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Isolation of *Babesia divergens* from carrier cattle blood using in vitro culture

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Abstract – *Babesia divergens*, the main causative agent of bovine babesiosis in Western Europe, was isolated from naturally infected cattle. Ninety-six blood samples were examined by means of an in vitro culture technique in sheep erythrocytes: 19 of them were collected from animals in the acute phase of the disease with visible parasitemia on blood smears, while the 77 remaining animals showed no microscopically detectable parasites. *B. divergens* was cultured from the 19 first blood samples as well as from 31 samples collected from asymptomatic animals. The time period before parasites could be detected in the culture varied in the latter samples from 6 to 20 days. The effects of sampling condition (anticoagulant used) and storage length were tested. A good correlation was obtained between immunofluorescent antibody test and culture, with identical results (positive or negative) for 89.6% of the samples collected from asymptomatic animals. The sensitivity of the in vitro culture method was determined and was about 10 parasites/mL of whole blood from three independent experiments performed with three different isolates, confirming its suitability to detect and culture diverse *B. divergens* isolates from carrier cattle. The parasites could indeed be isolated 9 months after the acute babesiosis phase in the blood of naturally infected animals. The 50 isolates collected in this study were successfully subcultured, cryopreserved and resuscitated using the same culture medium. The in vitro isolation of *B. divergens* from asymptomatic carrier cattle was achieved and will allow the analysis of parasite diversity within cattle herds.

***Babesia divergens* / carrier cattle / in vitro culture / isolation**

1. INTRODUCTION

Babesia divergens is an intraerythrocytic protozoan parasite, transmitted by the three-host tick *Ixodes ricinus* and is largely responsible for bovine babesiosis in Western Europe. In France, the mean clinical incidence of babesiosis in cattle is usually low (0.4% in France in the 90's)[17], even if the frequent high serological prevalence [6, 9, 18], and the maintenance of a carrier state over long periods [15] indicate the circulation of the parasite and hence its vector in the cattle environment. Carrier cattle constitute a constant source of parasites within the herd.

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Serological methods (IFAT: immunofluorescent antibody test; ELISA: enzyme-linked-immunosorbent-assay) are very useful to detect animals that have been in contact with *B. divergens* [3, 6], but they do not attest of its actual presence. Gubbels et al. [11] developed a reverse line blot hybridization assay able to simultaneously detect very low amounts of different blood parasites of cattle including *B. divergens*, *B. bovis* and *B. bigemina*. But none of the above mentioned methods allow the detection of carrier animals and subsequent characterization of *B. divergens* carried isolates. Gerbils are commonly used to test for the presence of *B. divergens* in bovine blood, but they have the drawbacks of a low sensitivity (10^3 to 10^4 inoculated parasites) [12, 16], and of the experimental use of animals.

Continuous in vitro cultivation of *B. divergens* parasites was first reported in 1982 by Väyrynen and Tuomi in bovine erythrocytes [20]. Later, erythrocytes from various animal origins corresponding to natural (bovine, human) or experimental hosts (rat, sheep) were proved to support *B. divergens* continuous in vitro growth [2, 4, 10, 21]. The culture system developed in our laboratory [4] with sheep erythrocytes allowed high in vitro parasitemia (50%) with only 10% serum (fetal calf or sheep). Sheep are not only a convenient source of red blood cells, but could also be experimentally infected with their own in vitro infected erythrocytes [4]. The in vitro isolation of *B. divergens* from carrier cattle which often have extremely low parasitemias has not, however, been described and would contribute to a better understanding of the relation between carrier status and serological status. It would also allow the isolation of several strains from one herd to first study their genetic diversity and then to compare them with the strains responsible for clinical episodes within the same herd. In this study, we used the already described in vitro culture in sheep erythrocytes [4] for the isolation and cultivation of *B. divergens* from naturally infected carrier cattle.

2. MATERIALS AND METHODS

2.1. Sample origin and collection

This study was carried out on 96 samples collected between May 2001 and August 2002 from dairy cows in the Ille-et-Vilaine department (Western France). Among them, 19 were obtained by veterinarians from animals expressing acute babesiosis symptoms (fever, anemia, hemoglobinuria, anorexia), while the remaining 77 originated from asymptomatic animals.

