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SHORT COMMUNICATION

High genetic diversity of *Babesia canis* (Piana & Galli-Valerio, 1895) in a recent local outbreak in Berlin/ Brandenburg, Germany

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Abstract

Canine babesiosis caused by *Babesia canis* (Piana & Galli-Valerio, 1895) is emerging in new regions in Europe since its vector *Dermacentor reticulatus* (Fabricius, 1794) is expanding its geographic range. In the Berlin/Brandenburg area in northeast Germany, *D. reticulatus* is highly abundant but in the past only one autochthonous *B. canis* infection was reported. Since 2015, autochthonous cases were occasionally diagnosed but numbers increased since autumn 2019. The aim of the study was to genotype autochthonous canine *Babesia* spp. infections from Berlin/Brandenburg. Between 04/2015 and 01/2022, 46 dogs with acute babesiosis were presented to the small animal clinic (one dog was infected twice resulting in 47 samples). There were 32 dogs that had never left Berlin/Brandenburg and 14 others that had not left the region in the 6 weeks prior to disease onset. PCRs targeting the 18S rRNA and the Bc28.1 merozoite surface antigen were positive in 47 and 42 samples, respectively. Sequencing of cloned PCR products identified all samples as *B. canis* with 17 18S rRNA and 12 Bc28.1 haplotypes. Based on network analysis for 18S rRNA sequences and a previously described polymorphic dinucleotide, samples were assigned to two distinct clusters. One contained 31 and the other 16 samples. Using network analysis, the Bc28.1 haplotypes could also be separated into two clusters differing by at least five polymorphisms. Analyses of sequences from multiple clones indicated the presence of up to five 18S rRNA and eight Bc28.1 haplotypes and thus high parasite variability in an individual host. The genetic diversity could suggest that the parasites in the region have multiple origins, but diversity in individual dogs and dog populations from endemic regions is unknown. The suitability of both markers for genotyping is questionable due to potential intragenomic diversity for the rRNA and high intergenomic variability for the Bc28.1 marker.

Barbara Kohn and Jürgen Krücken contributed equally.

KEYWORDS
autochthonous, *Babesia canis*, dog, genotyping, PCR

1 | INTRODUCTION

Babesia are classified as Apicomplexa and belong to the order Piroplasmida. In Europe, canine babesiosis is caused by four species, that is, *Babesia canis*, *Babesia vogeli* and rarely *Babesia gibsoni* and *Babesia microti*-like (synonyms *Babesia* (*Theileria*) *annae* and *Babesia vulpes*) (Solano-Gallego et al., 2016; Baneth et al., 2019). In infections with *B. canis*, the clinical presentation in dogs is variable and ranges from subclinical infections to multiorgan failure resulting in death (Köster et al., 2015; Solano-Gallego et al, 2016). It is often an acute infection with clinical cases mainly in spring and autumn (Solano-Gallego et al., 2016). The incubation period is 4–21 days (Boozer et al., 2003). In the acute course, the clinical signs are mainly lethargy, inappetence, fever, pale mucous membranes, pigmenturia, splenomegaly and hypovolemia (Köster et al., 2015). Common clinicopathological changes are thrombocytopenia, anaemia, leukopenia, increased renal and liver parameters and haemoglobinuria. Complications include hepatopathy, acute kidney injury, cerebral babesiosis, pancreatitis and myocardial dysfunction (Köster et al., 2015; Solano-Gallego et al., 2016). The chronic or subclinical course of babesiosis is poorly described; but may either be asymptomatic or may include non-specific signs such as intermittent fever, inappetence, loss of body condition, lymphadenomegaly and splenomegaly (Birkenheuer, 2014; Milanovic et al., 2020).

All *Babesia* species are vector-borne pathogens transmitted by hard ticks and in the case of *B. canis* the only known vector is *Dermacentor reticulatus* (Solano-Gallego et al., 2016). In addition to vector-mediated transmission, direct transmission through blood transfusions or contaminated cannulae (Solano-Gallego et al., 2016) as well as vertical transmission have been described (Mierzejewska et al., 2014; Adaszek et al., 2016). *Babesia canis* DNA has also been detected in mice and jirds after oral inoculation with *B. canis*-infected *D. reticulatus* ticks suggesting that oral transmission might also be possible (Corduneanu et al., 2020).

