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Epidemiological study of the prevalence of *Babesia divergens* in a veterinary practice in the mid-east of France

J. Devos[a], D. Geysen[b,*]

[a]chez Mathy", 42360 Panissières, France
[b]Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium

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Abstract

To assess the epidemiology of *Babesia divergens* in a veterinary practice based in the mid-east of France (“Monts du Lyonnais”), blood was collected from 254 cattle belonging to 24 herds. To assess the dynamics of the carrier state, six carriers were identified, treated with flumethrin and sampled once every 3 weeks during 6 months. Two different DNA extraction methods were compared. Each sample was tested for the presence of parasites using a PCR–RFLP test based on the 18S rRNA gene. The sensitivity of the test was equivalent to a parasitaemia as low as 10^{-5}% (in “Filter Paper” samples) and 10^{-6}% in 1 ml blood (extracted using “Matrix”). With the latter method, the rate of detection diminishes in the low parasitaemia range but could probably be improved. This test proved to be very useful in the detection of *B. divergens* carriers. Serology using IFAT showed 7% of the cattle seropositive, which is suggestive of a disease situation with a low clinical risk level. Analysis of the PCR results suggests a 20% prevalence rate of carriers in the cattle population. The use of the mean parasitaemia is proposed to serve as a babesiosis clinical risk indicator. This approach could also be used in other babesia infections provided the lowest detectable parasitaemia level (threshold level) could be resolved for each parasite species.
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Keywords: *Babesia divergens*; Diagnosis-protozoa; PCR; Epidemiology

* Corresponding author. Tel.: +32 3 216 1431; fax: +32 3 247 6264.
E-mail address: dgeysen@itg.be (D. Geysen).

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1. Introduction

Babesiosis (or pirolasmosis) is a parasitic infection due to the intraerythrocytic multiplication of protozoa of the genus *Babesia* (Apicomplexa, Sporozoea, Piroplasmida, Babesiidae) (Levine et al., 1980). All babesial parasites are transmitted by ixodid ticks and are one of the most common parasitic infections of domestic and wild animals. Four main species are known to infect cattle: *Babesia bovis*, *Babesia bigemina*, *Babesia divergens* and *Babesia major* (Persing and Conrad, 1995). In temperate Europe, only the last two are found and clinical cases are mainly due to *B. divergens* (Losson and Lefevre, 1989).

B. divergens is a micro-babesia (length smaller than the erythrocyte radius) with typical paired, but divergent forms and is considered to be a typical member of the Babesiidae (M'Fadyean and Stockman, 1911). Phylogenetic analysis indicated that *B. divergens* is strongly related to *Babesia canis* and less to *Babesia caballi, B. bigemina* and *B. bovis* (Mackenstedt et al., 1994). Like in most *Babesia* spp. infections, clinical cases are relatively rare (clinical prevalence is about 0.4% in France (L'Hostis et al., 1995)) as most of the infections are not apparent and development into a carrier state is the rule.

Various approaches have been developed to study the epidemiology of babesiosis. Serology, particularly the immunofluorescence antibody test (IFAT), is the most utilized technique (Böse et al., 1995; Losos, 1986; Gray and Kaye, 1991; Edelhofer et al., 1998; Chauvin et al., 1995; Losson and Lefevre, 1989; Adam and Blewett, 1978; de Vos and Potgieter, 1983). This test has been used until recently to estimate the endemicity and the clinical risk of babesiosis (Anon., 1984). Its main disadvantage relates to serological cross reactions between the various *Babesia* species. This complicates the interpretation of serological results in the presence of more than one *Babesia* species (Papadopoulos et al., 1996). Another drawback of serology is the inability to correlate positive results with the presence of live parasites.

Cell culture or experimental infections of splenectomized steers have been used for the detection of low parasitaemia (Böse et al., 1995; Joyner and Davies, 1967). These expensive and rigorous methods have been made relatively obsolete by the development of molecular tools (Figueroa et al., 1992, 1994; Gubbels et al., 1999; Kirvar et al., 2000; Sparagano et al., 1999; Calder et al., 1996). The latter tests still need to be validated on field samples.

In this paper results of a PCR–RFLP test based on the 18S rRNA gene are presented. This test can detect all *Babesia* species and differentiate them by their specific RFLP profiles. To confirm usefulness of the method, an epidemiological survey (cohort monitoring and cross-sectional survey) was carried out in a region where *B. divergens* clinical prevalence is low (0.1%). The PCR results were compared with IFAT serology data.

