

**Short note**

**Sheep as a new experimental host for *Babesia divergens***

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**Abstract** – *Babesia divergens* was cultivated in sheep erythrocytes in RPMI 1640 supplemented with 10% Fetal Calf Serum (FCS) or sheep serum. In vitro cultures in sheep red blood cells were initiated with human erythrocytes infected in vitro with *B. divergens* Rouen 1987 or with gerbil blood infected with several isolates from bovine origin. After the first subcultures on sheep erythrocytes, a ten-fold multiplication of the parasites was obtained within 48 h. Erythrocytes from three splenectomized sheep were infected in vitro with *B. divergens*; when parasitaemia reached 10%, the animals were inoculated with homologous parasitized erythrocytes. All sheep expressed hyperthermia with a peak between the 6th and the 9th day post-infection (p-i) and a transitory parasitaemia 10 days p-i. In vitro primary cultures were performed on two of these sheep, demonstrating the parasite persistence at very low parasitaemia in the infected animals. Splenectomized sheep can be used as a new model for *B. divergens* chronic infection.

***Babesia divergens* / sheep / in vitro culture / experimental infection**

**Résumé** – **Le Mouton : un nouvel hôte expérimental de *Babesia divergens*.** *Babesia divergens* a été cultivé in vitro dans des hématies de Mouton suspendues dans du RPMI 1640 supplémenté par 10 % de sérum de veau fœtal ou de sérum de Mouton. Les cultures en hématies de mouton ont été initiées avec des hématies humaines infectées in vitro par *B. divergens* Rouen 1987 ou avec des hématies de gerbilles infectées par différents isolats d'origine bovine. Après les premiers passages sur hématies de mouton, les parasites se multiplient 10 fois en 48 h. Les hématies de trois moutons splénectomisés ont été infectées in vitro par *B. divergens* ; lorsque la parasitémie in vitro a atteint 10 %, les animaux ont été infectés expérimentalement avec les hématies homologues. Tous les moutons ont présenté un pic d'hyperthermie entre le 6<sup>e</sup> et le 9<sup>e</sup> jour post-infection (p-i) et une parasitémie

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transitoire 10 jours p-i. Des cultures in vitro primaires réalisées sur deux animaux ont démontré la persistance du parasite pendant 28 semaines. Le mouton splénectomisé peut être utilisé comme un nouveau modèle d'infection chronique par *B. divergens*.

### ***Babesia divergens* / mouton / culture in vitro / infection expérimentale**

## **1. INTRODUCTION**

*Babesia divergens*, transmitted by the three host ticks *Ixodes ricinus*, is the main causative agent of bovine babesiosis in Europe. It appears to be less host specific than the other *Babesia* species from domestic animals. *B. divergens* is responsible for most cases of human babesiosis in Europe [6], particularly in splenectomized people. Splenectomized rats could be experimentally infected by *B. divergens* [10], but the main experimental model for *B. divergens* babesiosis remains the Gerbil (*Meriones unguiculatus*), which develops an acute and fatal babesiosis [9]. The in vitro culture of *B. divergens* has also been described in erythrocytes from different origins: rat [1] and humans [5]. This paper presents the in vitro culture of *B. divergens* in sheep erythrocytes and the first results on experimental infections of splenectomized sheep with *B. divergens*.

## **2. MATERIALS AND METHODS**

### **2.1. *B. divergens* isolates**

*B. divergens* Rouen 1987 was obtained from a man who had recovered from acute babesiosis [5]. Additionally, five *B. divergens* isolates were obtained from infected cattle in 1988: 1903B (Corrèze), 2212B (Côtes d'Armor), 2704A (Eure), 3606A (Indre), 5601A (Morbihan). The different isolates were intraperitoneally injected into two gerbils successively before in vitro culture.

