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Original article

Experimental in vitro transmission of *Babesia* sp. (EU1) by *Ixodes ricinus*

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Abstract – *Babesia* sp. (EU1), first characterized in 2003, has been implicated in human cases of babesiosis in Italy, Austria and Germany. It has been identified in roe deer and in its suspected tick vector, *Ixodes ricinus,* in several European countries. The aim of the present study was to validate the competence of *I. ricinus* as a vector of *Babesia* sp. (EU1) via experimental infections. For this purpose, a parasite strain isolated from roe deer was cloned in sheep erythrocytes. After experimental infections, parasite DNA was successfully amplified by PCR in both eggs and larvae originating from infected *I. ricinus* females and in the salivary glands of females exposed to *Babesia* sp. (EU1) as nymphs. We also demonstrate that infected females were able to transmit parasite DNA during a new blood meal. Together with previous epidemiological studies, these results validate *I. ricinus* as a competent vector for *Babesia* sp. (EU1).

zoonosis / *Babesia* sp. (EU1) / *Ixodes ricinus* / in vitro culture / experimental transmission

1. INTRODUCTION

Babesia sp. infections are caused by tick-borne protozoan parasites that infect animal and human erythrocytes. In animals, babesiosis is responsible for major economic losses around the world and, in humans, it is gaining increasing attention as an emerging disease [15]. Since the identification of the etiologic agent of the disease, hundreds of human cases have been reported in the USA, where babesiosis is mostly attributed to the parasite *Babesia* (*Theileria*) *microti* which is transmitted by the tick *Ixodes scapularis* [14]. In Europe, approximately 30 human cases of babesiosis have been reported over the last fifty years [14, 25]. These infections have been traditionally attributed to the bovine parasite *Babesia divergens* transmitted by *Ixodes ricinus* [2, 9]. However, in 2003, Herwaldt et al. described the first molecular characterization of a new *Babesia* species, *Babesia* sp. (EU1), provisionally named *Babesia venatorum*, and isolated from two human cases in Austria and Italy [12]. Since this time, another case of human babesiosis has been attributed to *Babesia* sp. (EU1) in Germany [11]. Thus, since molecular-based tests were often not performed to confirm infection by *B. divergens*, we can hypothesize that other cases of human babesiosis may have been caused by *Babesia* sp. (EU1) and that the prevalence of this parasite in humans has been underestimated. Recently

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reported serological cross-reactivity between *B. divergens* and *Babesia* sp. (EU1) support this hypothesis [8].

Babesia sp. (EU1) was identified from roe deer (*Capreolus capreolus*) in Slovenia in 2005 [6] and in Italy and France in 2007 [3, 22]. Such studies validate the potential of roe deer as wild reservoirs that could maintain the circulation of the parasite. In addition, the fact that roe deer are often heavily infested with *I. ricinus*, a tick that also frequently feeds on humans, suggests that this tick could be the vector of *Babesia* sp. (EU1).

Duh et al. reported the first detection of *Babesia* sp. (EU1) DNA in *I. ricinus* in Slovenia in 2005 where, among the 135 ticks analyzed, 2.2% were carrying this parasite [7]. Since then, other studies have revealed by PCR that 1–2% of *I. ricinus* ticks collected from wild and domestic animals in Switzerland [5, 13] and in the Netherlands [15] harbored *Babesia* sp. (EU1) DNA. Finally, we have also previously reported some epidemiological evidence that *I. ricinus* could be a potential vector of *Babesia* sp. (EU1); parasite DNA was detected in 26% of larval pools from female ticks collected on infected roe deer in France [3]. Finally, some recent data showed that *Babesia* sp. (EU1) sporozoites isolated from salivary glands (SG) of naturally infected ticks are able to invade erythrocytes in vitro [1]. Some experimental infection studies are now needed in order to definitively prove the competence of *I. ricinus* as a vector of *Babesia* sp. (EU1).

We report here the cloning of *Babesia* sp. (EU1), as well as a morphological description of the different parasite stages present in vertebrate blood cells and in ticks. Experimental infections of *I. ricinus* were performed with in vitro infected blood and demonstrate that parasite DNA was transstadialy and transovarialy transmitted between tick life stages, as well as from infected ticks to blood during a re-feeding step on non-infected blood.

