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Long-term follow-up of owned, free-roaming dogs in South Africa naturally exposed to *Babesia rossi*

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ABSTRACT

Babesia rossi is an important, tick-borne intraerythrocytic protozoan parasite; however, its natural history and epidemiology is poorly understood. *Babesia rossi* is the most virulent *Babesia* sp. in domestic dogs and is generally considered to cause severe babesiosis, which is fatal if left untreated. However, subclinical infections and mild disease from *B. rossi* have been reported, although the clinical progression of these cases was not reported. Therefore, to better understand *B. rossi* under field conditions, we evaluated its clinical progression and seroprevalence in an owned, free-roaming dog population in Zenzele, South Africa, where the parasite is endemic and prevention is not routine. The entire dog population in Zenzele was monitored intensively at the individual level from March 2008 until April 2014, primarily for a longitudinal study on rabies control. Subsequent evaluation of *B. rossi* comprised analyses of clinical and laboratory data collected from the Zenzele dog population during the 6 year study period. A substantial proportion (31% (n = 34)) of 109 dogs (randomly selected from every available dog in February/March 2010 older than ~6–8 weeks (n = 246)) tested by Indirect Fluorescent Antibody Test had seroconverted strongly to *B. rossi*. All 34 dogs were generally consistently healthy adults, determined from regular clinical examinations between March 2008 and April 2014. Blood smear examinations at multiple time points between July 2009 and February 2011 were also undertaken for almost all of these (34) seropositive dogs and all those tested were consistently negative for *Babesia* spp. Subclinical infections and mild disease were also the main findings for a separate group of 18 dogs positive for *Babesia* spp. on blood smear examination and confirmed to be infected with *B. rossi* by Polymerase Chain Reaction – Reverse Line Blot. Almost all of these dogs were positive at only one time point from repeat blood smear examinations between July 2009 and February 2011. We suggest that these observations are consistent with immunity acquired from repeated, low-level exposure to the parasite, generating transient subclinical infections or mild disease. Should this be the case, the use of tick control, particularly in adult dogs in free-roaming populations in *B. rossi* endemic regions, should be carefully considered.

1. Introduction

The intraerythrocytic protozoan *Babesia rossi* is an important cause of morbidity and mortality in domestic dogs throughout sub-Saharan Africa (Oyamada et al., 2005; Sasaki et al., 2007; Schoeman, 2009; Nalubamba et al., 2015; Proboste et al., 2015). In South Africa, the parasite is transmitted between dogs by the tick *Haemaphysalis elliptica* (Apanaskevich et al., 2007; Birkenheuer, 2014) and causes babesiosis in dogs throughout the year, with incidence peaking during the summer (Shakespeare, 1995; Collett, 2000; Birkenheuer, 2014). The disease occurs mostly in young dogs and a median age of 15 months has been reported, although mature dogs can also be affected (Schoeman et al., 2007; Schoeman, 2009).

Babesia rossi is the most virulent of the *Babesia* spp. in domestic dogs, often causing acute and peracute disease with severe clinical signs including anorexia, depression, weakness, fever, pale mucous membranes, tachycardia and tachypnoea in infected dogs (Schoeman and Leisewitz, 2006; Penzhorn, 2011; Leisewitz et al.,

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2019a). Clinical signs are attributed to haemolysis, precipitating anaemia and tissue hypoxia (Taboada and Lobetti, 2006), and an excessive pro-inflammatory response (Goddard et al., 2016; Leisewitz et al., 2019b), leading to hypotension and shock as described in *Babesia canis* infections (Matijatko et al., 2009; Zygner et al., 2014). Thrombocytopenia is also a very frequent finding (Kettner et al., 2003), including in the absence of anaemia (Taboada and Lobetti, 2006; Birkenheuer, 2014). The disease is often fatal even with treatment, with approximately 10% mortality in hospitalised dogs (Schoeman and Leisewitz, 2006; Birkenheuer, 2014). Indeed, infection in dogs left untreated is reported to be almost invariably fatal (Matjila et al., 2009). However, there are reports of dogs infected with *B. rossi* without overt clinical findings (Matjila et al., 2004; Birkenheuer, 2014), although the clinical progression of these cases, and treatment provided, was not reported. Consequently, it is unclear if these subclinical infections were incubatory or convalescent, preceding or following fulminant babesiosis, or were unassociated with severe babesiosis. Furthermore, the duration of an (original) infection with *B. rossi* is unclear. While "most infectious agents do not have the potential for persistent infection [chronic carrier state]" (Rothman et al., 2008, p. 551 and p. 559), a chronic carrier state is reported for *Babesia* spp. in domestic dogs (Irwin, 2009; Birkenheuer, 2014), although not for *B. rossi* specifically. Cases of mild disease from *B. rossi* have also been reported; but the clinical and treatment histories of these cases were also not reported (Adamu et al., 2014).

While the clinical progression of cases of fulminant babesiosis from *B. rossi* presented to veterinary facilities for treatment is well described, the natural history and epidemiology of *B. rossi* infections is not. Therefore, to better understand *B. rossi* infection under field conditions, we investigated the progression of infections with the parasite, and its seroprevalence, in an owned, free-roaming dog population in South Africa, where *B. rossi* is endemic and tick control and treatment for babesiosis are not routine.

