$ leucocytes/l (normal limits: $6–12 \times 10^9$/l).

Direct detection of B. canis

All but one dog were assessed for the presence of *Babesia* sp. by investigating Giemsa-stained blood smears of capillary blood samples. In 20 dogs, this test was positive for large *Babesia* species (Table 1). PCR was performed for all but one dog and revealed the presence of *Babesia* DNA in all 28 animals, among them 8 cases where the blood smear was negative (Table 1). Subsequent sequencing of the amplification products of 19 dogs showed 100% identity among each other. The sequence was deposited in GenBank under accession number JX678979. At 2 positions (129 and 130), clear double peaks were observed in each chromatogram; in both positions, an A and a G were shown in the sequencing reaction (Fig. 2). The sequence was 99% identical with *B. canis* sequences deposited in GenBank. The 1% difference originated from the 2 unresolved positions.

Serology

Of the 21 dogs that were assessed at the day of first presentation, 18 dogs were seronegative for *B. canis* (Table 1). Serological follow-up of 15 animals revealed seroconversion within 14 dogs (Table 1). Three dogs (nos. 13, 24, and 29; Table 1) were seropositive for *B. canis* at the day of their first presentation. Dog no. 13 had a borderline titre of 1:32, which increased to 1:160 2 weeks later. Dog no. 24 showed intermittent haemoglobinuria that was initially attributed to vesical calculi. It was diagnosed with *B. canis* infection about 1 month after it had been in the risk area (Table 1). Dog no. 29 was the dog retrospectively included into the study; in spring 2010, it suffered from a second episode of canine babesiosis.

Treatment and outcome

Dog no. 1 died undiagnosed and therefore without adequate treatment. All other dogs received treatment with imidocarb dipropionate, 6 mg/kg body weight, subcutaneously, repeated once after 14 days. Treatment was usually initiated within 4 days after the first presentation of the dog (Table 1). Ten dogs even received the treatment at the day of presentation, either upon clinical suspicion alone or after diagnosis with a blood smear done by the veterinarians themselves. In 25 dogs, the clinical signs disappeared 1–2 days after the first administration of imidocarb dipropionate. In these cases, *Babesia* DNA was not detected in the following blood samples, and the haematological values usually returned to normal (data not shown). Three dogs died despite appropriate treatment, and one dog (no. 26) required 3 treatments for full recovery. This dog showed neuromuscular signs with pain in the neck region and lameness at first presentation. It relapsed with clinical signs and parasitaemia 9 days after the initial treatment. Clinically, the dog

Table 1
Summarized information on the dogs affected during 2 babesiosis outbreaks, including a retrospectively considered dog.

Dog no.	1st pres	Area	Smear	PCR[a]	IF 1	IF 2	Vaccination	Tick prophylaxis	Days until therapy	Outcome	Travel history
1	2/27/2011	A	nd	*B. canis*	nd	nd	No	None	None	**Dead**	None
2	3/5/2011	A	Positive	*B. canis*	nd	nd	No	Imidacloprid/permethrin[b]	4	**Dead**	Germany/Austria, October 2010
3	3/7/2011	A	Positive	nd	nd	**1:80**	No	Fipronil	3	Good	None
4	3/18/2011	A	Negative	*B. canis*	nd	**1:128**	No	None	1	Good	None
5	3/21/2011	A	Positive	*B. canis*	nd	nd	No	Deltamethrin[b]	1	**Dead**	None
6	3/26/2011	A	Positive	*B. canis*	nd	nd	No	Deltamethrin[b]	0	Good	None
7	4/4/2011	A	Positive	*B. canis*	nd	nd	No	None	0	Good	None
8	4/26/2011	A	Positive	*B. canis*	nd	nd	No	None	0	Good	None
9	5/14/2011	A	Positive	*B. canis*	Negative	nd	No	Imidacloprid/permethrin	0	Good	None
10	9/8/2011	B	Positive	*B. canis*	Negative	**1:640**	No	Imidacloprid/permethrin	2	Good	None
11	9/21/2011	B	Positive	*B. canis*	Negative	**1:320**	No	Deltamethrin	0	Good	None
12	9/26/2011	B	Positive	*B. canis*	Negative	**1:320**	No	Fipronil	0	Good	None
13	9/27/2011	B	Negative	*B. canis*	**1:32**	**1:160**	No	Deltamethrin	8	Good	None
14	10/26/2011	B	Negative	*B. canis*	Negative	**1:320**	No	None	1	Good	None
15	11/9/2011	B	Negative	*B. canis*	Negative	**1:320**	No	Permethrin	1	Good	Hungary, May 2010
16	11/18/2011	B	Negative	*B. canis*	Negative	**1:80**	No	None	4	Good	None
17	11/26/2011	B	Negative	*B. canis*	Negative	nd	No	None	0	Good	None
18	2/21/2012	B	Negative	*Babesia*	Negative	**1:320**	No	None	0	Good	Hungary, 2010
19	3/12/2012	B	Positive	*B. canis*	Negative	Negative	No	Permethrin	1	Good	None
20	3/13/2012	B	Positive	*B. canis*	Negative	nd	No	None	0	**Dead**	None
21	3/19/2012	B	Positive	*Babesia*	Negative	**1:160**	No	Deltamethrin	1	Good	None
22	3/22/2012	B	Positive	*Babesia*	Negative	nd	No	None	2	Good	None
23	4/11/2012	B	Positive	*Babesia*	Negative	nd	No	None	0	Good	None
24	4/21/2012	B	Positive	*Babesia*	**1:1600**	nd	No	None	30	Good	None
25	5/6/2012	B	Positive	*Babesia*	Negative	**1:200**	No	None	1	Good	None
26	5/7/2012	B	Positive	*Babesia*	Negative	nd	No	Imidacloprid/permethrin	1	Good[c]	None
27	5/9/2012	B	Positive	*Babesia*	Negative	**1:100**	No	Deltamethrin	1	Good	None
28	5/9/2012	B	Negative	*Babesia*	Negative	**1:160**	No	Permethrin	1	Good	None
29[d]	4/22/2010	B	Positive	*B. canis*	**1:640**	nd	No	Permethrin	2	Good	Hungary, 2 days ago

