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Original article

The potential of *Rhipicephalus microplus* as a vector of *Ehrlichia ruminantium* in West Africa

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ABSTRACT

Heartwater, or cowdriosis, is a virulent tick-borne rickettsial disease of ruminants caused by *Ehrlichia ruminantium*, biologically transmitted by *Amblyomma* species (*A. variegatum* in West Africa). In West Africa, this bacterium was recently reported to naturally infect the invasive cattle tick, *Rhipicephalus microplus* (Rm) through trans-ovarian transmission from replete adult females to offspring. A 'sheep-tick-sheep' cycle was set up to determine whether feeding the progeny of these ticks on naïve sheep could lead to infection, and to compare clinical outcomes resulting from this transmission with those observed following infection by the natural *A. variegatum* (Av) vector. Using local strains of ticks (*KIMINI*-Rm and *KIMINI*-Av) and of *E. ruminantium* (BK242), we recorded, using the PCR technique, the presence of bacterial DNA in ticks (larvae for Av and females for Rm) engorged on sheep inoculated by BK242-infected blood. The bacterial DNA was also detected in the next stages of the lifecycle of *R. microplus* (eggs and larvae), and in sheep infested either by those *R. microplus* larvae or by *A. variegatum* nymphs moulted from larvae engorged on blood-inoculated sheep. Bacterial infection in these sheep was demonstrated by detecting antibodies to *E. ruminantium* using the MAP1-B ELISA and by isolation of the bacterium on cell culture from blood. The sequences of PCS20 gene detected in ticks and sheep were identical to that of the BK242 strain. Our results confirm that *R. microplus* can acquire and transmit *E. ruminantium* to the next stage. However, this transmission resulted in a mild subclinical disease whereas severe clinical disease was observed in sheep infested by *A. variegatum* infected nymphs, suggesting differences in the tick/bacteria relationship. Future studies will focus on replicating these findings with ticks of different isolates and life stages to determine if *R. microplus* is playing a role in the epidemiology of heartwater in West Africa. Additionally, studies will investigate whether sheep that are seropositive due to infestation by *E. ruminantium*-infected *R. microplus* are subsequently protected against heartwater. Such data will add to our understanding of the possible impact of *R. microplus* in areas where it has become recently established.

1. Introduction

Ehrlichia ruminantium is an obligate intracellular bacterium transmitted by ticks of the genus *Amblyomma*. It is the causative agent of heartwater, or cowdriosis, an infectious, virulent and non-contagious disease of ruminants (Camus et al., 1996; Allsopp, 2015). Domestic

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and wild ruminants can be infected, but the former are more susceptible while the later mainly display asymptomatic infections (Camus et al., 1996). Four clinical forms of heartwater are recognized in livestock: hyperacute, acute, subacute and subclinical. Animals overcoming the infection, thanks to previous (innate or acquired) immunity or early treatment, develop a protective immunity lasting at least 2 years (Stewart, 1987) which allows subsequent subclinical infection (and immunity boost) when animals are newly infested by the infected vector ticks. Small ruminants are more susceptible to heartwater than cattle, with high lethality (up to 80%) recorded in sheep (Stachurski et al., 2019). In cattle, losses can also be significant, for example when imported susceptible or exotic breeds are reared in or moved through heartwater-endemic areas. Cattle losses due to heartwater are far lower in West Africa where herds remain predominantly constituted of less susceptible local breeds, except in some dairy farms located in peri-urban areas around big cities (Adakal et al., 2013b). Moreover, the infection rate in the West African tick-vector is high enough, i.e. 9% to 26% (Faburay et al., 2007; Farougou et al., 2012), to allow frequent *E. ruminantium* infection of all cattle, even of less infested young calves for which the disease takes a subclinical form followed by protective immunity (Uilenberg, 1995; Deem et al., 1996). These characteristics lead to the establishment of enzootic stability for the disease in traditional farms (Ocaido et al., 2009; Kasaija et al., 2021).