### **2.2. Cryopreservation of *B. divergens* isolates**

Infected gerbil blood or in vitro culture were cryopreserved. Erythrocytes of parasitized gerbils or parasitized sheep erythrocytes were washed twice for 10 min at 1 200 g with RPMI 1640 (Eurobio, Les Ulis, France); the pellet was then resuspended at 0 °C to obtain a final dilution of 25% erythrocytes, 47.5% RPMI 1640, 7.5% DMSO (SIGMA, St Quentin Fallavier, France) and 20% Fetal Calf Serum (FCS, Eurobio); 0.5 mL aliquots in sterile ampoules were frozen to -80 °C in liquid isopropanol (SIGMA) for 16 h and stored in liquid nitrogen. To recover the frozen parasites, the ampoules were thawed in a 37 °C water bath and the parasites were washed for 10 min at 1200 g in RPMI 1640 before infection of gerbils or initiation of the in vitro cultures.

### **2.3. In vitro culture of *Babesia divergens***

In vitro culture of *B. divergens* Rouen 1987 was initiated with infected gerbil blood cells and was first performed in human red blood cells as previously described [2]. The parasites were grown in 25 cm<sup>2</sup> culture flasks, in O group erythrocytes suspended at 2.5% haematocrit in RPMI 1640 (Eurobio) pH 7.3 buffered by 25 mM HEPES, 2.1 g/L NaHCO<sub>3</sub> and supplemented with 10% human serum (AB Group). Cultures were performed in a humidified 6% CO<sub>2</sub> atmosphere at 37 °C. The media were changed every two days until the parasitaemia reached 10% and daily when

parasitaemia exceeded 10%. For the subcultures, the infected erythrocytes were diluted to 1% with normal human erythrocytes.

In vitro culture in sheep erythrocytes of *B. divergens* Rouen 1987 was initiated from in vitro culture in human erythrocytes. In vitro culture in sheep erythrocytes of the five isolates of bovine origin were directly initiated with infected gerbil blood. The culture method was similar but the media was supplemented with FCS or sheep serum.

#### 2.4. Experimental infection of sheep

Three 6-month-old male sheep were splenectomized under gas anaesthesia. The animals were infected at least three weeks after splenectomy. Infective material was produced by cultivating *B. divergens* Rouen 1987 on homologous red blood cells in sheep serum. An infective dose of  $10^9$  to  $5 \times 10^9$  parasitized erythrocytes diluted in NaCl 9‰ was intravenously injected. Sheep n° 1 and n° 2 were treated with dexamethasone at 0.5 mg/kg for 4 days after experimental infection. Rectal temperature and haematocrit were recorded daily for 15 days. For 2 sheep (n° 2 and n° 3), in vitro primary cultures were performed for 28 weeks: the blood of infected sheep was

collected on citrate dextrose phosphate (CPD, SIGMA) and centrifuged at 1 100 g for 10 min; 1 mL of packed red blood cells was suspended in culture media supplemented with 10% sheep serum and grown in 25 cm<sup>2</sup> culture flasks. The culture medium was changed every 2 days until parasitaemia reached 10%.

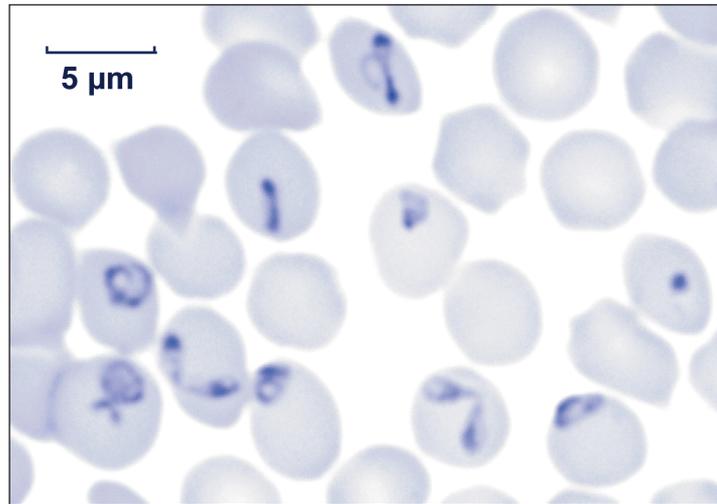
### 3. RESULTS

#### 3.1. In vitro culture

In vitro culture of *B. divergens* Rouen 1987 in sheep erythrocytes was initiated with 10% of human erythrocytes at 10% parasitaemia. During the first subcultures, the multiplication of *B. divergens* in sheep erythrocytes was lower than in human erythrocytes (Tab. I). After the 7th subculture in sheep erythrocytes, a 10% parasitaemia was obtained within 48 h. When in vitro culture was initiated with erythrocytes from parasitized gerbils, the 2nd passage was very long (Tab. I); after the 4th passage in sheep erythrocytes, a 10% parasitaemia was obtained within 48 h (Fig. 1). For all the *B. divergens* isolates, parasitaemia of 50–60% could be reached in vitro in 72 to 96 h of culture.