Several canine vector-borne diseases are spreading from Mediterranean and Eastern European regions into Central and even Northern Europe and this range expansion is at least partially driven by climate change (Baneth et al., 2012) but changes in land use as well as travelling of dogs to and import from endemic regions will surely also contribute. Prominent examples for this tendency are *Dirofilaria repens* (Capelli et al., 2018), *Leishmania infantum* (Maia et al., 2015), *Thelazia callipaeda* (Lebedewa et al., 2020) and *B. canis* (Mierzejewska et al., 2015). A prerequisite for the increasing incidence of autochthonous infections with *B. canis* is the high abundance of *D. reticulatus* ticks, which were found on dogs in the Berlin/Brandenburg area nearly as frequently as *Ixodes ricinus* (Beck et al., 2014). The combination of frequent import of infected dogs from endemic regions (Barutzki et al., 2007) and the high abundance of vectors make endemization of the pathogen very likely.

The spread of canine babesiosis depends largely on the occurrence of the ticks needed as vectors. In Germany, the occurrence of an adult *D. reticulatus* tick was first documented in 1973 (Immler, 1973). Since then, *D. reticulatus* has increased its geographic range in Germany considerably (Földvári et al., 2016) and is still continuing to do so (Drehmann et al., 2020). This expansion is thought to be driven by factors including climate change, changed land use and increased abundance of hosts such as red foxes, wild boars and roe deer (Földvári et al., 2016).

Autochthonous canine babesiosis was detected only 3 years following the first reported occurrence of its vector tick in Germany (Liebisch et al., 1976). Since then, several endemic foci of babesiosis have been found in southern and southwestern areas of Germany (Beelitz et al., 2012). In a retrospective study, clusters of autochthonous infections were reported in Breisgau (Beelitz et al., 2008) in southern Germany, while only isolated cases occurred in the surrounding regions (Rubel et al., 2016; Drehmann et al., 2020). Furthermore, autochthonous infections with *B. canis* have been observed in dogs from Saarland (Beelitz et al., 2012), Baden-Württemberg (Barutzki et al., 2007) and Rhineland-Palatinate (Kehl et al., 2005). However, individual cases have also been reported from Lower Saxony (Jensen et al., 2005). For a long time, only a single case report of an autochthonously infected dog was available from the Berlin/Brandenburg region, although *D. reticulatus* occurs in increasing numbers (Heile et al., 2006; Richter et al., 2013; Beck et al., 2014; Kohn et al., 2019).

By PCR, more than 2000 questing ticks and 197 *D. reticulatus* associated with dogs were examined but no *Babesia* spp. were detected in any of them (Schreiber et al., 2014; Kohn et al., 2019). However, the introduction of (chronically) infected animals into such areas is expected to lead to outbreaks through local transmission and eventually endemization. Unfortunately, data about the number of dogs imported into Germany, their geographic origin and health status are not systematically recorded and therefore not available.

For genotyping of *B. canis*, partial 18S rRNA gene (Seleznova et al., 2020) and the Bc28.1 merozoite surface antigen (Carcy et al., 2015) sequences have been used previously. For the 18S rRNA sequence, the variation is very limited and only two single nucleotide polymorphisms have been described and there was no association of genotype with severity of disease in samples from Latvia (Seleznova et al., 2020) suggesting the 18S rRNA gene is only poorly suitable for genotyping. Therefore, Carcy et al. (2015) used the Bc28.1 sequence and identified three major groups of this antigen sequence (Bc28.1A, B and G) that differed remarkably between different geographic regions within Europe.

Since 2015, only a few dogs with acute babesiosis were diagnosed annually in the Small Animal Clinic but from 2019 onwards cases occurred more regularly. The aim of this study was to describe the

molecular diversity of autochthonous *B. canis* infections in the area to determine if the parasites were derived from a single or multiple sources.

2 | MATERIALS AND METHODS

2.1 | Selection of patients

The examined cases of the study were exclusively patients of the Small Animal Clinic of the Freie Universität Berlin. Dogs diagnosed with acute canine babesiosis were only included if they had not left the Berlin/Brandenburg area at least 6 weeks prior to diagnosis. All 46 dogs included were diagnosed using a *Babesia* spp. specific PCR (Zahler et al., 1998) and for 40 dogs also by blood smears. One dog was diagnosed positive twice in 2019 and 2020 and therefore the total number of samples included was 47.