Peripheral blood was aseptically punctured from the cattle's tail veins into 5 mL evacuated glass tubes containing 0.7 mL of Citrate Phosphate Dextrose (CPD, Sigma, Saint Quentin Fallavier, France) as the anticoagulant. The time period between sample collection and processing varied from 1 to 25 days when sent by the postal services (cases of acute babesiosis), but samples from asymptomatic animals were processed within 4 days after sampling, except when storage length effects were tested.

2.2. The influence of sampling and storage conditions on the culture success

In order to compare the effect of the anticoagulant on the parasite survival, 34 blood samples were collected into CPD and Lithium Heparin, and also into EDTA tubes for only four of them. They were then processed the same way.

The effect of storage length on culture success was tested by comparing the culture results for seven IFAT positive samples collected from asymptomatic carriers (Lithium Heparin as the anticoagulant) kept at room temperature and used respectively 24 h and 9 days after sampling. The test was performed on Lithium Heparin because of its widespread availability and use by veterinarians.

2.3. Sample processing and parasite presence evaluation

Collection tubes were centrifuged at 800 *g* for 10 min at room temperature. The sera were stored at –20 °C until tested by IFAT (Indirect Fluorescent Antibody Test), and the buffy layer was discarded. Packed erythrocytes were then washed once with 10 mL RPMI 1640 (Cambrex, Belgium) and centrifuged for 10 min at 1 200 *g*. The pelleted packed erythrocytes were used to initiate the in vitro culture.

The presence of *B. divergens* in the samples was checked by light microscopy of May-Grünwald-Giemsa (MGG) stained thin blood smears. When parasites were not immediately visible, a minimum of 30 microscope fields (about 10^4 erythrocytes) was observed. Sera were tested by IFAT at three dilutions (1/80, 1/320, 1/1280) according to the protocol described by Chauvin et al. [3]. The *B. divergens* isolate Rouen 1987 from infected gerbil blood was used as the antigen. This technique, despite a low sensitivity drawback, allows the detection of antibodies for at least 7 to 8 weeks after the acute phase, and is therefore more suitable to evaluate the cattle carrier status than the ELISA test developed in our laboratory [3].

2.4. In vitro culture of parasites

Culture was carried out on 24-well culture plates in RPMI 1640, fetal calf serum (FCS, Cambrex, Belgium) 10%, heat-inactivated at 56 °C for 30 min before use, and RPMI 1640 washed sheep erythrocytes at a final concentration of 1.5×10^9 red blood cells/mL. Gentamicin at a final concentration of 50 µg/mL was added to prevent bacterial contaminations. Each 2 mL-well was inoculated with 150 µL of bovine packed washed erythrocytes in the case of non-parasitemic animals and with only 50 µL when parasites were visible on blood smears. Cultures were performed at 37 °C in a humidified 6% CO_2 atmosphere. Every 2 or 3 days, the cultures were fed by removing 1.5 mL of the medium overlying the erythrocyte layer and replacing it with the same volume of fresh medium (RPMI 1640 + FCS 10%). Parasitemia was checked regularly by pipetting small samples of erythrocytes to make thin blood smears subsequently MGG stained.

When parasitemia reached 1–5%, subcultures were carried out by pipetting 20 µL of the sedimented erythrocytes and transferring them into fresh medium. Cryopreservation and resuscitation from liquid nitrogen storage were performed according to Chauvin et al. [4].

In order to eliminate the possibility that the *Babesia* isolates originated from the sheep donor erythrocytes, one 2 mL-well per plate was kept uninoculated. A positive control (isolate Rouen 1987) [4] was cultivated in the same conditions through the entire culture period to ensure the growth condition reliability (medium and incubation).

2.5. Determination of the sensitivity of the in vitro culture method

Blood samples from three animals in the course of acute babesiosis were processed 2 to 4 days after their collection. Parasitemias, calculated by examining about 2 500 erythrocytes and red blood cell counts calculated using a Malassez cell were used to determine the number of parasitized erythrocytes/mL. The samples were then serially diluted in order to inoculate each 2 mL-well with a calculated amount of 10^4 to 0.5 parasites/well, with 14 to 19 different parasite amounts between these two extremes. Three cultures were inoculated for each dilution.

For one of the blood samples, a more precise calculation of the sensitivity was performed by initiating the culture in 96-well culture plates with 18 to 24 wells (150 µL) inoculated with each of the following calculated parasitized erythrocyte quantities (0.25, 0.5, 1, 2.5 and 5). To study the ability of the parasite to adapt from in vivo growth

to in vitro culture, the above mentioned parasitized bovine blood was inoculated to a gerbil which blood was collected when parasites were detected on blood smears, diluted and in vitro inoculated as described above. Culture was carried out the same way as previously, with fresh medium (1.5 mL or 100 μL) added to the sedimented erythrocytes every 2 or 3 days.