2. MATERIALS AND METHODS

2.1. Blood samples used in culture

Roe deer blood and the parasite strain used in the study were obtained from animals captured in the Wild Fauna Reserve of Chizé (Deux-Sèvres, France). Sheep blood samples used for cultivation of *B. divergens* came from one sheep commonly used as a donor for *B. divergens* cultures and reared at the National Veterinary School of Nantes (France). The *Babesia*-free status of this animal was confirmed by in vitro culture. All blood samples were collected in heparin containing Venoject tubes (Terumo), centrifuged 10 min at 800 *g* and the plasma and buffy coat were discarded. The pellet of red blood cells was then washed and diluted 1:3 with RPMI 1640 containing 50 µg/mL gentamicin and 0.25 µg/mL amphotericin B. This solution was then maintained at 4 °C until use in parasite cultures and for tick feeding. The autologous plasma was maintained at –20 °C until use in tick feeding. All media and antibiotics used in the study were from Cambrex (Charles city, Inc., IA, USA).

2.2. Ticks

I. ricinus ticks used for experimental feedings were collected from the forest of Princé (Loire-Atlantique, France) by flagging the vegetation as previously described [24]. Negative controls used for PCR amplification came from *I. ricinus* collected from Belle-Île-en-Mer, a babesiosis-free island in the Gulf of Morbihan (France). All ticks were reared and maintained in humidity chambers with a relative humidity (r.h.) of 80–90% at 22 °C.

2.3. Parasite isolation and cloning

The *Babesia* sp. (EU1) isolate used in this study, named C201, came from one single roe deer during a study carried out in January 2006 and was isolated as previously described [3, 18]. Briefly, parasites were first isolated from blood samples in autologous erythrocytes (7.5% hematocrit) in RPMI 1640 containing 50 µg/mL gentamicin, 0.25 µg/mL amphotericin B and 20% fetal calf serum (decomplemented 30 min at 56 °C). Cultures were performed at 37 °C in 5% CO_2-air with replacement of the medium every 2–3 days during 3 weeks (without disturbance to the erythrocyte layer), and were monitored periodically for parasitaemia by examination of thin blood smears stained with May-Grünwald Giemsa-based (Diff-Quick, Dade Behring, Deerfield, IL, USA) stain. Six subcultures were then carried out by a 10% dilution of parasitized culture with fresh complete culture media containing autologous roe deer erythrocytes (7.5% hematocrit) every 3 or 4 days. Parasites were then adapted to culture in sheep erythrocytes during 30 subculture rounds and cloned by

limiting dilution in 96 well microplates (up to 0.25 parasites per well). At last, direct sequencing was performed by Qiagen (Hilden, Germany) in order to obtain the complete 18S ribosomal RNA gene of the parasite. This was done using the amplification product of a 1 727 bp sequence obtained with the PCR primer set CRYPTO F and CRYPTO R and following the amplification conditions described by Herwaldt et al. [12].

2.4. Artificial feeding and infection of ticks

Ticks were infected with an in vitro culture of *Babesia* sp. (EU1) clone C201A as previously described for *B. divergens* [4]. Cultures were diluted daily with fresh sheep red blood cells to give a mean parasitaemia of 8% infected erythrocytes. Parasitized erythrocytes were pelleted by centrifugation for 10 min at 800 *g* and mixed with 1/3 (v/v) homologous plasma containing 50 μg/mL gentamicin and 0.25 μg/mL amphotericin B. Three mL of restored blood were put into the glass feeder and presented to the ticks until repletion, with two blood changes per day. Rabbit skins used in artificial feeding were obtained from animals previously used for rearing tick colonies in the laboratory and were treated as previously described [4]. Fifty-two nymphs and 9 adult females were used for artificial infection. Only fully engorged ticks that spontaneously detached were then retained for the study and placed in a humidity chamber. Since mating is required for the completion of female engorgement, an equal number of males and females were used to feed adult female ticks. The rearing was performed in an acclimatized incubator at 22 °C, 80–90% r.h. and an ambient CO_2 level.

2.5. *Babesia* sp. (EU1) DNA detection

Parasite DNA extraction from ticks was performed as previously described using the Promega extraction kit (Promega, Charbonnière, France) [4]. DNA was extracted from individual adult (SG) or from pools of around 50 eggs or larvae. During the final step, the DNA was rehydrated in 50 μL and 300 μL rehydration solution for respectively adult SG and pools of eggs and larvae, and stored at 4 °C until use. For all analyzed samples, the efficiency of DNA extraction was confirmed by a PCR assay with primers designed to amplify *Ixodes* spp. 16S rRNA gene [4]. For blood samples removed from the artificial feeder, DNA was extracted from erythrocyte pellets with the Promega extraction kit following the manufacturer's instructions and suspended in a final volume of 60 μL.