2.2 | Molecular identification of the *Babesia* species and genotyping

A 379 bp *Babesia* sp. 18S rRNA gene fragment was amplified using the Primers RLB-F (5′-GAGGTAGTGACAAGAAATAACAATA-3′) and RLB-R (5′-TCTTCGATCCCCTAACTTTC-3′) (Gubbels et al. 1999). For amplification of a 660 bp fragment from the merozoite surface gene Bc28.1 gene, primers F281&2a (5t′-ACTGAGGATGAGAAAAGG-GATAGT-3′) and R281 (5′-GTCCACAACCGCGCGACGGCGCAAC-3′) were used (Carcy et al. 2015).

The PCR mixture contained 0.25 mM dNTPs, 0.5 μM of each primer and 0.4 U Phusion™ High-Fidelity DNA Polymerase (ThermoFisher) in 20 μl 1× Phusion HF PCR buffer including 2 μl template DNA (60–280 ng/μl). After initial denaturation at 98°C for 30 s, 40 cycles of 98°C for 10 s, 65°C (18S rRNA) or 50°C (Bc28.1) for 30 s and 72°C for 30 s were performed before final incubation at 72°C for 5 min.

Positive and negative control reactions were always conducted in parallel with samples. The positive control reactions contained 200 copies of a *B. canis* plasmid DNA with the amplicon as insert. An 18S PCR product was generated in 47 samples (one dog was positive twice), while Bc28.1 PCR amplification was successful in 42 samples. All PCR products were purified with the DNA Clean & Concentrator™−5 from Zymo research, cloned into the p SC-A-amp/kan vector (Agilent) and analysed by Sanger sequencing at LGC Genomics (Berlin). Sequences were initially analysed using BLASTn (Altschul et al., 1990) searches in GenBank limiting the search to sequences from Piroplasmida. One sequence was generated for each PCR product. Furthermore, several clones of the 18S rRNA and Bc28.1 genes were picked and sequenced for two samples each. For 18S, 9 clones of sample 190 and 3 clones of sample 206 were analysed. In addition, 7 clones of sample 197 and 8 clones of sample 206 of Bc 28.1 were examined. These samples were selected without any special characteristics.

2.3 | Network analysis

The 18S rRNA sequences were aligned using MAFFT (Katoh et al., 2005) using the 'align gappy regions anyway' and the Q-INS-I options. The Bc28.1 sequences were aligned codon-wise using Muscle (Edgar et al., 2004) as implemented in Mega7 (Kumar et al., 2016). Phylogenetic analysis was done with popArt 1.7 (Bandelt et al., 1999) (http://popart.otago.ac.nz) to construct median joining networks from the obtained sequences.

3 | RESULTS

3.1 | Patients

Of the 46 dogs included, four cases were diagnosed with *B. canis* in 2015/2016 while 42 cases were included 2019–2021, predominantly between September and January (n = 38) but also in March and June (n = 7) (Table 1). In addition, one dog each was presented in August and February. One dog was diagnosed positive twice in 2019 and 2020. After treatment in 2019, the dog tested negative in the PCR. The age range of the dogs was 0.5–12 years (median 7, mean 6.4). The majority of the dogs was male (n = 30). Overall, 32 dogs had never left the Berlin/Brandenburg area and 14 others had not left the region in the 6 weeks prior to disease onset. Reasons for presenting dogs were clinical signs such as lethargy (46), fever (23) and 'reddish urine' (19). All dogs needed intensive care treatment and received imidocarb. Four dogs were euthanized, 3 dogs died.

3.2 | Babesia species identification

All 18S rRNA gene sequences showed 99.47%–100% identity to *B. canis* 18S rRNA gene sequences from GenBank. Identity to *B. vogeli* and *B. rossi* were approximately 95.5% and 92.4%, respectively (Table S1). All infections were therefore considered to be caused by *B. canis*.

3.3 | Genotyping of *Babesia canis*

All *B. canis* sequences deposited in GenBank and showing 100% query coverage in a Blast analysis were included in the network analyses. All sequences were aligned (Figure S1). The phylogenetic network containing 57 18S rRNA sequences plus 41 sequences from GenBank showed two main clusters separated by two single nucleotide polymorphisms (Figure 1). The rarer haplotype (represented by AY649326 in Figure 1) contained 9 sequences from Berlin/Brandenburg. Associated with this haplotype but separated by one or two polymorphisms were further sequences from GenBank and 11 additional sequences from Berlin/Brandenburg (cluster B). The more frequent haplotype (represented by AY072926) included 30 sequences from Berlin/Brandenburg and 21 sequences from other countries (cluster A). Seven sequences