3. RESULTS

3.1. In vitro isolation of *B. divergens* directly from cattle blood

Among the 96 samples collected and analyzed in this study, 19 came from animals in the acute phase of babesiosis. They were all microscopically positive, with parasitemia ranging from 1 to 15%, and serologically positive when tested by IFAT with *B. divergens* Rouen 1987 as the antigen. In vitro culture was successfully carried out for all of them.

The 77 blood samples collected from asymptomatic dairy cows were negative for *Babesia* parasites based upon the examination of a minimum 10^4 erythrocytes on MGG-stained thin blood smears. However, in vitro cultures were found positive for 31 of them, 27 (87.1%) of which were also positive by IFAT. A good correlation was observed between the IFAT and the culture results since 89.6% of the samples were either positive or negative for both tests. Only 10.4% of the blood samples gave positive results for only one of the two tests, the same proportion being identified as positive equally by one or the other technique (Tab. I).

The period between in vitro culture initiation and microscopically positive culture varied from 6 to 20 days (Tab. II) in the case of the asymptomatic carriers. Most of them (80.6%) could be detected within 12 days after culture initiation. Among the remaining 19.4%, most isolates were characterized by their slow growth, even after several subcultures (data not shown).

The 50 isolates collected in this study and successfully subcultured were all cryopreserved and resuscitated from liquid nitrogen storage.

3.2. Influence of sampling and sample storage conditions on the culture success

The blood from 34 asymptomatic dairy cows collected the same day in CPD and Lithium Heparin tubes was used to initiate the in vitro culture. Among them, 23 were culture negative and 10 were culture positive, whatever the anticoagulant used. In one case, the culture was successful only

Table I. *Babesia divergens* presence estimated by the immunofluorescence antibody test (IFAT) and in vitro culture in 77 blood samples from cattle with no apparent symptoms.

IFAT	In vitro culture	Number of samples (percentage)
–	–	42 (54.5)
+	+	27 (35.1)
+	–	4 (5.2)
–	+	4 (5.2)

Table II. Time span for a positive *Babesia divergens* culture diagnosis for the 31 culture positive blood samples collected from asymptomatic cattle carriers.

Days in culture	+ 6	+ 10	+ 11	+ 12	+ 14	+ 15	+ 19	+ 20
Number of positive samples (cumulative %)	9 (29)	6 (48.4)	4 (61.3)	6 (80.6)	1 (83.8)	3 (87.1)	1 (96.8)	1 (100)

Table III. Sensitivity of the *Babesia divergens* in vitro isolation method. Blood samples collected from 3 acute babesiosis cases (Ben 1, Pich 1 and Pich 2) were diluted and used to inoculate the culture with a calculated number of parasites. The culture results for each triplicate are indicated as + or – according to the presence or absence of parasites on MGG stained blood smears. Di = day of culture initiation.

Mean number of inoculated parasites per well	Blood sample and day of culture examination		
	Ben 1 at Di + 13	Pich 1 at Di + 12	Pich 2 at Di +14
0.5	+ – –	– – –	– – –
1	+ + –	+ – –	+ + –
2.5 and more	+ + +	+ + +	+ + +

from the blood collected in the Lithium Heparin tube. A comparison between EDTA, Lithium Heparin and CPD was performed on four samples. Three of them gave identical results with the three anticoagulants (two culture positive and one culture negative). One was found negative when collected on EDTA only.

To address the influence of storage length on in vitro culture success, seven blood samples from asymptomatic animals (parasitemia < 0.01%) were selected (IFAT positive) and used to initiate in vitro culture 24 h and 9 days after sampling. They were kept in the collection tube (Lithium Heparin) at room temperature before use. They all gave positive cultures whatever the duration between the sampling and culture initiation.

3.3. Sensitivity of the *B. divergens* in vitro isolation method

The sensitivity of the culture method was determined by inoculating 2 mL wells of medium with decreasing amounts of parasites from bovine infected blood. The results detailed in Table III show the high sensitivity of the method, since one to two parasites/well were enough to initiate the culture. The results were the same for the three blood samples, showing the reproducibility of the method with different isolates of *B. divergens*.