Note: Only Rothman's (2008, pp. 551–552; Supplementary Table S1) definitions for infection, disease and chronic carrier are used throughout this paper. Acquired immunity refers to "antigen specific immunity attributable to the production of antibody and specific immune T lymphocytes *following* exposure to an antigen" (Blood et al., 2007, p. 941); and results from repeated exposure to the pathogen (Bousema et al., 2014; Fowkes et al., 2016).

2. Materials and methods

2.1. Study population

Zenzele is an informal settlement approximately 10 km west of Johannesburg (26°15′27.19″S and 27°41′45.68″E) with an area of 0.72 km^2. All of the dogs resident in Zenzele were owned and the majority were allowed to roam freely. Almost all of the dogs in the settlement were mixed breed. For the period February 2008–February 2011, the mean population size for dogs ~8 weeks of age and older was 323, with a median population size of 321 and range of 285–362; at least 40% of new dogs were acquired from outside the community; and 81.6% of new dogs were acquired as puppies (0–13 weeks of age) (Morters et al., 2014b). Subjectively, the population size remained within the same range from March 2011 to April 2014. Other than vaccinations against rabies in October 2009 (by the Department of Agriculture, Forestry and Fisheries [DAFF], South Africa) and February 2010 (by the primary researcher), the vast majority of the study population did not have access to veterinary care before May 2011. Some dogs vaccinated in October 2009 may have received one dose of ivermectin with the vaccination. From May 2011, charity operated mobile veterinary services have been available in Zenzele.

2.2. Overview of data collection and analyses

The entire dog population in Zenzele was monitored intensively at the individual level from March 2008 until April 2014, primarily for a longitudinal study on rabies control. Details of the study on rabies control have been published elsewhere (Morters et al., 2014a,b). To evaluate the clinical progression of *B. rossi* infection in dogs in Zenzele, the longitudinal clinical and laboratory data generated from the rabies control study were reviewed for two separate groups of dogs whose blood samples had been stored during the rabies control study and were subsequently shown to be positive for exposure to *B. rossi*. The two groups of dogs comprised (i) all those positive by Polymerase Chain Reaction (PCR) – Reverse Line Blot (RLB) in July 2009, February 2010 and/or February 2011; and (ii) all those positive by Indirect Fluorescent Antibody Test (IFAT) in February or March 2010. Seroprevalence for *B. rossi* was also estimated from the second group of dogs. The collection and analyses of the clinical and laboratory data for the rabies control study and analyses of the stored blood samples for this evaluation of *B. rossi* are summarised below. Further details are provided in the Supplementary Methods S1–S3.

2.3. Demographic and clinical data collection

The dog population in Zenzele was highly dynamic, with new dogs frequently acquired into, and resident dogs lost from, the settlement during the 6 year rabies control study period (from March 2008 until April 2014). During this period, demographic and longitudinal clinical data were collected for each dog in Zenzele for the entire time it was in the settlement, which includes all of the dogs reported in this evaluation of *B. rossi*. The clinical data were collected by physical examination and owner questionnaire during household visits undertaken about every 7–15 weeks. Medical records from the mobile veterinary service and owners were checked from May 2011. See Supplementary Methods S1 for a detailed description of the demographic and clinical data collection.

2.4. Blood sample collection and laboratory analyses for the rabies control study

Blood samples were also collected during household visits. The sampling regime (i.e. sample size and sampling dates) was also designed specifically for the rabies control study. See Table 1. Blood smear examinations, complete blood counts (CBCs) and blood protein assays were done for all dogs sampled at four time points (July 2009, February 2010, August 2010 and/or February 2011). See Supplementary Methods S2 for a detailed description of blood sample collection and the laboratory methods for the tests noted above. The sera from all four time points, as well as from an additional time point in March 2010, were stored. The whole blood from all (15) dogs positive for *Babesia* spp. on blood smear examination in July 2009, February 2010 and/or February 2011 was stored. Three other dogs were positive for *Babesia* spp. on blood smear examination in August 2010, however their whole blood samples were inadvertently not stored. Blood samples from the dog used as the positive control for the IFATs (see Section 2.5) were also stored.

2.5. Laboratory analyses of the stored blood samples for the evaluation of *B. rossi*

All (15) dogs with stored whole blood samples (noted in Section 2.4) comprise the first group of dogs listed under Section 2.2. All 15 samples were tested by PCR-RLB for *B. rossi*, *Babesia vogeli*

Table 1
The laboratory analyses used to evaluate the clinical progression and seroprevalence of *Babesia rossi* in dogs in Zenzele, South Africa.

Samples collected, tests used and test results	July 2009	February 2010	March 2010	August 2010	February 2011
Number of dogs blood sampled (whole blood and serum)	135[a]	191[b]	63[c]	134[d]	103[d]
Number subjected to CBC, blood protein assay & venous blood smear exam	135	191	–	134	103
Number subjected to capillary blood smear exam	135	~120	–	–	–
Number positive for *Babesia* spp. on blood smear exam (n = 18)	3	8	–	3	4
Number blood smear positive tested for *B. rossi* by PCR-RLB	3	8	–	–[e]	4
Number positive for *B. rossi* by PCR-RLB (see Supplementary Table S4)	3	8	–	–	2[f]
Number negative for *Babesia spp.* on blood smear exam	132	183[g]	–	131	99
Number screened for exposure to *B. rossi* by IFAT (n = 109)[h]		85	24		
Number (strong) positive for exposure to *B. rossi* by IFAT (n = 34)		28	6		
Number borderline strong positive for exposure to *B. rossi* by IFAT (see Supplementary Table S3)		3	0		