In West Africa, the only known tick vector for *E. ruminantium* is *A. variegatum*, which maintains the bacterium only transstadially (from larvae to nymphs and adults, or from nymphs to adults). However, the recent establishment of *Rhipicephalus microplus* in West Africa may change the situation. Indeed, it has recently been shown that, based only on the detection and sequencing of bacterial DNA, 29% of *R. microplus* adults collected in various west-African countries appeared naturally infected by *E. ruminantium* with transovarial transmission occurring from infected *R. microplus* adult females to their offspring (Biguezoton et al., 2016b). As discussed in Biguezoton et al. (2016b), these results were surprising since the literature reported only African *Amblyomma* species as tick vectors that had coevolved with *E. ruminantium* and rare events of transovarian transmission of the bacteria in only one of those, *A. hebraeum,* present in southern Africa (Bezuidenhout and Jacobsz, 1986). Moreover, confirmation that *R. microplus* may contribute to the circulation of *E. ruminantium* in West Africa could have important epidemiological consequences given the recent changes in the tick communities present in cattle and sheep herds in this region. The rapid dispersal of *R. microplus* in West Africa in recent years (Madder et al., 2007; Adakal et al., 2013a; Githaka et al., 2022) has resulted in higher abundance of *R. microplus* than *A. variegatum* in many areas where both species co-occur (Biguezoton et al., 2016a). This shift in relative vector density together with coinfestation of the two tick species has increased the relative probability of *R. microplus* to be infected by *E. ruminantium* (Biguezoton et al., 2016b). Moreover, similar to *A. variegatum, R. microplus* can feed and develop successfully on small ruminants in addition to cattle (Ghafar et al., 2020; Niu et al., 2016). Taken together, these observations raised diverse questions regarding current epidemiology of *E. ruminantium* in West Africa, two of which have been investigated in this study. Firstly, are *R. microplus* larvae from infected females able to transmit the *E. ruminantium* bacteria they acquired trans-ovarially to naïve sheep? Then, once the bacteria have been transmitted, what are the sheep reactions to such infections? Herein, we present findings from a study aimed at testing whether *R. microplus* may become a competent vector for *E. ruminantium* in West Africa. To investigate the second question, we compared sheep health after exposure to putatively infected *R. microplus* larvae with those of sheep exposed to the infection by the same bacterial strain, either via direct inoculation of infected blood or infestation by infected *A. variegatum* nymphs.

2. Materials and method

2.1. Sheep

Naïve sheep of the Bali-bali breed, 12-18 months old, were brought to CIRDES from a heartwater-free area in northern Burkina Faso (Dori, 14° 03′ N, 0° 03′ W) where both *A. variegatum* and *R. microplus* are absent. They were first quarantined in the barn, confirmed sero-negative for *E. ruminantium* by ELISA test (see 2.2.4 below) and then kept separately in compartments with one sheep per compartment. To prevent possible contamination between animals, experiments with the two tick species were implemented at different periods (from 07/13/2019 to 10/28/2019 for *R. microplus* trial and from 10/09/2019 to 12/30/2019 for *A. variegatum* experiment) and in different parts of the barn. Sheep were allocated to study groups as follow:

- **group#0:** 2 sheep for the amplification of the *E. ruminantium* strain BK242, with resulting aliquots of infected blood stored in liquid nitrogen until used. The presently described experiments used aliquots from a single animal (see below);
- **group#1** (relative to *A. variegatum* experiment, see Fig. 1)**:** 5 experimental sheep + 2 control sheep (uninfected);
- **group#2** (relative to *A. variegatum* experiment, see Fig. 1)**:** 5 experimental sheep + 2 control sheep (infested with uninfected ticks);
- **group#3** (relative to *R. microplus* experiment, see Fig. 2): 5 experimental sheep + 2 control sheep (uninfected);
- **group#4** (relative to *R. microplus* experiment, see Fig. 2)**:** 5 experimental sheep + 2 control sheep (the 2 same control sheep as in **group#3**, infested with uninfected ticks).

2.2. Tick strains

The *R. microplus* ticks used in the experiments belong to a colony maintained at CIRDES since 2011 (Biguezoton et al., 2016b). The ticks were initially collected from cattle in Kimini village (10° 06′16′′ N, 4° 46′42′′ W), close to the Côte d'Ivoire border. The colony, hereafter referred as *KIMINI*-Rm strain, is maintained by feeding 60 day-old larvae regularly on cattle. The *KIMINI-Av* strain of *Amblyomma variegatum* was derived from engorged females collected in the same Kimini village in 2018. This strain was maintained since 2018 by regular infestation of sheep by young larvae, nymphs and adults. Eggs and larvae of both strains are maintained in incubators at 27±1 °C with a relative humidity of 85-95%.