All cultures were positive when a calculated quantity of 2.5 parasites were inoculated in a 2 mL-well (Tab. III). The mean amount of erythrocytes in the 150 µL inoculated bovine packed erythrocytes was calculated and was around 3.5×10^9 (average calculated on eight samples with results ranging from 3.27 to 3.94×10^9). So, the lowest detectable parasitemia with our in vitro method was about 10^{-7}%, corresponding to 10 parasites/mL of blood.

To study the effect of the infected blood source on the parasite in vitro adaptation and therefore method sensitivity, the same isolate was used directly from bovine blood or after its inoculation into the gerbil. The results shown in Figure 1 reveal no significant differences in the number of parasites necessary to initiate the culture according to the blood source (Fisher's Exact Test with 2-Tail *p*-values of respectively 1, 0.716, 1, 0.208 and 1 when 0.25, 0.5, 1, 2.5 and 5 parasites/well were inoculated). The sensitivity was still around 10 parasites/mL of blood.

3.4. Application of the isolation method to follow cattle carrier status after acute babesiosis

Blood from six dairy cows was collected at three time points after the acute babesiosis phase. The last sampling was performed late in February, after the winter period characterized by a low tick activity

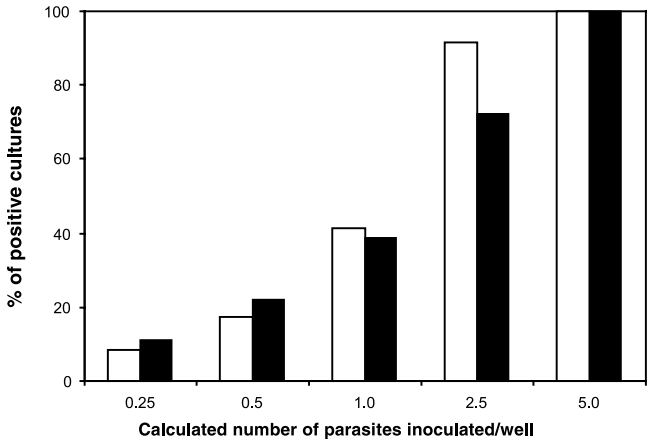

Figure 1. Sensitivity of the *Babesia divergens* in vitro isolation method according to the host of origin. The culture was initiated either directly from the infected bovine blood (□), or from gerbil blood first infected with the same stock (■). The culture was performed in 96-well plates with 18–24 wells/parasite concentration. Parasite presence or absence was controlled on MGG-stained blood smears 15 days post-inoculation.

and hence probably limited parasite reinfections. The results from the IFAT and in vitro culture are detailed in Table IV. All the examined animals were seropositive and still carried parasites before the winter period, namely 2 to 6 months after the acute phase. Three months later, at the end of the winter period, *B. divergens* could not be isolated from three of them, despite a positive IFAT result in two cases. The three other animals still carried parasites, indicating its possible persistence in the peripheral blood for at least 9 months after the acute phase.

4. DISCUSSION

A *B. divergens* culture system using sheep erythrocytes developed by Chauvin et al. in 2002 [4] was standardized and tested for its suitability for detecting and directly isolating *B. divergens* from babesiosis acute, postacute, and carrier stages on cattle.

Table IV. Follow-up of six dairy cows at three sampling times after the acute babesiosis events.

	Days after acute babesiosis	IFAT[a]	Culture	Days after acute babesiosis	IFAT[a]	Culture	Days after acute babesiosis	IFAT[a]	Culture
Cow 1	132	320	+	184	320	+	288	80	+
Cow 2	130	nd	nd	182	1280	+	286	320	+
Cow 3	127	320	+	179	320	+	283	80	–
Cow 4	15	1280	+	67	1280	+	171	1280	+
Cow 5	14	1280	+	66	1280	+	170	320	–
Cow 6	14	1280	+	66	1280	+	170	–	–

[a] Reciprocal values of the titers determined with the immunofluorescent antibody test (IFAT).
Nd: blood was not collected.

The results described in Tables I and IV underline the ability of the in vitro culture method to isolate *B. divergens* directly from cattle in the acute babesiosis phase (19/19 cases) or in the carrier stage (31/35 suspected carriers). A good correlation between IFAT and the culture was obtained, with 89.6% of the samples giving the same results with both techniques. The fact that IFAT showed negative results whilst parasites were isolated (5.2% of our cases) could be explained by a lack of sensitivity or by the absence of serological cross-reactions between different isolates with the serological method. On the contrary, the failure to isolate the parasite while antibodies are still circulating (5.2% of the cases) may be due to the sensitivity of the culture method, the sporadic nature of the chronic parasitemia, the absence of circulating parasites, the storage conditions, the persistence of antibodies after parasite clearance by the host, or the inability of certain parasite isolates to grow in our medium.