[a] Random sample (of 135 dogs) taken from every available dog older than ~6–8 weeks of age in Zenzele in July 2009.
[b] Every available dog (i.e. the entire population) older than ~6–8 weeks of age in Zenzele in February 2010 not vaccinated by the Department of Agriculture, Forestry and Fisheries (DAFF), South Africa, in October 2009; includes those dogs sampled in July 2009, not vaccinated in October 2009 and still present in Zenzele in February 2010.
[c] Every available dog vaccinated by the DAFF in October 2009 still present in Zenzele in March 2010. Vaccination by the DAFF was not observed by the primary researcher, therefore dogs vaccinated by the DAFF were excluded from the rabies control study laboratory analyses (only sera were collected to evaluate antibody responses to the vaccine, including in March 2010).
[d] Of the 191 dogs blood sampled (and concurrently vaccinated) in February 2010 by the primary researcher, 134 and 103 dogs were still present in Zenzele in August 2010 and February 2011, respectively, and sampled; therefore, the dogs sampled the February 2010 were also sampled up to three more times (in July 2009, August 2010 and/or February 2011) according to their availability.
[e] The whole blood samples for the three dogs blood smear positive in August 2010 were inadvertently not stored by the laboratory. One other dog (Supplementary Table S4, dog 9) positive for *B. rossi* by Polymerase Chain Reaction (PCR) – Reverse Line Blot (RLB) in February 2010 was also positive on blood smear examination for *Babesia* spp. in August 2010 – the August 2010 whole blood sample for this dog was also inadvertently not stored.
[f] Very weak positive for the genus *Babesia* (species not determined) for one sample and no nucleic acid detected for one sample.
[g] Excludes the eight dogs PCR-RLB positive for *B. rossi* because the exposure status was already determined by PCR-RLB.
[h] Random sample of 109 dogs selected from the total pool of 246 (183 dogs in February 2010 and 63 dogs in March 2010 (note: excludes the positive control dog's March 2010 sample)).

and *B. canis* using a previously described method (Matjila et al., 2004) with a sensitivity of at least 99% for each *Babesia* sp. There was no evidence of *B. vogeli* or *B. canis* in Zenzele; the samples tested positive for *B. rossi* only. This agrees with previous reports of the geographic distribution of *B. vogeli* in South Africa (Matjila et al., 2008), and no reports of *B. canis* in South Africa. Therefore, antibodies restricted to *B. rossi* antigen were measured by IFAT using the Onderstepoort Veterinary Institute, South Africa, protocol described in Supplementary Methods S3. To obtain the second group of dogs listed under Section 2.2, IFATs were undertaken on a sample of the aforementioned stored sera. The sample consisted of 109 sera (from 109 dogs), which were randomly selected from the 183 dogs *Babesia* spp. negative on blood smear examination in February 2010 and the 63 dogs vaccinated by the DAFF in October 2009 still present in Zenzele in March 2010 (see Table 1). This pool of 246 sera comprised the largest number of samples available when titres to *B. rossi* were expected to peak (at the end of the summer babesiosis season). The arbitrary sample size of ~100 sera was considered large enough (considering the exploratory nature of this study) to gauge seroprevalence in Zenzele.

The negative control for the IFATs was residual serum obtained from a blood donor dog registered with the Department of Veterinary Medicine, University of Cambridge, UK, who had recently undergone a standard health check, including routine haematology and biochemistry, to evaluate fitness for blood donation (Supplementary Table S2). This dog was an 11 year old, female neutered Labrador that had not travelled outside of the UK, a country where *Babesia* spp. affecting dogs are not endemic and where sporadic cases of *B. canis* have only recently been recognised in southeastern England (Fernandez de Marco et al., 2017). The negative control, owned by a veterinarian, had no clinical history of babesiosis, was negative for *Babesia* spp. on PCR and had mostly normal haematological and biochemical parameters. This dog's serum consistently fluoresced negative on IFAT, i.e. it had no intraerythrocytic piriform bodies visible or very dull fluorescence of intraerythrocytic piriform bodies (Supplementary Fig. S1).

The positive control serum for the IFATs was provided by a young adult, male mixed-breed dog born in Zenzele in July 2008 (Supplementary Table S2). This dog was diagnosed with severe babesiosis in November 2009, recovered fully without treatment and was still present in Zenzele in April 2014. Babesiosis caused by *B. rossi* was confirmed by blood smear examination and PCR-RLB for January 2010. Intraerythrocytic piriform bodies consistently fluoresced (strong) seropositive (i.e. bright apple green; Supplementary Fig. S2) on IFAT using serum collected at the same time in January 2010 (when his antibody titre peaked).

The (109) February and March 2010 test sera were first tested in batches of 10 at 1:40 dilution, i.e. each of the 10 wells on the antigen slide contained the serum of a different dog and the remaining two wells contained the positive and negative control sera. Thus, each dog's serum was tested once at 1:40 dilution for seroconversion to the antigen. In general, any test serum at a 1:40 dilution that fluoresced more strongly than the negative control was then tested at a dilution of 1:80. The degree of fluorescence was gauged at the time of microscopy but subsequently more objectively determined by careful evaluation of all of the recorded images for each test serum (at least six high resolution, representative digital images were recorded for each well, including the control wells). Only digital image titres are reported in Section 3. Any test serum that fluoresced at least as intensively as the positive control at 1:80 dilution on the majority of the digital images was defined as (strong) seropositive (Birkenheuer, 2014). The degree of fluorescence on the digital images was broadly categorised as seronegative (fluorescence $\leq$ the negative control), weak seropositive, moderate seropositive, borderline strong seropositive and strong seropositive (fluorescence $\geq$ the positive control).