2. Materials and methods

2.1. Study area and bovine breeding description

This study was carried out in a veterinary practice on the west side of the "Monts du Lyonnais" (France). The vegetation can be classified as pasture land of the shrub type with

a lot of woodland. Farms have an average of 27 heads of cattle (mainly Prim'Holstein and Montbéliard breeds) over 1 year old, with a maximum number of 110. The main farm activity consists of milk production with an average quota of 150,000 l of milk per farm. The pasture grazing period usually begins in March and ends in December. Some animals, mainly breeding heifers and beef cows, are kept on pasture all year round.

2.2. Sampling protocols

2.2.1. Cohort sampling

To analyse the dynamics of the carrier state, six animals, including one cow (no. 445) treated for babesiosis in September 2001 were identified as carriers and sampled ($N = 45$ samples) once every 3 weeks from October 2001 to March 2002. These animals all came from a small dairy herd of 20 heads in a babesiosis endemic area. They were treated every 6 weeks with flumethrin pour-on (Bayticol®, Bayer) to prevent tick reinfestation.

2.2.2. Two stage sampling for cross-sectional survey

For the cross-sectional survey a two stage sampling was used. Within a list of 126 regular clients of the practice 24 of the farms were sampled by random number generation without knowledge of their babesiosis clinical history. These 24 farms contained approximately 900 cattle over 1 year of age. A random list of 300 numbers was established constituting the cattle to sample. As some animals were not present on the sampling day, the total number of sampled cattle was 254. Blood samples were collected in 5 ml Venoject tubes with EDTA from the caudal vein during the period of 2 November–15 December 2001.

Each sample was divided over 1 ml of whole blood, kept in an Eppendorf tube at −20 °C and 0.5 ml of whole blood spotted onto filter paper (Whatman® no. 3), air-dried during 24 h and stored at 4 °C, separated by plain filter paper in plastic bags with silica gel. The rest of the sample was centrifuged during 10 min at 6000 × *g* and 1 ml plasma was stored in Eppendorf tubes at −20 °C.

To determine the PCR detection threshold of the two extraction methods, blood was sampled from two cows with clinical babesiosis showing a parasitaemias of 1.5% (cow A) and 7% (cow B). Blood was diluted serially to a 10^6 using blood of a newborn naive calf.

2.3. Immunofluorescence antibody test (IFAT)

We used *B. divergens* infected blood cells from a gerbil (*Meriones nguiculatus*) with a parasitaemia of 50% as antigen for the IFAT method as described by Gray and Kaye (1991).

Negative control samples consisted of serum from a calf bred outside a *B. divergens* infected area. Positive control serum came from cow no. 445 sampled 3 weeks after clinical babesiosis.

2.4. Extraction of DNA

Two DNA extraction methods were compared. The "Matrix" method (method M) was derived from Ansell et al. (2000). One milliliter of each defrosted blood sample was added

to 0.5 ml of 0.5% saponin in phosphate-buffered saline (PBS), kept 2 h at room temperature and centrifuged at 12,879 × *g* for 3 min with removal of supernatant. The pellet was diluted with 1 ml PBS, vortexed at 1000 rpm for 1 min and centrifuged at 12,879 × *g* for 3 min. Following removal of supernatant 200 μl InstaGene™ Matrix (Bio-Rad, CA, USA) was added to the pellet (20–30 μl) and treated following the manufacturer's instructions. The detection threshold was also determined using this method on 6 μl blood instead of 1 ml.

The "Filter Paper" method (method F) is derived from the description of Plowe et al. (1995). Four circular, 6 mm pieces of filter paper (Whatman® no. 3 equivalent to 40 μl of blood) were cut out with a puncher, eluted with 1 ml of 0.5% saponin in PBS, shaken, incubated at 4 °C for 4 h and centrifuged at 8451 × *g* for 5 min. The supernatant was removed and replaced with 1 ml PBS. The tubes were vortexed, then incubated at 4 °C for 2 h and centrifuged at 8451 × *g* for 5 min. The supernatant was removed and replaced with 100 μl of a solution of 10% Chelex 100 (Bio-Rad). The tubes were vortexed at 95 °C for 10 min and centrifuged at 8451 × *g* for 5 min. This time, the supernatant was removed to a new tube and centrifuged at 8451 × *g* for 5 min. The supernatant (100 μl) from this centrifugation was then removed carefully to a new tube, without disturbing the Chelex 100 pellet and stored at −20 °C until use.