Area A: Dotzigen; area B: Baldegg; IF 1: antibody titre of the immunofluorescence assay performed at first presentation of the dog; IF 2: antibody titre of the immunofluorescence assay performed as follow-up, normally 10–14 days after the first treatment. Nd: not done.

[a] *Babesia*: diagnosis of *Babesia* sp. by PCR; *B. canis*: diagnosis by PCR followed by sequencing.

[b] Product on dog for less than a week before presentation at the veterinarian.

[c] After 3rd treatment.

[d] The same dog had suffered from a first episode of canine babesiosis diagnosed by blood smear in November 2009, five days after its return from a journey to Hungary.

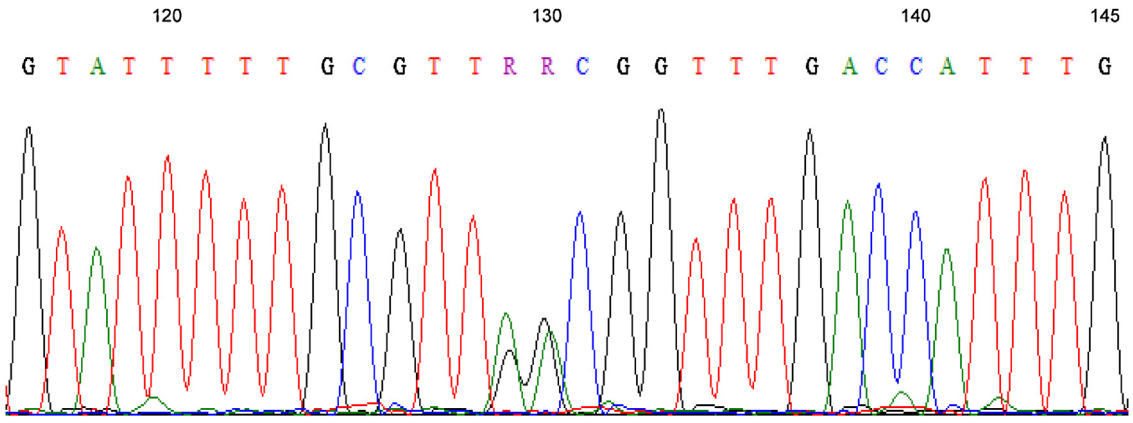

Fig. 2. Partial chromatogram of the *B. canis* sequence amplified from adult *D. reticulatus* and dogs. Clear double peaks indicating either a G or an A at positions 129/130 of the partial 18S rRNA gene were identified in all samples from dogs and ticks sequenced in this study. The positions correspond with the positions 610/611 of the whole-length ssrRNA gene.

improved rapidly after the second treatment, but totally recovered only after the third treatment.

Prophylaxis

None of the dogs had been vaccinated against canine babesiosis. However, 16 dogs had received a tick prophylaxis either as a spot on or as a collar (Table 1). In 3 dogs, the prophylaxis had been administered within one week prior to presentation at the veterinarian (Table 1).