Stored sera belonging to the first group of dogs listed in Section 2.2 were tested at 1:40 dilution only to determine which were seronegative.

3. Results

Overall, the vast majority of the dogs in Zenzele confirmed as exposed to *B. rossi* by IFAT or PCR-RLB were generally consistently healthy or had transient mild disease as a result. The longitudinal clinical and laboratory data for each of these exposed dogs are presented in their entirety in Supplementary Tables S3 and S4 to allow verification of this finding. The contents of Supplementary Tables S3 and S4 are summarised below, with reference to the Tables for specific cases. The group of dogs positive for exposure to *B. rossi* by IFAT is reported first; and the group positive on PCR-RLB is reported second. Note: Normal ranges for haematocrits and platelet counts are 0.37–0.55 l/l and 200–500 × 10^9/l, respectively.

3.1. Seroprevalence and the clinical progression of B. rossi in the dogs confirmed positive for exposure by IFAT in February and March 2010

The prevalence of dogs seropositive for *B. rossi* in February and March 2010 was 31% (34 dogs seropositive (fluorescing at least as strongly as the positive control at 1:80 dilution against *B. rossi* antigen) out of a total of 109 dogs tested). All (34) of these dogs also fluoresced strongly against *B. rossi* antigen at 1:40 dilution. See Table 1. From this observation, there is 95% certainty that the true seroprevalence in the wider population in early 2010 was between 22% and 40% (Kirkwood and Sterne, 2003, p143). The 34 (strong) seropositive dogs are numbered 1–34 in Supplementary Table S3 (column A). Supplementary Table S3 also shows the date and age each dog was registered (i.e. first observed in Zenzele) (columns AO and AP); the date and reason each dog left the study population or the date it was last evaluated (column AQ); and each dog's clinical and laboratory findings during the intervening period (columns AS-AU and D-AN, respectively). Note: All of the dogs in Supplementary Table S3 were acquired as puppies by their owners, except for dogs 3, 11, 16, 27 and 30 which were acquired as juveniles, and dogs 21 and 34 which were acquired as adults.

The majority of the 34 (strong) seropositive dogs were monitored for at least 12 months before, and until at least 7 months after, blood sampling in February or March 2010. All 34 dogs were generally healthy throughout the period they were monitored, with no clinical signs observed or reported consistent with serious disease caused by *B. rossi*. Haematocrits and platelet counts were evaluated in July 2009, February 2010, August 2010 and/or February 2011 for 31 of these (strong) seropositive dogs. Values ranged between 0.28–0.58 l/l and 137–786 × 10^9/l, respectively, consistent with no clinical findings of severe babesiosis. Representative examples of dogs strongly seropositive include dogs 5 and 7, which were registered in March 2008 as adults, left Zenzele in July 2012 and about September 2013, respectively, were healthy throughout the intervening period and had normal haematocrits and platelet counts (and negative blood smear examinations, see below) at all four time points. Another representative example is dog 3, which was registered in November 2008 as a juvenile, was present in Zenzele in January 2014, had four episodes of non-specific clinical signs during the intervening period, including slightly pale mucous membranes in May 2010 and February 2011, and had normal haematocrits and platelet counts (and negative blood smear examinations) in February 2010, August 2010 and February 2011, except for a haematocrit of 0.31 l/l in February 2011. The exception to this overall trend in the data was dog 30, which died in February 2010, the same month it was blood sampled. A neighbour reported that dog 30 died of injury, however this could not be confirmed with the owner. Given the laboratory results in February 2010 (haematocrit 0.30 l/l, platelet count 112x10^9/l and positive for *Anaplasma platys* on blood smear examination), the possibility that dog 30 died from a tick-borne disease cannot be ruled out.

Thirty-seven dogs (as listed in Supplementary Table S3) were recorded as (strong) seropositive at the time of microscopy; however, three of these dogs (dogs 35–37) were subsequently recategorised as borderline strong seropositive following careful examination of their digital images. These three dogs were also monitored in Zenzele for an extended period (from at least 14 months before, and until 6 months after, being blood sampled in February 2010). The clinical and laboratory findings for these (three) dogs are similar to dogs 1–34 discussed above.

Thirty-one of the 37 seropositive dogs were blood sampled in February 2010 and six were sampled in March 2010. As described in Section 2.5, all 31 dogs were negative on blood smear examination for *Babesia* spp. in February 2010. Blood smears were examined for blood parasites at least twice for 28 of the 31 dogs sampled in February 2010 (i.e. 28 dogs were also sampled in July 2009, August 2010 and/or February 2011). All 28 dogs were also negative for *Babesia* spp. at all of the other time points (Supplementary Table S3 columns D/E, N/O, X and AG).

Figs. 1 and 2 show the age distribution of the 109 seropositive and seronegative dogs determined by IFAT, including the 37 seropositive dogs detailed in Supplementary Table S3. No dog was (strong) seropositive at a dilution of 1:80 below the 11th month of life. Dogs seronegative at a dilution of 1:40 and, thus, not tested at a dilution of 1:80 were treated as seronegative in Fig. 1.