2.5. Polymerase chain reaction (PCR)

The primers pairs BabF3/BabR2 and BabF3/BabR3 (Table 1) were used for a nested-PCR. Amplification was carried out in 250 μl tubes. Each tube received 25 μl of a PCR mix consisting of 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.6 mM $MgCl_2$, 200 μM of each of the four dNTPs, 20 pM of each primer, 0.5 U Taq polymerase, 1 μl Yellow Sub™ (GENEO BioProducts, Hamburg, Germany) and 5 μl DNA template for the first round, whereas 0.5 μl from the first round mixture was used as template for the second round. Samples were overlaid with fine mineral oil. The samples were placed in a thermocycler (PTC100, MJR, USA). The amplification program was as follows: a first round of 40 cycles consisting of three steps (30 s denaturation at 94 °C, 45 s annealing at 55 °C and 60 s polymerization at 72 °C); a second round of 25 cycles consisting of the same steps. Each round began with a step of 3 min denaturation at 94 °C and ended with an extension step of 8 min at 72 °C.

A 5 μl of each PCR product was loaded with 2 μl of loading buffer onto a 2% agarose gel and submitted to electrophoresis during 20 min at 100 V. The gel was stained in

Table 1
Primer sequences used for the *Babesia divergens* and the GAPDH study in bovine blood DNA extracts

Name	Sequence	Length[a]
Bab F3	5′ ATG TCT AAG TAC AAG CTT TTT ACG GT 3′	26
Bab R2	5′ TTG ATT TCT CTC AAG GTG CTG AAG GAG TCG 3′	30
Bab R3	5′ AAA GGC GAC GAC CTC CAA TCC CTA GT 3′	26
GAPDH1	5′ GATGCTGGTGCTGAGTATGTAGTG 3′	24
GAPDH2	5′ ATCCACAACAGACACGTTGGGAG 3′	23

[a] Length in nucleotides.

ethidium bromide, washed under running tapwater and photographed under UV illumination.

Positive samples were submitted to restriction with *Dde*1. Restriction enzyme digestion was done according to the manufacturer's specifications (Gibco, UK) using 10 U μg^{-1} DNA (0.6 U μl^{-1} PCR product) on 5 μl of amplified DNA in 15 μl total volume. The reaction was left overnight in a water bath at the specified temperature. A 6 μl of restricted sample mixed with 2 μl loading buffer was transferred onto a 10% polyacrylamide gel together with a 100 bp DNA ladder (MBI, Fermentas, Lithuania) for fragment size determination. DNA fragments were separated by horizontal electrophoresis in 0.5× TAE buffer at 100 V for 2.5 h. The gel was stained using a commercial silver staining kit (Silver staining kit DNA plusone, Pharmacia Biotech, Uppsala, Sweden) and mounted for storage. Positive control samples were derived from *B. divergens* infected blood, taken from a heifer sampled during the clinical course of the experimental infection; the negative control was distilled water.

2.5.1. Other tests

Negative cohort samples were submitted to the bovine housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification to test the extraction quality (DNA presence and absence of inhibition factors) using the primers GAPDH1 and 2 (Mertens et al., 1999 and Table 1) in a single round PCR approach (same conditions as first round *B. divergens* amplification).

Theileria parva and *B. bigemina* DNA extracts were included to test the specificity of the amplification. The ''Amplify'' program of Engels (1993) was used to test the primers against all known 18S rRNA sequences of Apicomplexa.

2.5.2. Data analysis

Analyses were conducted using Stata 7 software (Stata Corporation, 2001).

3. Results

Positive PCR results were characterised by the presence of a DNA fragment of 934 base pairs spanning a variable region of the 18S rRNA gene (GenBank no. Z48751). To confirm the specificity of the amplicons, a *Dde*1 digestion was performed on all positive PCR samples and the resulting RFLP profile analysed. Table 2 shows the expected lengths of the fragments after restriction with *Dde*1 for *B. divergens*, *B. bigemina*, *B. bovis* and *T. parva*.