TABLE 1 GenBank accession numbers, summary of phylogenetic analysis and signalment for all dogs included in the study

ID	Date*	18s rRNA GenBank†	Bc28.1 GenBank†	Cluster 18S rRNA‡	Cluster Bc28.1§	Breed	Sex¶	Age (years)#
131	11/2019	ON152329	ON167604	B	1	Small Münsterländer	mn	10
143	11/2019	ON152330	ON167605	A	1	Weimaraner	m	4
148a	11/2019	ON152331	ON167606	A	1	Malinois	fn	8
148b	11/2020	ON152344	ON167618	A	1	Malinois	fn	9
153	12/2019	ON152332	ON167607	A	2	Lhasa Apso	mn	6
160	11/2019	ON152333	ON167608	A	1	Old German shepherd dog	f	3
161	05/2020	ON152334	ON167609	B	1	German shepherd	m	
162	09/2020	ON152335	ON167610	A	2	Jack Russel Terrier	mn	11
163	09/2020	ON152336	ON167611	B	1	Cocker Spaniel	fn	12
164	10/2020	ON152337	ON167612	B	1	Wire-haired dachshund	m	0.5
165	10/2020	ON152338	ON167613	B	1	Small Münsterländer	m	1
166	05/2016	ON152339	ON167614	A	2	Labrador	m	11
167	04/2015	ON152340	ON167615	A	2	Ovtcharka	f	9
168	05/2015	ON152341	ON167616	A	2	Dogue de Bordeaux	m	2
169	06/2015	ON152342	n.a.	A	n.a.	Miniature schnauzer	m	6
170	11/2020	ON152343	ON167617	B	1	Shepherd-Mix	mn	11
172	11/2020	ON152345	ON167618	B	1	Malamute	m	8
173	12/2020	ON152346	ON167619	B	1	Akita Inu	f	7
174	12/2020	ON152347	ON167620	B	1	Jack Russel Terrier-Mix	m	8
175	01/2021	ON152348	ON167621	A	1	Goldendoodle	f	1
176	01/2021	ON152349	ON167622	B	1	Labrador	f	9
177	01/2021	ON152350	ON167623	B	1	Shih Tzu	mn	9
178	03/2021	ON152351	ON167624	B	1	Wire-haired dachshund	m	3.5
179	04/2021	ON152352	n.a.	B	n.a.	Labrador	m	9
180	08/2021	ON152353	ON167583	B	2	Old German shepherd dog	f	3
182	02/2022	ON152354	n.a.	B	n.a.	Old German shepherd dog	f	11
184	09/2021	ON152355	ON167584	B	1	Cattle dog	f	8
185	09/2021	ON152356	ON167585	B	1	Weimaraner mix	m	7
186	09/2021	ON152357	ON167586	A	1	Labrador	m	5
187	09/2021	ON152358	ON167587	A	2	Swiss mountain dog	f	8
188	09/2021	ON152359	ON167588	B	1	Eurasier	m	3
189	10/2021	ON152360	ON167589	B	1	Mongrel	f	8
190	10/2021	ON152361	ON167590	B	1	Rottweiler	m	8
191	10/2021	ON152362	ON167591	B	1	AC Sheepdog	m	4
192	10/2021	ON152363	ON167592	A	2	Swiss Mountain Dog	m	0.9
193	10/2021	ON152364	ON167593	B	1	Mongrel	m	1
194	10/2021	ON152365	ON167594	B	2	AC Sheepdog	fn	5
195	10/2021	ON152366	ON167595	B	2	Border Collie	mn	9
196	10/2021	ON152367	n.a.	B	n.a.	DSH mongrel	fn	10
197	10/2021	ON152368	ON167596	A	2	Labrador	f	4
198	10/2021	ON152369	ON167597	B	1	Mongrel	m	3
199	10/2021	ON152370	ON167598	B	2	Collie mongrel	m	11

(Continues)

TABLE 1 (Continued)

ID	Date*	18s rRNA GenBank†	Bc28.1 GenBank†	Cluster 18S rRNA‡	Cluster Bc28.1§	Breed	Sex¶	Age (years)#
200	10/2021	ON152371	ON167599	A	2	Labrador	m	6
201	11/2021	ON152372	ON167600	B	1	Border collie mongrel	m	12
202	11/2021	ON152373	ON167601	B	2	Mongrel	fn	10
203	12/2021	ON152375	n.a.	B	n.a.	Weimaraner	m	0.5
206	01/2022	ON152376	ON167603	B	2	King poodle	m	0.6