PCR amplification was performed on 10 μL and 20 μL of extracted DNA samples from tick and blood samples, respectively, using the primer pair EU1up and EU1down [13] designed for the *Babesia* sp. (EU1) 18S rRNA. Reactions were performed in a final volume of 30 μL or 50 μL, for either tick or blood samples, and contained 0.33 mM dNTPs (Eurobio, Lille, France), 2 mM $MgCl_2$, 1× PCR buffer, 1 U Taq polymerase (Eurobio) and 1 μM of each primer. PCR conditions included 94 °C for 5 min, 45 cycles of 30 s at 94 °C, 30 s at 61 °C, 30 s at 72 °C, and a final elongation step of 10 min at 72 °C. The 362 bp amplification product was then visualized on an ethidium bromide-stained 1.5% agarose gel.

2.6. *Babesia* sp. (EU1) detection in ticks after an infectious blood meal

In order to show parasite ingestion by the ticks, 6 engorged nymphs and 2 adult females were dissected the day *post repletion*. The whole intestine was removed and dissected in sterile PBS in order to detect parasites in the blood meal by microscopic examination of Giemsa-stained smears. Remaining engorged nymphs were then allowed to molt to adults (around 2–3 months p.r.) and at least 1 month after molting, SG of 3 females infected as nymphs were dissected in order to detect parasite DNA by PCR amplification. This procedure allowed us to detect parasites that could be transmitted by ticks and not simply those that might have persisted in the tick from the previous blood meal. Engorged females were allowed to lay eggs during 10–30 days and 3 broods were analyzed. Part of the eggs was subjected to DNA extraction to test for the presence of parasitic DNA by PCR amplification, and the remaining eggs were allowed to hatch into larvae. These larvae were also subjected to DNA extraction for parasite DNA detection by PCR amplification.

2.7. *Babesia* sp. (EU1) DNA detection after transmission from infected ticks to host blood

Eight female ticks, fed on *Babesia* sp. (EU1) infected blood as nymphs, were successfully subjected to a partial re-feeding step for 8 days in order (1) to detect parasite DNA in their SG after the stimulus of a re-feeding step, and (2) to evaluate their ability to transmit DNA of the acquired parasite to the blood contained in the artificial feeder. Three milliliters of non-infected restored sheep blood were

used in the feeder and changed once a day. At day 3 post fixation, 3 females were detached and dissected in order to perform DNA extraction and PCR amplification on SG, whereas the remaining females were allowed to continue their meal. Each day, from 0 to 9 days post-fixation, a Giemsa-stained blood smear was prepared for microscopic examination using blood removed from the feeder; the remaining blood was centrifuged for 10 min at 800 *g* and the pellet of red blood cells was maintained at –20 °C until DNA extraction. This blood was tested for parasite presence as described above.

3. RESULTS

3.1. *Babesia* sp. (EU1) cloning

Subcultures of C201 samples, positive for *Babesia* sp. (EU1) in homologous erythrocytes, were successfully performed in sheep erythrocytes. Morphological aspects of *Babesia* sp. (EU1) are typical of a small *Babesia* species as shown in Figure 1. Two clones were obtained from the 10 wells containing 0.25 parasite per well. From the 30 wells with 0.5 parasite per well, 6 were positive, demonstrating a good cloning efficiency.

Sequencing of the PCR-amplified 18S rRNA gene revealed 100% identity with that of the complete 18S rRNA gene sequence published for the *Babesia* sp. (EU1) isolate from a human [12].

3.2. *Babesia* sp. (EU1) transmission between tick life stages

Efficient parasite acquisition by ticks was validated by microscopic examination of the gut contents after feeding on *Babesia* sp. (EU1) C201A infected blood (Fig. 2). *Babesia* were ingested over a period of time and the development of the parasites was therefore not synchronous. As a result, several different parasite stages lying free of erythrocytes could be observed in the tick midgut. Line 1 of Figure 2 shows a polymorphic population of individual elongated Strahlenkörper with only one nucleus and some protrusions. Line 2 shows examples of two Strahlenkörper cojoined; this close adherence of the two gametes represents the

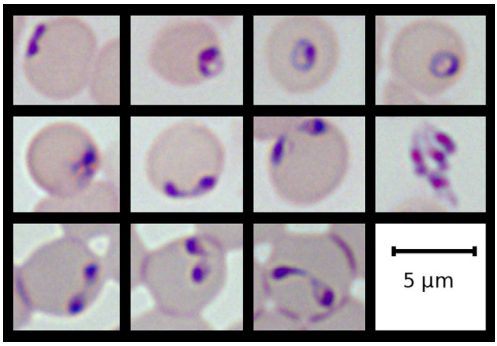

Figure 1. Light micrographs of developmental stages of *Babesia* sp. (EU1) from in vitro culture (May-Grünwald Giemsa based stain). The first two lines show C201 isolate cultured in roe deer red blood cells. Free merozoites, trophozoites, dividing forms and small geminate merozoites at the border of the erythrocyte membrane are very similar to *B. capreoli* in roe deer erythrocytes or *B. divergens* in bovine erythrocytes. The last line shows geminate merozoites of the C201 clone in sheep erythrocytes; morphological aspects are very similar, but geminate forms appear more variable in shape and localization.