Table 2
Expected lengths of fragments, in base pairs, after nested-PCR with primers BabF3/BabR2 and BabF3/BabR3 before and after restriction with *Dde*1 enzyme

	Babesia divergens[a]	*Babesia bigemina*[a]	*Babesia bovis*	*Theileria parva*[a]
Whole length	934	907	875	923
Fragments after restriction	407	434	418	463
	308	297	299	174
	151	151	151	151
				115

[a] Results confirmed by experimentation.

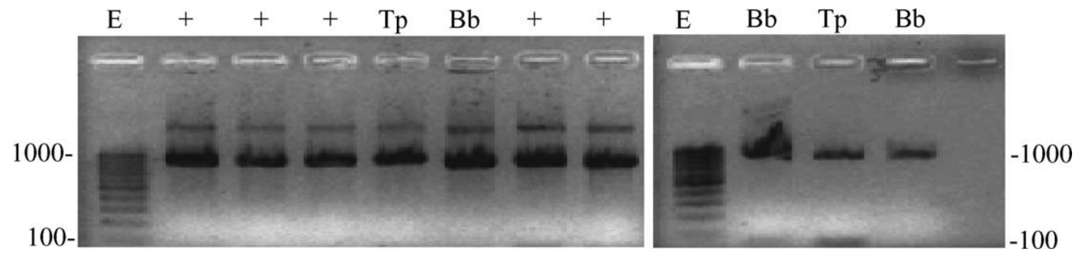

Fig. 1. PCR results of *Babesia divergens* (+), *Babesia bigemina* (Bb) and *Theileria parva* (Tp) samples. The PCR test amplifies a similar length fragment for the three parasites (E: scale graduated from 100 to 1000 base pairs).

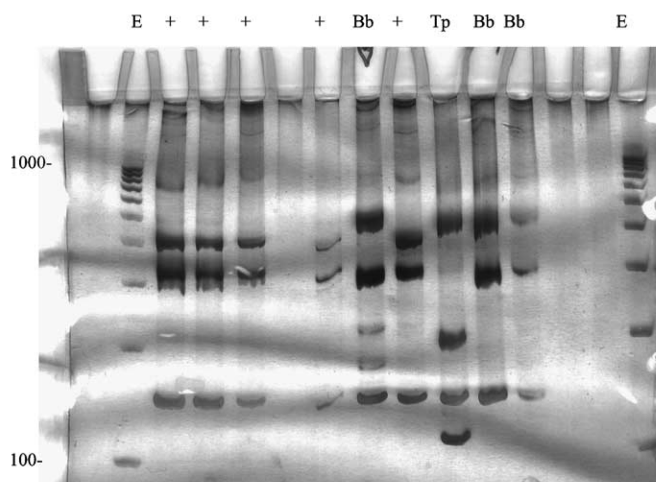

Fig. 2. RFLP results of *Babesia divergens* (+), *Babesia bigemina* (Bb) and *Theileria parva* (Tp) samples. The parasites are differentiated by the use of the RFLP with *Dde*1 (E: scale graduated from 100 to 1000 base pairs).

Figs. 1 and 2 show the PCR and RFLP results obtained with *T. parva* and *B. bigemina* DNA samples in addition to different *B. divergens* positive samples. DNA from the *Babesia* species and from *T. parva* gave PCR positive results. The length of the amplicons was around 1000 bp as predicted (see Table 1) but small size differences could be observed among some of the fragments. All these RFLP profiles were in agreement with the theoretical values, computed from the expected PCR fragment length and presence of the restriction sites (see Table 1).

The detection threshold results are summarised in Fig. 3. These were obtained by analysing PCR results of diluted blood samples of known parasitaemias from cows A and B. The bloodsamples of these cows were diluted with negative blood and extracted. Extracts were either used undiluted or diluted 1/10 or 1/100 to evaluate possible inhibition. Method F gave positive results as low as 1.5×10^{-5}% (cow A) and 7×10^{-6}% (cow B). The analysis of 6 μl blood with method M gave positive results as low as 1.5×10^{-4}% (cow A). This method applied on 1 ml blood also gave positive results as low as 1.5×10^{-4}% (cow A). With cow B, results were positive as low as 7×10^{-6}% with the crude extract and 7×10^{-4}% with the extract diluted 10- and 100-fold. Samples extracted by the same method followed by GAPDH amplification always showed positive results.