*Date (month/year) of presentation in the Small Animal Clinic.
†GenBank accession number.
‡Cluster according to Figure 1.
§Cluster according to Figure 2.
¶m, male; mn, male neutered; f, female; fn, female neutered.
#Age of dog 161 was not documented.

from Berlin/Brandenburg and the sequence of the subspecies *Babesia canis presentii* parasitizing cats (Baneth et al., 2004) were associated with this haplotype but separated by one or three polymorphisms. The assignment to one of the clusters is in agreement with the presence of a dinucleotide sequence motif at position 160/161 in Figure S1. All sequences assigned to cluster A have a AG motif at this position while all sequences from cluster B show the GA sequence. One sequence from Berlin/Brandenburg (B148b) was separated from both by two polymorphisms and has the sequence AA at position 160/161.

For two samples, multiple clones were sequenced to see whether identical sequences would be identified. For sample 206, the three obtained sequences were identical. However, for the nine sequences from sample 190, five different haplotypes (two associated with each cluster and one outside of cluster A) were found and the maximal distance between haplotypes was seven substitutions. Remarkably, the sequence B190.8 was separated from the major haplotype by three substitution, which is the same distance as the sequence of *B. canis presentii* obtained from a cat that was proposed to represent a separate subspecies (Baneth et al., 2004).

The DNA and protein alignments of all available Bc28.1 haplotypes are shown in Figures S2 and S3, respectively. The Bc28.1 data from Berlin/Brandenburg revealed 12 different haplotypes; none of them was identical with Bc28.1A, B or G from the study performed by Carcy et al. (2015). The phylogenetic network analysis identified two major clusters separated by at least five polymorphisms (Figure 2). Cluster 1 contained 10 haplotypes in total, 5 haplotypes from 29 dogs from Germany and in addition Bc28.1A and 5 sequence from dogs from Latvia and Lithuania, all derived from GenBank. Cluster 2 contained 18 haplotypes altogether, with 7 haplotypes from 28 German dogs plus Bc28.1B and Bc28.1G from GenBank and additional 11 haplotypes from Latvia and Lithuania. Two haplotypes contained sequences from Germany and Latvia. Sequences obtained in this study and Bc28.1A, Bc28.1B and Bc28.1G differed by at least two, two and three polymorphisms, respectively.

For two samples, multiple clones were analysed and the results revealed for both samples that they contained multiple haplotypes. For sample 197, three haplotypes (all from cluster 2) were identified in seven clones while in sample 206 two haplotypes (from clusters 1 and 2) were found in eight clones.

4 | DISCUSSION

The molecular investigations of the 18S rRNA gene showed a separation of the sequences into two groups. Separation of *B. canis* 18S rRNA gene haplotypes with particular emphasis of the dinucleotide at position 160/161 has previously been reported (Paulauskas et al., 2014). Originating from Latvia, only 9% of the samples showed the GA motif in position 160/161 (cluster B) while 91% showed the AG motif as in cluster A. From Poland, a few samples were reported to carry a TT motif at this position (Lyp et al., 2015) but the sequences were not deposited in GenBank and could therefore not be included in the network analysis presented here. Recent studies from Iran showed that both genotypes are present in dogs from the country but numbers of positive dogs that were analysed was too small to speculate about their frequency (Ghasemzade et al., 2021; Khanmohammadi et al., 2021). However, Hrazdilová et al. (2019) reported that in many cases direct sequencing of PCR products produced double peaks indicating simultaneous presence of different haplotypes in the sample. Since rRNA genes are typically multicopy genes, they concluded that different haplotypes correspond to different paralogs in the same genome. However, mixed infections with parasites of different 18S rRNA would be an alternative explanation. Knowledge about 18S rRNA copy number and intro genome variability would be helpful to discriminate between both options but unfortunately the published *B. canis* genome (Eichenberger et al., 2017) is not available so far on PiroplasmaDB. The *Babesia bovis* genome contains three rRNA units (Brayton et al., 2007) and Blastn searches with the 18S sequences from the present study against the genomes of *Babesia divergens* (Jackson et al., 2014), *Babesia bovis* (Brayton et al., 2007) and *Babesia bigemina* (Jackson et al., 2014) identified two, three, and three 18S rRNA loci, respectively. This at least suggests that the high number of five 18S rRNA haplotypes in sample 190 is not only due to diversity of paralogs within a genome but that parallel infections with multiple genotypes also contribute to diversity. The fact