If successful cultivation of *B. divergens* is indeed due to the better adaptation of a subpopulation to the imposed conditions, then the full diversity of the parasite population is not apprehended by this technique. In their study on the use of in vitro culture methods to isolate *B. equi*, Holman et al. were unable to subculture 30% of the isolated parasites [14]. However, several results indicate that such a strong selection did not occur in our conditions. Firstly, all the detected *B. divergens* isolates from the present and latter studies (about 220 different isolates) were successfully subcultured and continuously grown. Secondly, the experiments used to determine the sensitivity of the culture method indicated that for the three tested blood samples, approximately one inoculated parasite was enough to initiate a culture. Thirdly, the good correlation between IFAT and the in vitro parasite isolation also argued against a strong population selection. The failure to isolate the parasite when the IFAT was positive was probably due to the low circulating parasite concentration in the peripheral blood. The follow-up of six acute babesiosis cases over 9 months (Tab. IV) highlighted two such cases. They were characterized after the winter period by a drop of their antibody titer and a negative culture. This is probably indicative of a simultaneous drop in the concentration of the hosted parasite that was successfully isolated three months earlier, hence demonstrating its ability to grow in our culture conditions.

The culture method sensitivity mainly depends on the ability of the parasite to adapt to the new imposed conditions, to invade probably never encountered erythrocyte types and to grow properly from the supplied nutriments. The sensitivity of our method was calculated by serially diluting blood samples with microscopically detectable parasitemia from 3 acute babesiosis cases. We cannot rule out that the physiological state of the parasites in this situation (actively growing parasites stored for 2 to 4 days before culture initiation) is more favorable to its rapid adaptation to new environmental and nutritional conditions. On the contrary, parasites from the peripheral blood of carrier animals, exposed to the defense mechanisms of the host, could grow readily once freed from these constraints. The sensitivity of our culture technique was in the same range as the PCR-based methods described for *B. divergens* or other *Babesia* species [1, 5, 7, 8, 11]. A frequency of detection of about 15% at a 10^{-7}% parasitemia was achieved for *B. bovis* [5] compared to 100% in our study at the same parasitemia. In the case of *B. divergens*, Gubbels et al. designed a reverse line blot technique with a similar detection limit (10^{-6}%) [11]. The isolation of *B. divergens* from carrier animals up to 9 months after the acute babesiosis phase as demonstrated here could be very useful to characterize the diversity within this species at the herd level.

The incubation time required for detecting *B. divergens* on thin cultured blood smears ranged from 6 to 20 days for the carrier animals, compared to the 12–14 days

necessary when inoculation was done with one single parasitized erythrocyte (sensitivity assay). These varying lag times may be due to the differential number of parasites in the initial blood samples (time lag < 12 days) and to slow growing parasites (time lag > 15 days).

An in vitro isolation procedure of *Babesia* from carrier animals has been reported in the case of *B. equi* [14, 22] and *B. caballi* [13] from horses and *Babesia* sp. from free-ranging artiodactylids [19]. In one of these studies, 2.5 µL of packed erythrocytes were sufficient to initiate the *B. equi* culture, demonstrating the good sensitivity of the method [22]. However, the selective effect of the in vitro culture method on the parasite is sometimes very high [14].

From the data gathered in this paper, we can propose a procedure to isolate *B. divergens* from asymptomatic carriers. Samples can be collected on CPD or Lithium Heparin and stored up to 9 days at room temperature without dramatic effects on culture results, allowing samples to be sent by mail to the laboratory. However, the samples should be kept at 4 °C and processed as soon as possible (1 to 3 days after sampling). Cultures could be initiated only from serologically positive samples. However, the sensitivity of the IFAT and the possible lack of serological cross-reactions between particular *B. divergens* strains increase the risk of bias in the sampling by putting aside certain variants. An improvement of the IFAT could be provided by the use of mixed antigens representing different *B. divergens* antigenic types. Culture monitoring (blood smears) can be restricted to two time points, respectively at 14 and 24 days post inoculation.

In conclusion, the method described herein allows the identification of carrier animals, isolation of *B. divergens* and in vitro propagation of sufficient parasites from carrier cattle so that these parasites may be characterized and used in comparative studies of various field isolates.

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