3.2. The clinical progression of B. rossi in the dogs confirmed infected by PCR-RLB in July 2009, February 2010 or February 2011

As noted under Section 2.4, 18 dogs were positive for *Babesia* spp. on blood smear examination (see Table 1). Blood smear examination was undertaken for 11 of the dogs at more than one of the (July 2009, February 2010, August 2010 and February 2011) time points and for seven of the dogs at only one of the time points (Supplementary Table S4 columns D/E, N/O, X and AG). Of the 11 dogs tested at multiple time points, 10 were positive at only one of the time points and were negative at all other time points tested, and one dog (Supplementary Table S4, dog 9) was positive at two of the time points tested. Of the 15 dogs positive for *Babesia* spp. on blood smear examination subject to PCR-RLB, 13 were positive for *B. rossi* only, one was very weak positive for the genus *Babesia* but the species could not be determined and nucleic acid was not detected for one (Supplementary Table S4 column AO). Poor sample quality, from prolonged storage (at −80 °C) and the small volume of DNA remaining from the PCR-RLB assays initially undertaken in June 2012, may have been responsible for the repeat negative PCR-RLB results in April 2018. Laboratory error is suspected for the negative PCR-RLB results in June 2012. As expected, most (11) of the dogs were positive on blood smear examination in the summer, six were positive in the winter and one (dog 9) was positive in the summer and winter.

The demographic, clinical and laboratory data for each of the 18 dogs positive for *Babesia* spp. on blood smear examination are reported in Supplementary Table S4. This includes the date and age each dog was registered (i.e. first observed in Zenzele) (columns AR and AS); the date and reason each dog left the study population or the date it was last evaluated (column AT); and each dog's clinical and laboratory findings during the intervening period (columns AW-AY and D-AP, respectively). Note: All of the dogs listed in Supplementary Table S4 were acquired as puppies by their owners, except for dogs 11 and 15 which were acquired as juveniles. Overall, laboratory and clinical findings for the 18 dogs vary from subclinical infection to fatal babesiosis caused by *B. rossi*, but are predominately consistent with subclinical infection or mild disease.

Fatal babesiosis was confirmed in one juvenile (dog 10) and suspected in one other juvenile (dog 15) and one (probably) very old

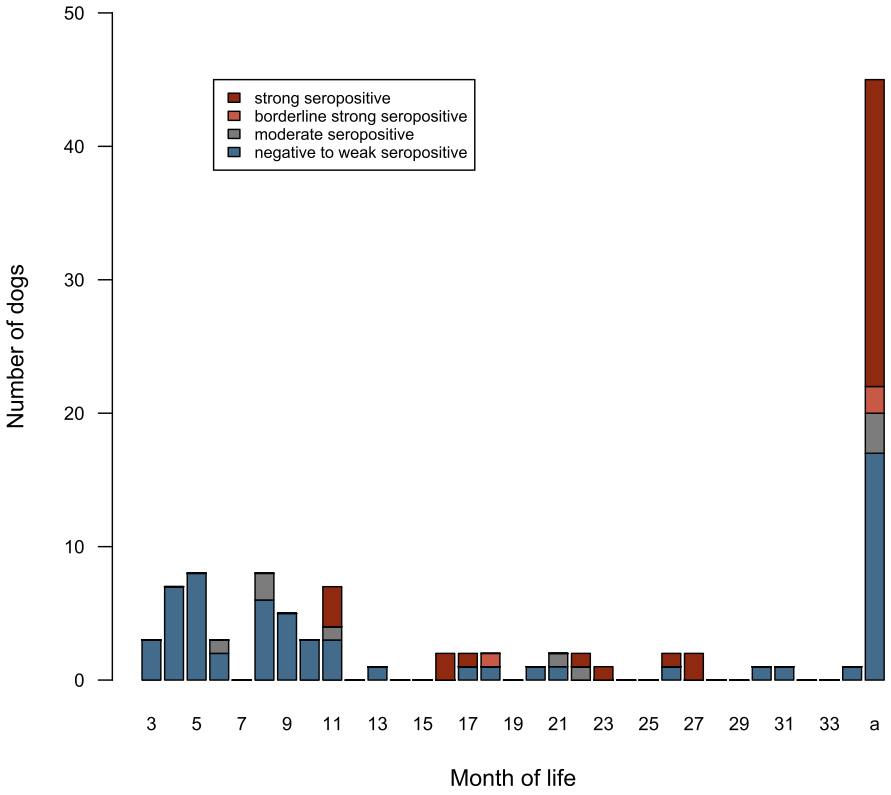

Fig. 1. Age distribution of the fluorescence for the 109 dogs from Zenzele, South Africa, from which (109) samples were taken and subjected to immunodiagnostics by IFAT at a dilution of 1:80. 'a' on the X-axis refers to adult dogs that were >52 weeks of age when registered into the study population, so their true age could not be determined by direct observation.