Travel history

We received the recent travel history of all 29 dogs affected during the outbreaks. One dog (no. 2) had travelled to Germany and Austria 5 months before the onset of clinical signs, and 2 other dogs (nos. 15 and 18) had travelled to Hungary in 2010, i.e. more than 12 months before the onset of the clinical signs (Table 1). The remaining 25 dogs had not been travelling abroad in the 6 months before the onset of the clinical signs.

Retrospectively included dog

Dog no. 29 (Table 1) is a young dog that frequently travelled to Hungary and had suffered from babesiosis twice: the first time at the end of November 2009 and a second time in April 2010. Both times, the clinical signs began a few days after its return from Hungary. Because the owner travelled to Hungary frequently, the dog received 6 prophylactic injections with imidocarb dipropionate within a period of 15 months after the first episode of babesiosis. Despite these treatments, it suffered from a second episode of babesiosis. Thereafter, it was vaccinated against babesioisis with Pirodog™ (Merial, France). PCR and sequencing was retrospectively performed on DNA extracted from blood taken during the second episode. PCR detected an 18S rRNA *B. canis* gene sequence identical to the one found in the other dogs.

Ticks

Flagging on 2 different days in area A resulted in the collection of 238 ticks, all of them the hard tick *Ixodes ricinus*. *Babesia* PCR was performed on all of them and yielded *Babesia* sp. *EU1* (also known as *B. venatorum*) in 7 ticks, but no *B. canis* DNA could be amplified. Flagging in area B resulted in the collection of 23 adult *D. reticulatus* ticks, 10 females and 13 males. All *D. reticulatus* were collected within a few square meters. *Babesia* DNA was found in 19 of the *D. reticulatus* (8 females and 11 males). Sequencing of the 18S rRNA gene amplification products identified *B. canis* in 9 of these ticks. For the remaining 10 ticks, sequencing did not yield an interpretable result, most probably due to a low DNA content and/or inhibitory effects in the sequencing reaction. Sequence comparison with the *B. canis* samples of the dogs demonstrated 100% identity.

Additionally, we received one tick collected on a diseased dog. It was a *D. reticulatus* female found on dog no. 14 (Table 1) that harboured *B. canis* with an 18S rRNA gene sequence identical with the *B. canis* present in the PCR-positive blood samples and in the free-living ticks. Furthermore, 5 *Ixodes* sp. ticks were collected on healthy dogs in area B, which were all found negative in the *Babesia* PCR.

Discussion

To the best of our knowledge, this is the first time that free-living *D. reticulatus* ticks harbouring *B. canis* DNA were identified in Switzerland. Interestingly, all of them were collected within a few square meters, and 9 ticks harboured *Babesia* DNA that could be identified as *B. canis*. The aggregated presence of vector ticks was not surprising, since this has been previously described for *I. ricinus* infected with *Babesia microti* in Switzerland (Foppa et al., 2002) and for *Dermacentor variabilis* infected with *Francisella tularensis* in the U.S. (Goethert and Telford, 2009). The reasons for these clustering events are not entirely understood, but microhabitat-related factors, as well as assembly pheromones, have been mentioned as a possible cause of the formation of such tick microfoci (Goethert and Telford, 2009; Li and Dunley, 1998).

The sequence of *B. canis* shown in this study and identified in dogs and ticks has not yet been described for Switzerland. In GenBank, only 2 *B. canis* sequences originating from 2 diseased Swiss dogs have been found. These sequences display GA (AY 648872) or AG (AY 648874) at positions 129/130 of the partial 18S rRNA gene, respectively (Casati et al., 2006). These isolates therefore correspond to other European *B. canis* isolates, where all combinations of A or G at the same positions were described (positions 129/130 in our PCR correspond to positions 610/611 of the whole-length ssrRNA gene) (Beck et al., 2009; Ionita et al., 2012). Importantly, Beck et al. (2009) also amplified sequences that displayed the A/G double peaks at those positions, representing either a double infection in the respective dogs or the possibility of genetic heterogeneity amongst the different copies of the ssrRNA genes of *B. canis*. As it seems fairly unlikely that all dogs from 2 unrelated areas and all ticks in our study were simultaneously infected with 2 different *B. canis* isolates, we suspect the presence of different alleles of the ssrRNA gene in the genotype of *B. canis* described here. Sequencing of *Babesia* sp. present in questing *I. ricinus* ticks confirmed the presence of *Babesia* sp. EU1/*B. venatorum* in these vectors as had

been formerly described for other Swiss areas (Casati et al., 2006; Hilpertshauser et al., 2006; Gigandet et al., 2011; Burri et al., 2011).