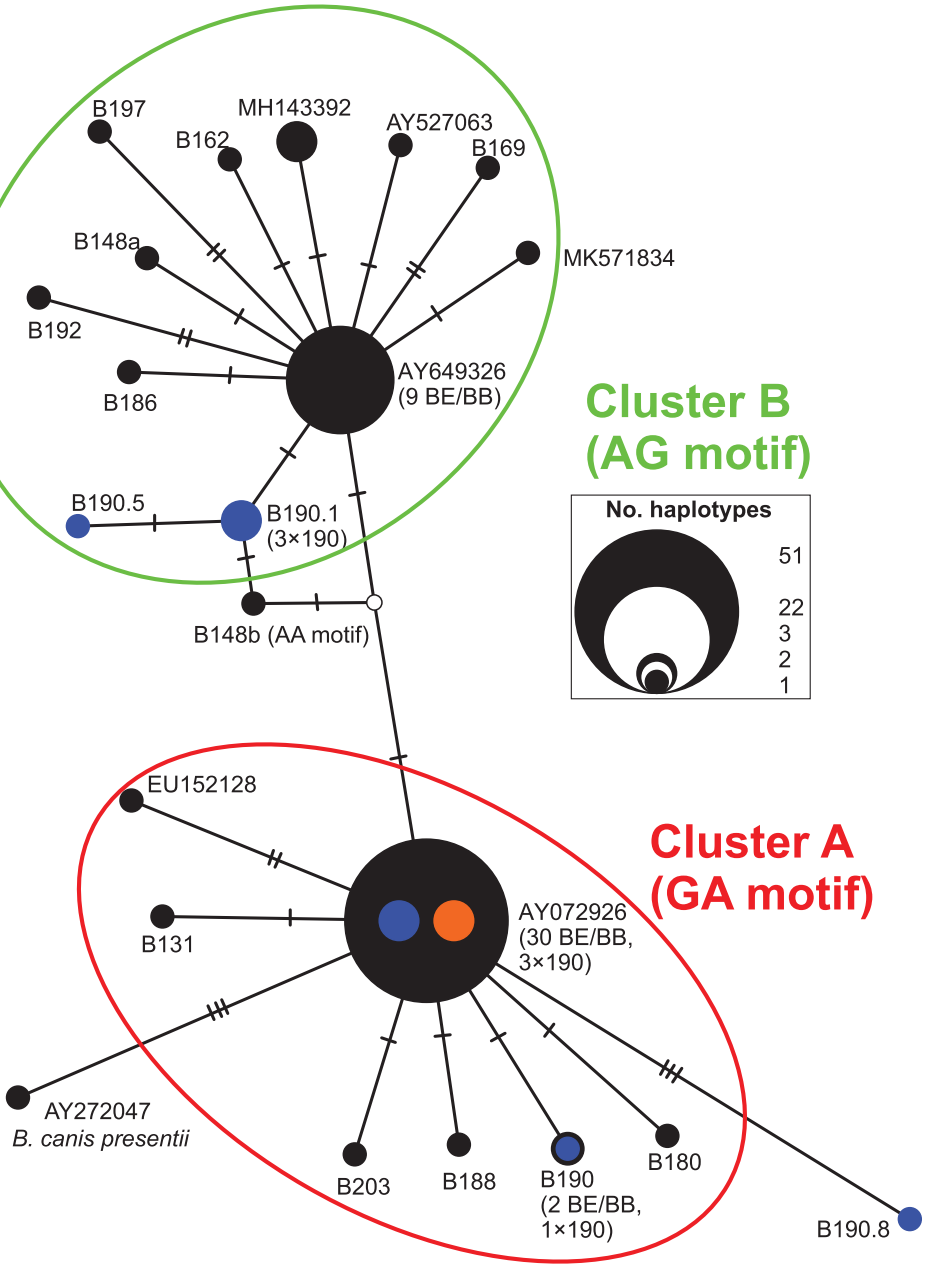

FIGURE 1 Median joining network for partial 18S rRNA sequences (379 bp) from *Babesia canis*. The size of the circles (see legend) corresponds to the number of sequences with identical haplotypes. Haplotypes are labelled with GenBank accession numbers or specimen IDs of one of the sequences. For haplotypes from the present study identical to previously described haplotypes, the number of samples for each haplotypes from Berlin/Brandenburg (BE/BB) is indicated in brackets if there is more than one sample in the haplotype. The number of polymorphisms by which two haplotypes differ is indicated by the number of hatches on the connecting line. For samples 190 (blue) and 206 (orange), eight and three clones were sequenced and labelled e.g. 190.1, 190.2, etc. The number of samples for these samples is also provided in brackets

that intragenome and intergenome variation of 18S rRNA gene copies remain unknown limits to usefulness of ribosomal markers for studies on variation between populations of the same species. However, this does not affect their high power to identify species (Roony, 2004).