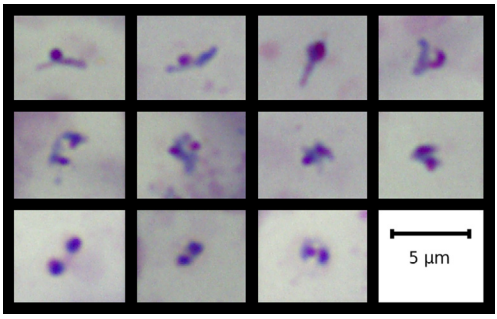

Figure 2. Light micrographs of developmental stages of *Babesia* sp. (EU1) from the midgut of *I. ricinus* females after an infectious blood meal with in vitro cultured parasites (May-Grünwald Giemsa based stain). Line 1: individual elongated Strahlenkörper with only one nucleus and some protrusions. Line 2: examples of two Strahlenkörper cojoined with close adherence. Line 3: bi-nucleated parasites, possibly representing zygotes from the fusion of two Strahlenkörper.

beginning of the fusion, which is illustrated in Line 3, where some bi-nucleated parasites possibly represent zygotes.

Among the 52 nymphs fed on *Babesia* sp. (EU1)-infected blood, 17 molted to female adults. Three of these were dissected and their SG was analyzed for *Babesia* sp. (EU1) by PCR: two harbored parasite DNA in their SG. Eight other females were subjected to the re-feeding step on non-infected sheep blood. Amongst these, 3 were dissected 3 days after the start of the meal. PCR amplification on DNA extracted from their SG revealed that all of them harbored parasite DNA.

Among the 9 adult females fed on *Babesia* sp. (EU1) infected blood, 3 laid eggs and successive larvae in which parasite DNA was searched for. Two broods, as well as the corresponding pools of larvae harbored *Babesia* sp. (EU1) DNA.

No positive DNA amplifications were obtained from control ticks from the *Babesia*-free colony.

3.3. *Babesia* sp. (EU1) transmission to blood

Among the 8 females infected with *Babesia* sp. (EU1) as nymphs and subjected to a re-feeding step, 5 fixed from day 2 to day 5 and were allowed to feed until day 9 on non-infected sheep blood. PCR amplifications performed each day on the blood removed from the feeder revealed the presence of parasite DNA from day 6 to day 9, indicating that ticks transmitted *Babesia* sp. (EU1) DNA via their saliva at least 4 days after their attachment to the membrane. No parasite was observed in microscopic examination of the prepared blood smears (in the 5 μL of blood removed each day).

4. DISCUSSION

Babesia sp. (EU1) was previously identified from human cases, as well as from roe deer and *I. ricinus,* in different areas in Europe, and represents a potential agent of an emerging zoonotic disease. In order to evaluate this potential, it is essential to define its biology and distribution, including the determination of its vector and vertebrate hosts. The aim of the present study was to validate, via experimental infections, the competence of *I. ricinus* to transmit *Babesia* sp. (EU1). For this purpose, a parasite strain isolated from roe deer was cloned in sheep erythrocytes, allowing us to make a morphological description of the parasite in both mammalian cells and in ticks. We then report an experimental demonstration that *I. ricinus* is able to acquire the parasite, to allow its sexual development, and to transmit the parasite to successive tick life stages and to vertebrate blood via saliva.

The *Babesia* sp. (EU1) clone used in the present study was isolated from roe deer blood and was successfully cultivated both in autologous roe deer and sheep red blood cells. To our knowledge, this study represents the first cloning of *Babesia* sp. (EU1) in culture and in heterologous erythrocytes from other ruminants, which may represent a possible range of vertebrate reservoir hosts. Until now, *Babesia* sp. (EU1) has been detected only in roe deer [3, 6, 22], which appears to be a wild reservoir host of the parasite, and in humans [11, 12]. A postulated strict host and vector specificity is traditionally accepted for *Babesia* spp., but *B. divergens* can infect a wide range of animals with or without splenectomy including bovines, gerbils, sheep, some cervid species, various non-human primates and humans (see review by Zintl et al. [25]). Additional vertebrate hosts can therefore not be excluded for *Babesia* sp. (EU1).