**Table I.** Number of days needed to obtain a 10% parasitaemia during the first in vitro subcultures (SC) for the different *B. divergens* isolates; SC were initiated with 1% of parasitized erythrocytes (in vitro parasitized human erythrocytes for the SC1 of *B. divergens* Rouen 87, infected gerbil erythrocytes for the SC1 of the other isolates, in vitro parasitized sheep erythrocytes for the other subcultures).

	SC 1	SC 2	SC 3	SC 4	SC 5	SC 6	SC 7
<b>Rouen 87</b>	4	4	4	4	4	3	2
<b>1903B</b>	2	14	11	3	2	2	2
<b>2212B</b>	7	14	3	2	2	2	2
<b>2704A</b>	2	14	6	2	2	2	2
<b>3606A</b>	2	18	8	2	2	2	2
<b>5601A</b>	2	14	6	2	2	2	2



**Figure 1.** In vitro culture in sheep erythrocytes of *B. divergens* Rouen 1987.

The cultures could be stored frozen in liquid nitrogen and reactivated in vitro without any reinfection of gerbils (data not shown).

### 3.2. Experimental infection of sheep

The 3 sheep expressed hyperthermia with a peak between the 6th and the 9th day post-infection (p-i). A transitory parasitaemia was observed on stained blood smears the 10th day p-i for sheep n° 2 and n° 3 (1‰) and between the 9th and the 12th day p-i for sheep n° 1 (maximum 3% on day 10 p-i). The in vitro primary cultures performed on sheep n° 2 and 3 were positive during the 28 weeks; a 10% parasitaemia was reached after 6 to 10 days of culture between the 8th and the 12th day p-i; from the 2nd to the 28th week p-i, 10% parasitaemia was reached in  $16.2 \pm 0.8$  days of culture (9 to 20 days).

## 4. DISCUSSION

The continuous culture of *B. divergens* was first described in bovine erythrocytes using the candle jar system [3, 12] and a medium supplemented with 40% bovine

serum. The culture achieved parasitaemia of 5–15%. The in vitro culture of *B. bovis* has been described in sheep, goat, horse and rabbit erythrocytes [11]. Similarly, Ben Musa and Phillips [1] and Gorenflot et al. [5] described continuous culture of *B. divergens* in rat erythrocytes and human erythrocytes respectively; they both used RPMI 1640 supplemented with 20% FCS and 10% human serum respectively. The culture in sheep erythrocytes described in this paper is closely related to these protocols. After 4 to 7 subcultures in sheep red blood cells, this culture system allowed high in vitro parasitaemia (50–60%) and used only 10% FCS and a convenient source of red blood cells.

The culture could also be adapted to sheep serum and allowed the experimental infection of sheep with their own erythrocytes infected in vitro with *B. divergens*. The primary cultures from erythrocytes of experimentally infected animals demonstrated the parasite persistence at very low parasitaemia in the infected animals for at least 28 weeks. After experimental infection, gerbils develop an acute and fatal illness; they are commonly

used as models for *B. divergens* babesiosis, notably to test the efficacy of vaccines [4, 7]. After natural infection, cattle do not always develop acute babesiosis; furthermore, they stay chronically infected [8]. In this study, after experimental infection, splenectomized sheep also became asymptomatic carriers of the parasite. Splenectomized sheep could then be used as a complementary model to evaluate the parasite persistence after administration of drugs or vaccine preparation. Furthermore, splenectomized sheep will be an efficient model of chronic babesiosis. This will enable the study of the in vivo interactions between different parasitic isolates and the transmission of *B. divergens* to *Ixodes ricinus*, since sheep are a normal host of the adult stage of these ticks.

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