In the investigations on the Bc28.1 gene, it is particularly noteworthy that a very high variability in haplotypes was found and none of the haplotypes was identical to the previously published haplotypes by Carcy et al. (2015). Obviously, the restriction fragment length polymorphism analyses leading to only three restriction patterns that were used to assign genotypes to samples (Carcy et al., 2015; Eichenberger et al., 2017), is not able to adequately describe the haplotype diversity. The variability of the Bc28.1 marker (12 haplotypes from 42 dogs) was particularly high when one considers the fact that it was observed in a small geographic area where *B. canis* was only recently introduced. This suggests that the marker is either too polymorphic for informative genotyping to correlate haplotypes with geographic origins or that parasites from very different origins were introduced into the Berlin/Brandenburg area and contributed to the current outbreak.

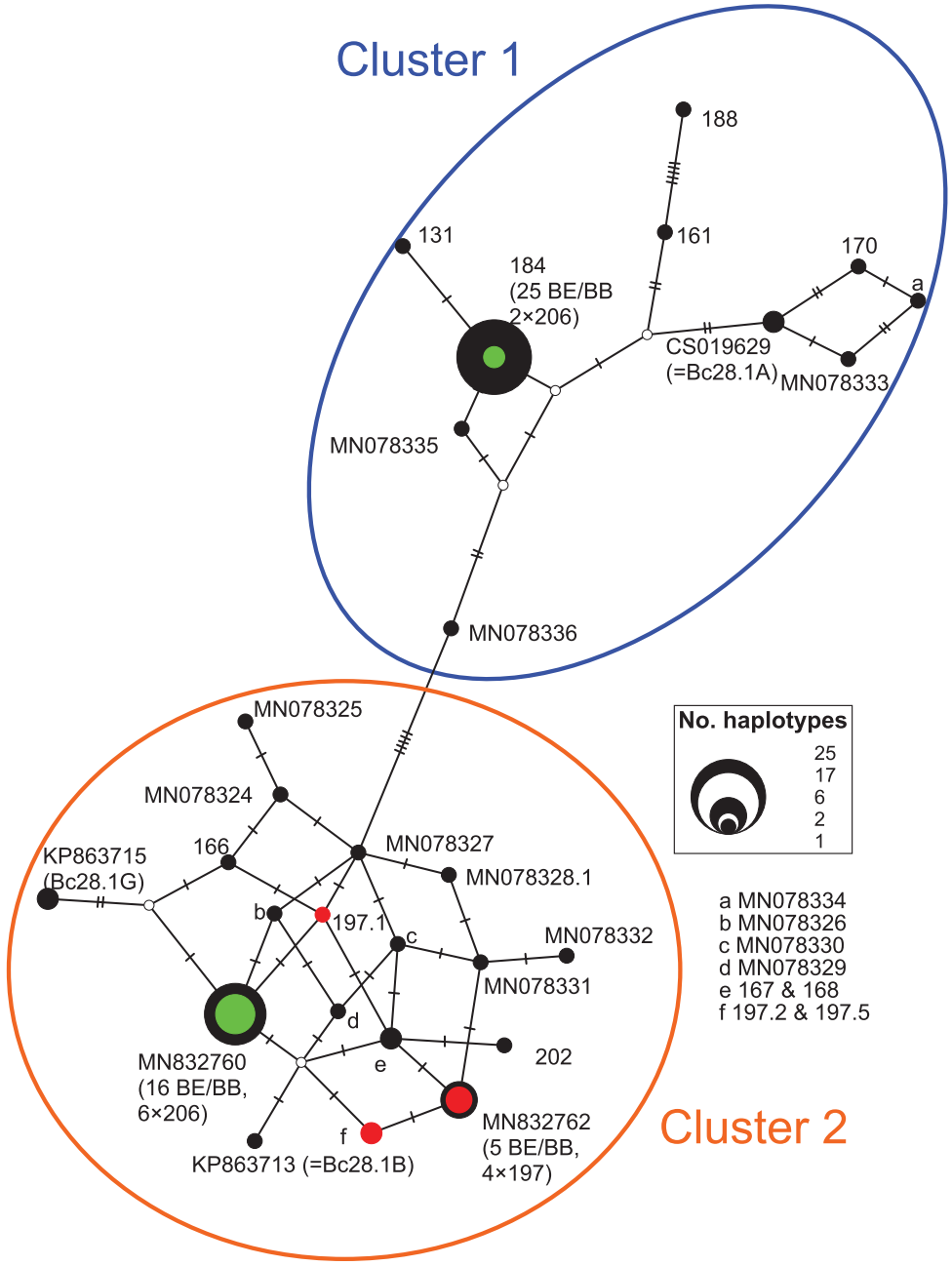

FIGURE 2 Median joining network of Bc28.1 surface antigen sequences (660 bp). The size of the circles (see legend) corresponds to the number of sequences with identical haplotypes. Haplotypes are labelled with GenBank accession numbers or specimen IDs of one of the sequences. For haplotypes from the present study identical to previously described haplotypes from Latvian and Lithuanian dogs, the number of samples for each haplotypes from Berlin/Brandenburg (BE/BB) is indicated in brackets if there is more than one sample in the haplotype. For samples 197 (red) and 206 (green), seven and eight clones were sequenced and labelled, for example, 197.1, 197.2, etc. The number of samples for these samples is also provided in brackets

For Bc28.1, multiple haplotypes were also identified from the same sample/dog. Since Bc28.1 is a single copy gene (Yang et al., 2012), these data strongly support that genetically diverse *B. canis* genotypes co-infect individual dogs. The haplotypes identified within a single dog were very diverse, particularly for dog 206. Therefore, the usefulness of Bc28.1 for genotyping *B. canis* isolates and correlate genotypes with geographic origin or virulence appears to be highly doubtful.

Even if none of the markers could be assigned to a certain geographic origin, the high genetic diversity of the samples at two different genetic loci in a previously non-endemic area is remarkable. In the absence of data regarding variability of these loci in individual dogs and dog populations in endemic countries, it remains unclear if this is exceptional or if *B. canis* populations show such high variation in general. The facts that the 18S rRNA marker is potentially problematic due to intragenomic variability between paralogs and the Bc28.1