I. ricinus is the most prevalent and widely distributed tick in Western Europe and represents the main vector of several zoonotic diseases including *B. divergens* which, until now, was believed to be the main etiological agent of human babesiosis on this continent [25]. DNA of *Babesia* sp. (EU1) has been detected by PCR from *I. ricinus* ticks in Slovenia [7], Switzerland [5, 13], and in the Netherlands [21]. However, such reports are not necessarily proof of the vectorial capacity of the tick, but only of its infection status. In fact, we have previously demonstrated that some parasite DNA acquired during the last blood meal remains in the carcass of the arthropod, in addition to those present in the SG, the only location

from which *Babesia* can be transmitted to a vertebrate host [4].

Observations by light microscopy immediately after the tick feeding on infected blood showed various parasitic stages of *Babesia* sp. (EU1). These different stages are similar to those observed for *B. bigemina* [10, 20, 23], *B. divergens* [4], and *B. canis* [19] and suggest sexual reproduction of the parasites. Even if sexual development of *Babesia* species in their vector has long been a matter of controversy, direct proof of such an event has been obtained by DNA quantification of *B. bigemina, B. canis* [17] and *B. divergens* [16], and it is now a generally accepted phenomenon. Microscopic observations reported here for *Babesia* sp. (EU1) lead to the same conclusion, as the fusion of the cell membrane and the close proximity of the nuclei reported in Figure 2 (Line 3) clearly suggest a sexual phase in development of the parasite. The apparition of sexual parasite stages was only found to occur in the tick vector, suggesting that some, as of yet, unidentified factors associated with engorged *I. ricinus* ticks may be required.

In a preceding study, we reported some field evidence of transovarial transmission of *Babesia* sp. (EU1) in *I. ricinus*; parasite DNA was detected in eggs and larvae issued from females collected on *Babesia* sp. (EU1) infected roe deer in France [3]. The results of the present study validate the transovarial transmission of parasite DNA, from artificially infected adult females to eggs and larvae. The existence of transovarial transmission is distinctive of the *Babesia* genus and means that the ticks themselves can represent a reservoir of the pathogen in the field. In addition, it is noteworthy that some *Babesia* sp. (EU1) negative eggs and larvae came from adults engorged with infected blood. This demonstrates that transovarial transmission is not a systematic event, as previously reported for naturally infected ticks [3].

In our previous study, we also reported that some *Babesia* sp. (EU1) positive eggs and larvae could come from adults engorged on non-infected roe deer. This finding suggested that, in addition to vertical transmission, there was transstadial transmission of the parasite, at least from the nymph to the adult. The same conclusion can be drawn from the study of Hilpertshauser et al. since *Babesia* sp. (EU1) was also detected in one adult male *I. ricinus*, a stage that is not hematophagous [13]. Here we demonstrate that *I. ricinus* nymphs and adults are able to acquire *Babesia* sp. (EU1) and that when nymphs are fed with infected blood, parasite DNA can be recovered in the SG of the succeeding female life stage. These females were then able to transmit it to vertebrate blood during a new meal on non-infected blood. In this case, transmission occurred at least four days after the attachment of the female to the membrane. Such a delay is not surprising given the time it takes for tick attachment, cement synthesis, and probably multiplication of the parasite in the SG. Indeed, two hypotheses are predicated concerning the effect of blood meal stimulus: the blood meal stimulates pathogen multiplication in the SG or pathogen migration to this area. Our results show that some parasite DNA could be detected in the SG without a refeeding step and thus, we assume that parasites were already present in tick SG before the stimulus of the blood meal. Here we report DNA transmission from *I. ricinus* SG to blood and not infectious parasites. However, it is more than likely that this DNA was carried by viable parasites to both SG within ticks and to vertebrate blood during tick feeding. Further investigations are now needed to optimize culture conditions in order to obtain the in vitro development of sporozoites, the parasite stage transmitted by ticks. Alternatively, some laboratory animal models also have to be developed that could be used to test this assumption and to study the life cycle of this parasite in more detail.

In conclusion, *Babesia* sp. (EU1) is a recently characterized parasite that may be responsible for an unaccounted number of human cases of babesiosis, and therefore needs increased attention and vigilance because of its important potential as an emerging zoonotic pathogen. In vitro cloning of *Babesia* sp. (EU1) is a very promising method for further

studies to analyze the biology of this parasite, along with mechanisms of infection and transmission by ticks.

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