In the cohort study, seven samples gave a PCR positive result for both extraction methods, four only with method M, five only with method F and 29 were always negative. Total number of positive samples after method M extraction was 11/45 (24%) and 12/45 (27%) after method F extraction. Twenty-six serology samples were IFAT positive with important variations related to the different cows (1/9 for cow no. 837, 9/9 for cow no. 445). When comparing the serology with the PCR results, we found eight samples PCR+/IFAT+, 8 PCR+/IFAT−, 14 PCR−/IFAT+ and 15 PCR−/IFAT.

In the cross-sectional study, 26 cattle were positive in at least one of the tests (IFAT or PCR; Table 3). They originated from 12 out of a total of 24 different farms. All the PCR+

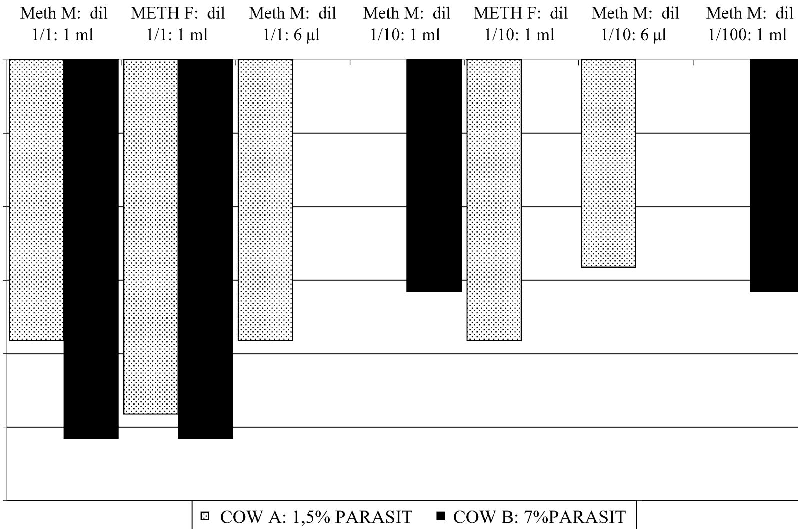

Fig. 3. Results of the PCR detection thresholds from samples submitted to the two extraction methods. Two bovines were sampled during a clinical *B. divergens* infection (parasitaemias: cow A, 1.5%; cow B, 7%). Blood was diluted 10-fold with blood from a newborn naïve calf and extracted using the methods M (matrix) and F (filter paper). Extracts were amplified undiluted or diluted 10- or 100-fold. *Y*-axis: percentage corresponding parasitaemias detected (10^{-1} to 10^{-6} parasitaemias).

cattle showed the specific *B. divergens* profile in the RFLP test. Three animals gave a nonspecific amplification product of the same (2) or different (1) length as a *Babesia* specific product, but were differentiated easily on their RFLP profiles (Fig. 4).

The theoretical study demonstrated that other bovine Apicomplexan parasites (*Neospora caninum*, *Besnoitia besnoiti*, *Cytauxzoon felis*, *Hepatozoon canis*, *Frenkelia microti*, *Toxoplasma gondii*, *Theileria buffeli*, *Cryptosporidium parvum*, *Sarcocystis mucosa*) would not give an amplification product with the primers used. Only *B. major*

Table 3
PCR and IFAT results obtained in the cross-sectional survey, using the two DNA extraction methods

	IFAT+	IFAT−	Total No. samples
Method M+			
Method F+	1	3	4
Method F−	3	4	7
Method M−			
Method F+	0	1	1
Method F−	14	228	242
Total samples PCR+	4	8	12
Total samples PCR−	14	228	242
Total No. samples	18	236	254

Method M+: matrix based extraction; F+: filter paper based extraction method.