markers shows high intergenomic variability even within an individual dog calls for the development of additional markers with intermediate variability and/or the application of deep sequencing approaches to characterize the overall complete variability of these markers on individual dog and tick host level and on parasite population level.

The high number of *B. canis* cases and their increased frequency since autumn 2019 highlight the considerable risk of widespread endemization of this highly pathogenic disease into currently unaffected geographical regions (Bajer et al., 2022). Earlier investigations of *D. reticulatus* activity in Berlin/Brandenburg demonstrated that the ticks show activity throughout the winter months as long as it is not freezing (Kohn et al., 2019). In contrast, almost complete inactivity occurred in the warm summer months July and August (Kohn et al., 2019). This fits with occurrence of clinical signs of the 47 autochthonous *Babesia* infections presented here, with new cases being detected in all month while only one case each was diagnosed in February (coldest month in the region) and August, and none in July (Table 1). The fact that seasonality of acute disease in dogs and of *D. reticulatus* ticks show the same pattern further suggests that transmission is mediated by the local tick population although a direct evidence of *B. canis* in questing *D. reticulatus* from the region is still missing (Dwużnik-Szarek et al., 2021; Dwużnik-Szarek et al., 2022).

This data set demonstrates the autochthonous occurrence and high genetic diversity of *B. canis* infections in dogs in Berlin/Brandenburg. Screening of blood donors and a year-round tick protection with products having repellent or fast killing effects in order to prevent/reduce the risk of transmission of *B. canis* is strongly recommended and must be communicated to dog owners in new endemic foci (Beck et al., 2014; Wardrop et al., 2016; Otranto et al., 2021).

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. All samples used for this study were collected for clinical purposes in the Small Animal Clinic of the Freie Universtät Berlin, Germany, and all patient owners agreed to use samples for research purposes in written form.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank and accession numbers are available in Table 1.

CONFLICT OF INTEREST

Ingo Schäfer is an employee and Elisabeth Müller the CEO of Laboklin, Bad Kissingen, Germany, a company that offers diagnostic tests for animals including tests for infection of dogs with babesiosis. Apart from that, the authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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