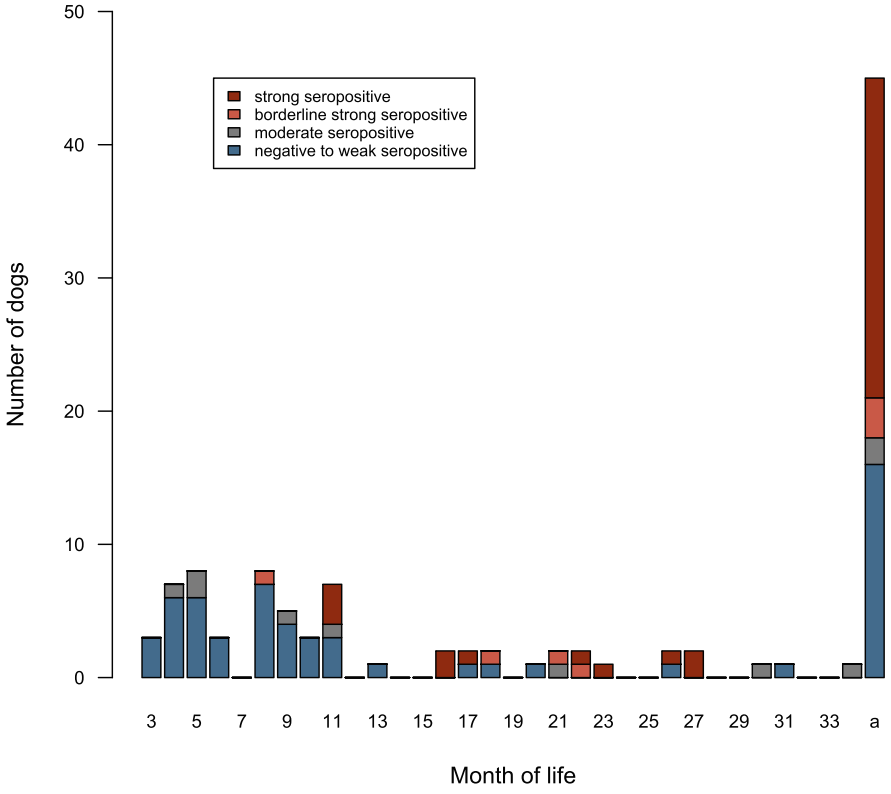

Fig. 2. Age distribution of the fluorescence for the 109 dogs from Zenzele, South Africa, from which (109) samples were taken and subjected to immunodiagnostics by IFAT at a dilution of 1:40. 'a' on the X-axis refers to adult dogs that were >52 weeks of age when registered into the study population, so their true age could not be determined by direct observation.

adult (dog 4). All three dogs were positive for *B. rossi* by PCR-RLB shortly before their deaths, coinciding with pathognomonic clinical signs and/or low haematocrits (0.20–0.28 l/l) and platelet counts (34–114 × 10^9/l). Three adults (dogs 1, 2 and 5) had transient clinical signs indicative of mild babesiosis, and dogs 2 and 5 also had low haematocrits (0.14 and 0.20 l/l) and platelet counts (~75 × 10^9/l). These findings coincided with a positive blood smear for *Babesia* spp. (but negative PCR-RLB result) for dog 1, PCR-RLB confirmation of *B. rossi* for dog 2 and PCR-RLB confirmation for *Babesia* genus for dog 5; all in February 2011. All three dogs were monitored for at least 12 months before, and 7 months after, diagnosis. Other than occasional slightly pale mucous membranes for dog 2, there were no clinical or laboratory findings consistent with repeat episodes of babesiosis.

The remaining nine adults and three juveniles were monitored for several months to years before and after being found positive for *B. rossi* by PCR-RLB or (for those dogs sampled in August 2010) positive for *Babesia* spp. on blood smear examination; with eight of the dogs monitored for at least 6 months before, and nine for at least 6 months after, diagnosis. During their observation periods, all 12 dogs had either no clinical signs or non-specific, mostly occasional clinical signs, including slightly pale mucous membranes for three of the dogs. These clinical signs occurred at the same time point as a positive PCR-RLB for *B. rossi* or blood smear examination for *Babesia* spp. for only two dogs (8 and 9). Haematocrits and platelet counts corresponding to the time points the dogs were positive on PCR-RLB or blood smear ranged from 0.17-0.40 l/l and 33-280x10^9/l, respectively. Seven of the (12) dogs were blood sampled at more than one of the four time points. Haematocrits and platelet counts corresponding to the time points the dogs were negative on blood smear ranged from 0.27–0.51 l/l to 74–772 × 10^9/l, respectively, but were mostly normal. Of note is dog 12, which had an apparently subclinical infection of *B. rossi*. This dog was registered in the study population as an adult 2 years prior to testing positive for *B. rossi* on PCR-RLB in February 2010, was still present in the study population in December 2013, had no clinical findings consistent with babesiosis throughout the intervening period, had normal haematocrits and platelet counts (other than a haematocrit of 0.31 l/l in February 2011) and negative blood smear examinations for *Babesia* spp. in July 2009, August 2010 and February 2011.

Of the dogs evaluated for seroconversion to *B. rossi* by IFAT at a dilution of 1:40 (Supplementary Table S4 column AP), the three dogs (16, 17 and 18) positive for *Babesia* spp. on blood smear examination in August 2010 that were not tested by PCR-RLB were (strong) seropositive (in August 2010); and three dogs (6, 10 and 13) PCR-RLB positive for *B. rossi* did not seroconvert or mounted only a weak humoral response to infection. Of these, the juvenile dog 10 was positive for *B. rossi* by PCR-RLB and had a haematocrit of 0.28 l/l and platelet count of 37 × 10^9/l on 9 February 2010, but had no clinical findings of babesiosis and was negative by IFAT at this time point. By mid-March, this dog had developed severe babesiosis but was only moderately seropositive shortly before being euthanized upon clinical deterioration. Similarly, the adult dog 6 tested positive for *B. rossi* by PCR-RLB and had a parasitaemia of 3+ (i.e. approximately 3% parasitaemia) by blood smear examination in February 2010 but was simultaneously negative by IFAT and had no clear clinical findings of babesiosis throughout the 3 years it was monitored.