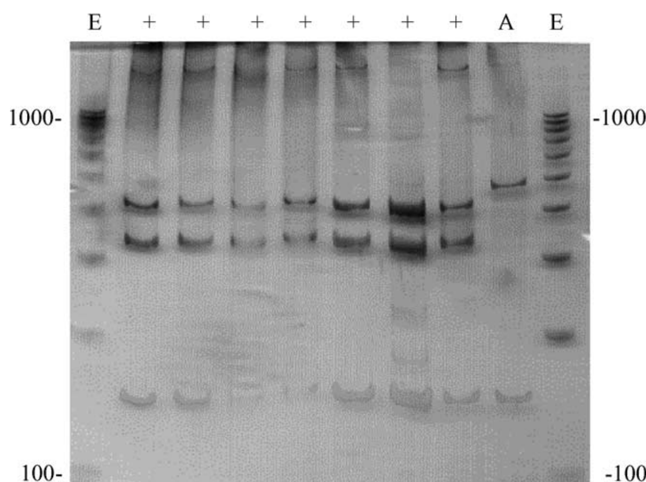

Fig. 4. *Dde*1 RFLP test with PCR+ samples. False positive results were found with the PCR test. The restriction with *Dde*1 allows easy differentiation. The nine "+" bands show the *B. divergens* typical profile. The A band shows a non-specific profile which does not correspond to a known organism (E: scale graduated from 100 to 1000 base pairs).

could not be tested as no sequence data could be found and we did not have access to any strain to be included in the assay.

The statistic analysis showed that IFAT+ animals were older ($6y\ 6m \pm 3y\ 7m$, $P < 0.05$) than the average ($5y\ 1m \pm 3y\ 1m$). Beef cattle and dairy cattle from other breeds than Montbéliard and Prim'Holstein were also more often IFAT+ (29% versus 5%, $P < 0.05$). We did not find any breed or age effect when analysing the PCR results.

4. Discussion

Our results demonstrate the specificity of the PCR–RFLP developed assay. The different *Babesia* species, as well as *T. parva*, yielded PCR fragments of comparable length, but were differentiated by the use of *Dde*1 RFLP (Table 2). The theoretical sensitivity of PCR tests has often been calculated, but confirmation using samples of known parasitaemia has not always been done (Sparagano et al., 1999). When documented samples were included, detection thresholds were found to be in the range of 10^{-4} to 10^{-6}% according to the test used (PCR, nested-PCR, PCR followed by Dot Blot) (Figueroa et al., 1994; Figueroa et al., 1992; Gubbels et al., 1999; Fahrimal et al., 1992; Kirvar et al., 2000). These results were based on single analysis and reproducibility has never been tested. Calder et al. (1996) showed that the frequency of detection diminishes in a logarithmic way from 100% detection with a 10^{-4}% parasitaemia (2000 parasites in the sample) to 15% detection level when a 10^{-7}% parasitaemia sample (equivalent to the presence of two parasites) was used.

The two extraction methods differ in the amount of blood examined. Method M analyses DNA extracted from 33 μl blood (equivalent to 2×10^8 erythrocytes), whereas only 2 μl blood (12×10^6 erythrocytes) is used in method F. This gives a volume difference

ratio of 16.5 between the two methods. Theoretically method M should give more positive results as this method examines more erythrocytes. However, it is well known that the presence of erythrocytes in the samples might inhibit PCR amplification due to the interactions of haemoglobulin with the polymerase enzyme (Wilson (1997)).

Theoretically, the different extraction protocols should detect parasitaemias of about 10^{-4}% (using 6 μl volume in method M), 10^{-5}% (method F) and 10^{-6}% (using 1 ml volume in method M). Method F gave results corresponding with this theoretical calculation.

Method M, applied to 1 ml blood did not result in a consistent DNA recovery although no PCR inhibition was detected given the positivity of the GAPDH test. When applied to 6 μl blood, DNA recovery seems to be correct as the results correspond to the theoretical estimation detecting two parasites in the DNA template (Table 4). Further tests are needed to optimise this extraction method using 1 ml blood volume in order to obtain consistent results.

In the cohort study, we found seven positive samples with the two methods, four positive with method M only, five positives with method F only and 29 persistently negative samples. The 12 positive samples extracted with method F should have been positive with the other method as well, but only seven were found positive. These results allow us to estimate the positive detection rate at 58% (7/12) for extraction method M corresponding with detecting parasitaemias equal or higher than 10^{-5}% but only at 24% (11/45) when considering the total number of cohort samples. We propose to use this latter rate as an estimation of the number of carriers in the survey population. The animals from the cohort can be considered as *B. divergens* carriers since *B. divergens* persists in cattle for 18 months to 4 years. This is longer than our observations with flumethrin treatment (Joyner and Davies, 1967). It is therefore likely that animals within the negative group (76%), may have had parasitaemia levels lower than the detection threshold due to the undulating nature of the carrier parasitemia. The deduction of the carrier rate from these results will allow us to calculate the prevalence for the whole population without knowledge of the real parasitemias.