2.3. Bacteria strain

A local strain of *E. ruminantium*, BK242, was isolated from *A. variegatum* ticks collected from sheep reared in Bekuy village (11°37'58''N, 3°54'12''W), central Burkina Faso, during a vaccine trial (Adakal et al., 2010). It was then isolated and cultured in bovine aortic endothelial cell cultures, and used for various vaccine or cross-protection studies (Adakal et al., 2010). Infected blood collected from inoculated and hyperthermic sheep was mixed with 10% DMSO and stored in liquid nitrogen at CIRDES. To obtain further experiment material, the BK242-infected blood sample was thawed at room temperature and inoculated into the two sheep in group#0, each receiving 2ml. Ten days post-inoculation, as the animals experienced hyperthermia (≥39.5 °C), blood was collected and preserved as aliquots with DMSO (10% DMSO + 90% of blood) in liquid nitrogen for later use as an inoculum for the experimental animals.

2.4. Experiments with ticks

The first aim of these experiments was to feed ticks of both studied species, during the bacteraemia, on sheep inoculated with blood

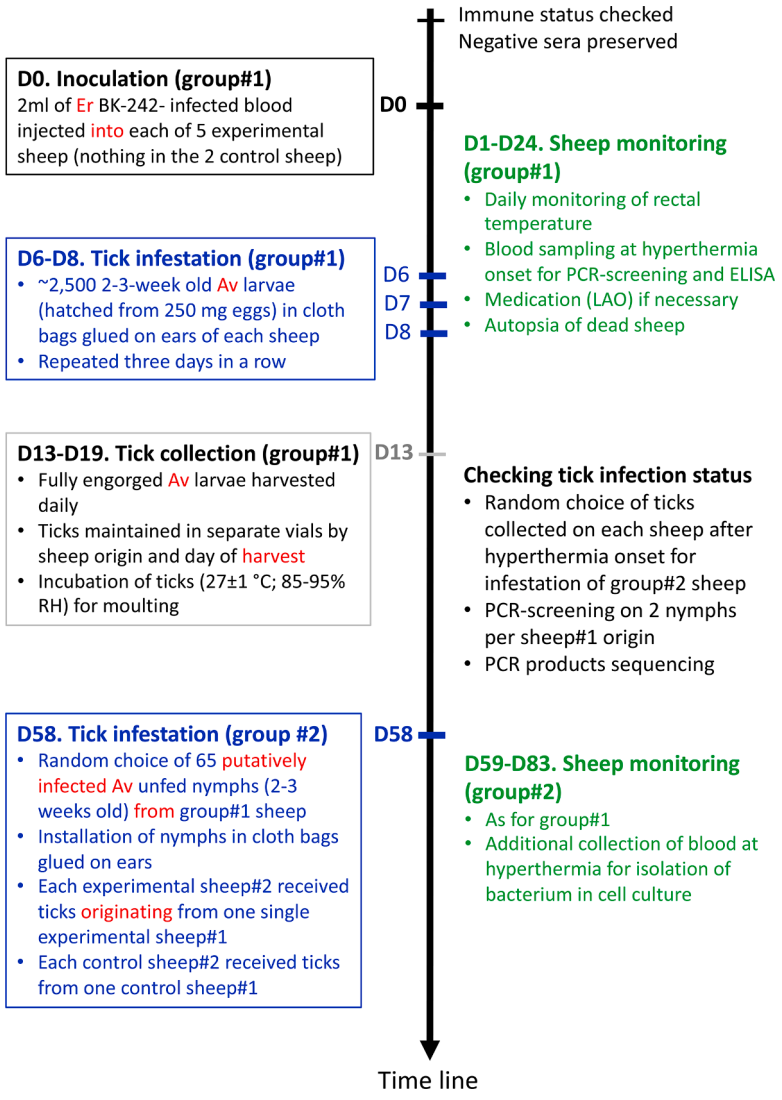

Fig. 1. Schematic description of the *Amblyomma variegatum* experiment. D stands for day whereas Er corresponds to *Ehrlichia ruminantium*. The chronology of the investigation steps detailed in 2.1-2.8 subsections is presented in reference to the day of bacterial inoculation into group#1 experimental sheep (D0 corresponding to July 13th 2019). LAO stands for the long-acting oxytetracycline used to protect sick sheep from death (cf. point 2.5).