3.3. Treatment provided by the mobile veterinary services from May 2011

Thirty-seven dogs, including the positive control dog, were still present in Zenzele in May 2011 (Supplementary Tables S2, S3 column AQ and S4 column AT). According to the owner, dog 28 in Supplementary Table S3 was diagnosed with *Babesia* spp. infection by the mobile veterinary services in February 2012; however, there is no clinic record of this or that the dog received any treatment from the mobile clinic. No other dog reported in Supplementary Tables S3 and S4 was diagnosed with or treated for *Babesia* spp. infection by the mobile veterinary services from owner reports and clinic records.

Occasional tick prophylaxis was provided for some of the 37 dogs while they were still present in Zenzele from May 2011 until April 2014. Ten dogs were treated once with topical permethrin and two of these dogs were treated again, 1–2 months after the first dose. Seventeen dogs received one dose of ivermectin; six of these dogs received one more dose and three received two more doses. The interval between doses of ivermectin varied from one to about 11 months. With one exception, permethrin was given at the same time as ivermectin.

3.4. Frequency of tick infestations

In general, ticks were a very common finding on examination throughout the study period, with ticks observed at least once for 89% of the borderline strong and strong seropositive and 84% of the negative to moderate seropositive dogs examined (Fig. 1).

4. Discussion

The longitudinal clinical and laboratory data and blood samples collected for the rabies control study in Zenzele between March 2008 and April 2014 were sufficient to allow a reasonably comprehensive clinical picture of *B. rossi* in the free-roaming dogs in the settlement to be generated retrospectively. Although the design of the rabies control study constrained the laboratory data to four time points, in combination with the longitudinal clinical data we observed a range of clinical and laboratory findings in dogs exposed to *B. rossi* in Zenzele, the majority consistent with subclinical infection and mild disease in adult dogs. This is a novel finding, contrary to the prevailing view that infection with *B. rossi* is invariably highly virulent.

One possible explanation for our observations is that the majority of the mature dogs in our study population had acquired immunity to *B. rossi* through repeated, low-level exposure to the parasite from a young age. We suggest immunity to *B. rossi* acquired through repeated, transient infections for several reasons. Firstly, acquired immunity has been observed under experimental conditions for *B. rossi* (Lewis et al., 1995) and *B. vogeli* (Brandao et al., 2003) and under field conditions for *B. canis* (Martinod et al., 1985). Secondly, it is well established that humans in malaria endemic areas acquire immunity to *Plasmodium* spp. through repeated exposure to the pathogen (Langhorne et al., 2008; Doolan et al., 2009; Bousema et al., 2014; Fowkes et al., 2016; Triller et al., 2017); and *Plasmodium* spp. are virulent, intraerythrocytic protozoa comparable to *B. rossi*. And thirdly, the resultant epidemiological picture of malaria in endemic regions is similar to that for *B. rossi* in Zenzele.

In malaria endemic regions, non-sterilising acquired immunity to the parasite increases with age, from repeated exposure to the pathogen and the establishment of immunological memory (Langhorne et al., 2008; Doolan et al., 2009; Bousema et al., 2014; Fowkes et al., 2016). Consequently, severe disease from malaria primarily affects young children, with an estimated case fatality rate of 2% in African children under 5 years of age (Doolan et al., 2009). While immune individuals in malaria endemic areas are generally continually parasitaemic from continual re-infection, “in the absence of continual exposure solid immunity against severe disease is apparently relatively short lived” (Doolan

et al., 2009 p. 14 and p. 17). Similarly, in Zenzele repeated exposure to *B. rossi* and the acquisition of immunity with age is plausible. With respect to repeated exposure, the vast majority of dogs resident in Zenzele (including those reported in this study) were acquired as puppies by their owners (Morters et al., 2014b) and ticks were commonly found during the physical examinations; confirmed by the observed seroprevalence, which shows a large (31%) proportion of the dog population had been bitten by infected ticks (probably also recently given the strength of seroconversion). On this basis, it is unlikely the seronegative adult dogs reported in this study were never exposed to the parasite. Titres may have waned in these dogs through lack of recent exposure, with an anamnestic immune response expected following re-infection. With respect to age-related immunity, mature dogs reported in this study were apparently unaffected, or not seriously affected, by exposure to *B. rossi*; none of the (34) dogs (strong) seropositive to *B. rossi* were young juveniles (Figs. 1 and 2); and fatal babesiosis was suspected only in young juveniles (Supplementary Table S4, dogs 10 and 15) and what may have been a very old dog (Supplementary Table S4, dog 4), consistent with the aforementioned general literature (Schoeman et al., 2007; Schoeman, 2009).

With respect to possible drivers of acquired immunity to *B. rossi* in dogs, the seasonal trough in exposure to *B. rossi* might act as a driver, with challenge from the pathogen during the winter months being sufficient to induce immunity but insufficient to induce clinical disease (Doolan et al., 2009). Concomitant acquired immunity to the ticks and/or grooming might also disrupt transmission and, thus, reduce the number of sporozoites transmitted per tick bite (Martinod et al., 1985). Lastly, given that female adult dogs in free-roaming dog populations do not routinely receive tick prophylaxis, protection from maternally derived antibody, boostered through concurrent environmental exposure culminating in life-long acquired immunity, should be considered.