The finding of questing adult *D. reticulatus* harbouring *B. canis* DNA, the presence of an infected tick on a dog, as well as the lack of a recent travel history together with the seroconversion of many dogs in our study indicate an autochthonous transmission of *B. canis* in the described outbreaks. Furthermore, the seasonal occurrence of the clinical cases coincided with the activity peaks of adult *D. reticulatus* (Bartosik et al., 2011). However, the final proof for the autochthonous cycle, i.e. the demonstration of infectious sporozoites in the local ticks, is missing in our study.

Interestingly, all described autochthonous cases of canine babesiosis in Switzerland occurred within the same biogeographic region Swiss Midlands. In this region, the area around Lake Geneva, which has more Mediterranean influence than the remaining Swiss Midlands (Gonseth et al., 2001), was the only one previously considered as endemic for canine babesiosis (Jacquier, 1974; Pfister et al., 1993; Porchet et al., 2007). Outside Geneva, only 4 outbreaks of canine babesiosis have been described so far (Sager et al., 2005; Schaarschmidt et al., 2006; this study). The 2 outbreaks described here differ from the previous ones by a higher number of dogs involved, by the presence of fatal cases, and by the occurrence of new infections in the following tick season. The unusually warm and dry (i.e. Mediterranean-like) climatic conditions during the 2 most recent outbreaks might have favoured the establishment of a local transmission cycle.

We may speculate about the way of introduction of *B. canis* to the 2 areas. Travelling dogs for instance may come in contact with various parasites, as has recently been demonstrated for dogs in Germany (Hamel et al., 2012). Either local *D. reticulatus* could have infected themselves on dogs infected with *B. canis*, or alternatively, ticks harbouring *B. canis* might have travelled with a dog to Switzerland. The occurrence of clinical babesiosis in a dog that frequently travelled to Hungary, a country endemic for *B. canis* (Földvári et al., 2005), as well as the travel history to Hungary of 2 more dogs of the same region, provide evidence that dog travelling did occur in this area and that it resulted in clinical babesiosis in at least one dog. Therefore, dogs travelling to or originating from endemic regions can be a source of introduction.

The outbreaks presented here showed characteristics of a disease emergence in an unprepared area. Despite the presence of the classical signs of canine babesiosis in the affected dogs, this disease did not rank very high on the differential diagnosis list in the first cases. Dog no. 1 which was affected by babesiosis in February 2011 was referred from the private veterinarian to an animal clinic, but died undiagnosed. Dog no. 2 was correctly diagnosed, but because imidocarb diproprionate was not constantly available in the pharmacies of the local veterinarians, treatment could be administered only 4 days after first presentation. After dog no. 2 had died, the local veterinarians alerted their colleagues via an e-mail forum provided by the Swiss Society of Veterinarians. Simultaneously, some practitioners held informative meetings for dog owners to increase the awareness in the local population, and press reports were published in the local newspapers. These actions increased disease awareness and led to more rapid diagnosis and adequate treatment of the diseased dogs identified later on. A similar dynamic of a *B. canis* outbreak in a seemingly non-endemic region was described in the Netherlands (Matjila et al., 2005).

None of the dogs in our study was vaccinated against *B. canis*. This is not surprising, as most veterinarians only recommend vaccination against babesiosis for dogs that frequently travel to traditional risk countries. The fact that 16 of the dogs assessed in our study became infected with *B. canis* despite tick prophylaxis is rather worrying. However, we do not know whether the tick repellent had always been correctly administered; at least in 3 dogs it was not applied early enough in order to reach its full efficacy before the dogs became ill. Therefore, we cannot conclude that tick prophylaxis per se is inefficient.

Our study confirmed the higher sensitivity of PCR compared to the blood smear for the detection of *B. canis* (Schaarschmidt et al., 2006). A negative blood smear in a dog with typical clinical signs of babesiosis can therefore not exclude infection with *Babesia* sp.

Taken together, we report canine babesiosis outbreaks in 2 areas that had been considered free from this disease and from where no reports of free-living *D. reticulatus* existed. Sequencing either revealed double infection with *B. canis* in all dogs and ticks or, more likely, a genotype of *B. canis* that displays heterogeneity of the ssrRNA gene copies. The trend to a warmer climate in non-endemic tick habitats together with other factors such as increased dog travelling activities might further favour the long-term establishment of *B. canis* and/or new outbreaks of babesiosis. Regular tick monitoring should be performed to assess possible changes in tick population and establishment of *B. canis*. However, the most important factor for animal welfare is the constant disease awareness of veterinarians in endemic and especially in seemingly non-endemic regions.

Competing interests

The authors declare that they have no competing interests.

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