infected with *E. ruminantium*. Once engorged, the collected ticks were placed in incubators for oviposition (*KIMINI-Rm* females) or moulting (*KIMINI-Av* larvae) and assessment of their relative efficacy to acquire bacterial infection. A second aim of these experiments was to compare the efficiency of bacterial transmission from tick to naïve sheep between tick species, hence between infected *KIMINI-Rm* larvae that received the bacteria from their mother and infected *KIMINI-Av* nymphs that acquired bacterial infection when blood-feeding as larvae. A third aim was to evaluate the possibility of differences in the disease outcome among sheep that acquired infection from bacteria of the same aliquots of infected blood but via different transmission routes: direct inoculation, via infected *KIMINI-Rm* larvae or via *KIMINI-Av* nymphs. Experimental protocols required thus a pairwise association between two groups (N and N+1) of sheep so that (i) investigation of the bacterial transmission from inoculated sheep to ticks was carried out with the sheep of group N, and (ii) transmission from possibly infected ticks to naïve sheep was performed with sheep of group N+1. The protocol had to be adapted to the particular characteristics of the cycle of each tick species (see below and Figs. 1 and 2 for its adaptation to *A. variegatum* or *R. microplus*, respectively). Monitoring of sheep health status and of seroconversion, assays of bacterial isolation from sheep blood as well as PCR-screening and ELISA-testing for the presence of bacterial DNA in ticks or sheep-blood remained constant across experiments as well as among experimental and control sheep.

2.4.1. *Amblyomma variegatum experiment*

A. variegatum is a three-host tick whose larvae engorge in about 5-8 days (Yonow, 1995). Given the incubation period of heartwater following inoculation with infected blood (expected to last 10-14 days), tick larvae were placed on the inoculated sheep 6, 7 and 8 days after the inoculation with infected blood. Larvae and nymphs of *A. variegatum* were put on sheep in cloth bags glued on ears. The different steps of the experiment are detailed in Fig. 1.

2.4.2. *Rhipicephalus microplus experiment*

R. microplus is a one-host tick. The unfed larva is therefore the only questing stage. Moulting from engorged larva to nymph, and from engorged nymph to adult both occur on the infested host, on which mating of ticks and female feeding also take place. The parasitic phase of the lifecycle lasts about 19-25 days (Hitchcock, 1955). *R. microplus* larvae should therefore be placed on sheep before the inoculation of infected blood. The larvae were placed on sheep's backs and allowed to feed freely. Preliminary data predicted high efficiency in transovarial transmission of the bacteria in the *KIMINI-Rm* tick strain (100%