Table 4
Comparison of the minimal detected parasitemias with the two extraction methods

Method	Blood volume submitted to extraction	Extract volume (μl)	Extract dilution	Blood volume submitted to extraction (μl)	Minimal detected parasitemia (%)	Estimation of parasites detected
Method M on 6 μl	6 μl	150	1	0.2	1.5×10^{-4}	2
	6 μl	150	10	0.02	1.5×10^{-3}	2
Method M on 1 ml	1 ml	150	1	33	1.5×10^{-4}	300
	1 ml	150	1	33	7×10^{-6}	14[a]
	1 ml	150	10	3.3	7×10^{-4}	140
	1 ml	150	100	0.33	7×10^{-4}	142
Method F on 1 ml	40 μl	100	1	2	1.5×10^{-5}	2
	40 μl	100	10	0.2	1.5×10^{-4}	2
	40 μl	100	1	2	7×10^{-6}	1

[a] 7×10^{-7}% parasitemia were not tested.

In the cross-sectional survey, the difference between the two extraction methods can be explained by an inconsistency in the DNA extraction yield and the different detection thresholds. It is likely that the inconsistent results of the seven method M extracted positive samples (but negative when extracted by method F) were due to a parasitaemia level under the detection threshold of 10^{-5}%. On the contrary, samples positive by method F and negative with method M probably related to the low detection rate of the method.

Until now, IFAT was the routine method used to estimate babesiosis endemicity (Anon., 1984). The clinical risk for babesiosis has been found to be low when there are less than 20% seropositives present in a population, whereas a stable endemic situation is correlated with more than 80% seropositives. Between these levels, the clinical risk is considered to be high. In the cross-sectional survey, 7% of the animals are IFAT positive. For the dairy cattle, this ratio is only 5% whereas it is 29% for the beef cattle. The latter herd segment is at risk of clinical illness and cohort animals (58% IFAT positives) are even in a more dangerous situation.

The comparison between the cohort and survey results highlights the difference in mean parasitaemia according to the epidemiological status. In the cohort, representing animals from an high clinical risk herd, we observed 27% of the animals with a parasitaemia superior or equal to 10^{-5}% (method F detection threshold). In a previous study in an high clinical risk herd of 36 animals, we observed 22% of the animals with the same parasitaemia level (unpublished data). Only four (1.6%) of the 254 survey animals had a parasitaemia superior or equal to 10^{-5}%, even though the carrier prevalence was estimated at 20% representing a population under low clinical risk. These results suggest a direct correlation between the clinical risk estimator and the mean parasitaemia.

These observations are in line with the classical notions of infection pressure. This might allow us to redefine the clinical risk situation which had been characterised on the basis of the percentage of IFAT positives (Anon., 1984). Until now the clinical risk of babesiosis had been estimated by indirect indicators: the bovine serological titers estimated by IFAT (Anon., 1984) or the vector infection pressure estimated by whole body tick counts (de Vos and Potgieter, 1983).

The collection and interpretation of these data is difficult (Adam and Blewett, 1978). IFAT is the reference test for epidemiological babesiosis surveys. The results of our study agree well with the traditionally adopted criterion. We found 7% of the animals positive on IFAT, which corresponds to a low clinical risk situation (the clinical prevalence is estimated to be about 0.1% in France). However, comparison with the PCR results indicated that this method underestimates the number of carrier animals. Based on the PCR results we can estimate the *B. divergens* prevalence to be 20%.

The follow up (cohort study) of naturally infected animals allowed us to estimate the detection rate of carriers to be 24% and to propose the use of the mean parasitaemia as an estimation factor of clinical risk. The estimation factor could be based on the mean parasitaemia or the percentage of PCR positives at a certain parasitemic level. The PCR advantage lies in its direct estimation of the parasite numbers. The described PCR–RFLP test has demonstrated its specificity as well as its usefulness to study other *Babesia* species than *B. divergens*, even in situations of mixed infections. Development of this approach would involve the determination of a realistic threshold by analysis of data from multifocal

surveys after optimalisation of the extraction method. This approach could also be used in other *Babesia* infections, provided that the thresholds could be resolved for each parasite species.

This study provides data for an alternative and easier method Tp define clinical risk situations, but more survey results need to be gathered from regions where various epidemiological situations prevail to confirm the usefulness of this approach.

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