Our observations further suggest that infections with *B. rossi* in Zenzele were transient, consistent with Rothman's view (Rothman et al., 2008, p. 559); rather than persistent in the absence of re-exposure, indicative of a chronic carrier state (as defined by Rothman et al., 2008, p. 551). Firstly, the 31 seropositive dogs also tested for *Babesia* spp. by blood smear examination were consistently negative on blood smear examination across multiple time points, including at the time they were seropositive in February 2010 (Supplementary Table S3). Given that blood smear examination detects parasitaemia as low as 0.0002% (Woolley et al., 2017), and the expertise of the laboratory staff, it is highly likely these dogs were not infected with *Babesia* spp. when they were tested, rather than being persistently infected with infection being persistently missed. Similarly, all of the dogs that were positive for *Babesia* spp. by blood smear examination were only ever positive at one of multiple time points tested (Supplementary Table S4). The only exception was dog 9, where re-infection between successive (blood smear positive) time points could have occurred. As discussed above, an effective immune response rapidly clearing infection upon re-exposure (including shortly before February 2010) cannot be discounted. Nonetheless, blood smear examination was limited to up to four time points, with an interval of at least 6 months between time points; the dogs listed in Supplementary Table S3 were not tested by PCR-RLB which is more likely to detect *B. rossi* (Takeet et al., 2017; Woolley et al., 2017), either viable organisms or residual break-down products following infection; and variations in titres with time and laboratory and clinical findings was not evaluated; therefore a chronic carrier state cannot be ruled out. Conversely, purported chronic carrier states are generally associated with convalescence (Goering et al., 2013). The high prevalence (31%) of (strong) seroconversion to *B. rossi* in Zenzele suggests that if this were the case, a large fraction of the dog population survived severe babesiosis to develop a convalescent carrier state. This scenario suggests persistent infection generates strong humoral responses for up to 2 years (between registration into the study population in March 2008 and immunodiagnostics undertaken in February 2010). For the parasite to have persistently replicated at levels below microscopic detection but sufficient to stimulate continually strong humoral responses following recovery from severe disease before March 2008 may be less plausible than acquired host immunity rapidly clearing re-infections which occurred after registration. In this respect also, our findings do not provide clear evidence of "a chronic carrier state which occurs when the host is cured of disease but has persistent infection and the infectious agent continues to replicate leading to continued infectiousness"; with infection defined as when "an agent is present in host tissues without signs, symptoms or laboratory evidence of tissue damage" (Rothman et al., 2008, p. 551 and p. 552).

Alternative explanations for *B. rossi* causing subclinical infection and mild disease should also be considered, not least because of laboratory findings consistent with mild babesiosis in three juveniles in Zenzele infected with *B. rossi* (Supplementary Table S4, dogs 7, 13 and 16). The prognosis of *B. rossi* infections requiring treatment varies with the genotype of the species (Matjila et al., 2009) and breed of dog (Reyers et al., 1998; Mellanby et al., 2011). Both of these factors may contribute to the mixed picture of infection by *B. rossi* in populations. Firstly, the prognosis of fulminant babesiosis from *B. rossi* in dogs in South Africa has been associated with the genotype of the erythrocyte membrane antigen gene (BrEMA1) (Matjila et al., 2009); and the absence of the BrEMA1 gene has been associated with mild babesiosis from *B. rossi* in dogs in Nigeria (Adamu et al., 2014). However, the absence of the BrEMA1 gene from *B. rossi* has yet to be demonstrated in South Africa in order to differentiate acquired immunity from genotype as the cause of mild disease. Secondly, the predominantly mixed breed dogs in Zenzele may be genetically less prone to severe babesiosis from *B. rossi* than the pure and first cross breed dogs often presented to clinicians with severe babesiosis (Mellanby et al., 2011; Liu et al., 2017).

In conclusion, our observations warrant two important future lines of enquiry regarding the natural history and epidemiology of *B. rossi* infection in owned, free-roaming dog populations in endemic regions where babesiosis is not routinely prevented. The first is the role of repeated exposure generating acquired immunity against the pathogen, attenuating the severity and duration of infection. Should this prove to be the case, we may also learn from observations of control measures such as insecticides in malaria endemic regions where reductions in exposure to *Plasmodium* spp. to below levels necessary to maintain acquired immunity resulted in serious rebound epidemics (Romi et al., 2002; Doolan et al., 2009). Consideration should be given to the effect of reductions in acquired immunity through the widespread use of tick prophylaxis in mature free-roaming dogs. Equally, the value of sub-therapeutic tick prophylaxis in dogs with acquired immunity to *B. rossi* should also be considered; not least because from May 2011 27% of the (37) dogs positive for *Babesia* spp. on blood smear examination and immunodiagnostics, including the positive control, still present in Zenzele had received at least one dose of topical permethrin and 46% had received ivermectin subcutaneously at least once. The second important line of enquiry is with regard to the absence of the BrEMA1 and related genes from *B. rossi* in South Africa and its association with the clinical progression of babesiosis. The absence of the BrEMA1 gene may prove to be indicative of the prognosis for *B. rossi* infections in general.

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Appendix A. Supplementary data

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