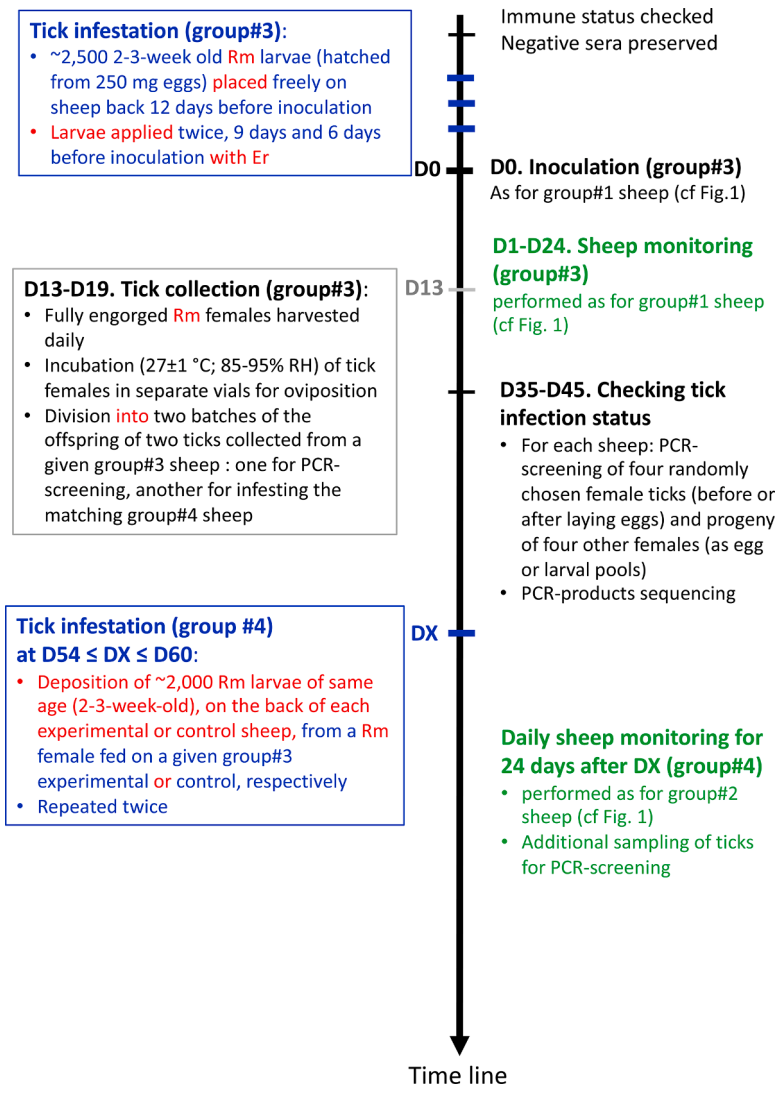

Fig. 2. Schematic description of the *Rhipicephalus microplus* experiment. D stands for day whereas Er corresponds to *Ehrlichia ruminantium*. The chronology of the investigation steps detailed in 2.1-2.8 subsections is presented in reference to the day of bacterial inoculation into group#3 experimental sheep (D0 corresponding to October 10th 2019).

according to Biguezoton et al. 2016b), so that we restricted the PCR-screening of the progeny from females fed on a given experimental group#3 sheep to four siblings. The detailed experiment is presented in Fig. 2.

2.5. Clinical survey

Rectal temperature and putative disease symptoms were recorded daily at 7 a.m. for each animal (experimental and control) in all groups. The clinical signs monitored in studied animals were the following: fever, anorexia, depression, coughing, lack of coordination, tremors, hyperesthesia, dyspnea, opisthotonos, lateral recumbency with paddling or galloping movements and sudden death (Nair et al., 2021). Blood samples were collected in EDTA tubes when hyperthermia was noticed ($T° \geq 39.5$ °C), preserved in ethanol (1/4 ethanol + 3/4 of blood), and later screened for *E. ruminantium* DNA. Serum samples were collected before infection, during and after hyperthermia for analysis by ELISA. Animals displaying tremors and hyperthermia for 3-5 consecutive days were treated with long-acting oxytetracycline (LAO) 20% (1ml/10kg body weight) to prevent death.

2.6. Detection of E. ruminantium by PCR and ELISA test

Ticks, blood and cell culture samples collected on each of the 5 animal groups were processed for DNA extraction and nested PCR amplification of *E. ruminantium* DNA at the PCS20 locus as previously described (Biguezoton et al., 2016b; Martinez et al., 2004) with minor modifications. These modifications consist of the use of 5% DMSO and 0.1% TWEEN 20 in the Master Mix and 1.5 µl of pure DNA or PCR product. Samples of PCR products were sent for purification and sequencing by AZENTA, formerly GENEWIZ (Germany) and phylogenetic analyses performed.

The MAP1-B ELISA test of *E. ruminantium* was performed as previously described (Faburay et al., 2007; Mboloi et al., 1999; Van Vliet et al., 1995) with samples diluted at 1:100 on volume to volume basis in 0.1 M PBS, pH 7.2 and supplemented with 0.1% Tween 20 and 3% skim milk. Homologous sera collected during the animal pre-screening stage and right before the sheep were infected by blood or infected ticks were used as duplicate negative controls for the ELISA test carried out with the serum samples collected during hyperthermia and at the end of the study. Peroxidase-conjugated sheep-specific IgG antibodies were used. ELISA optical densities were measured using an ELISA reader from a CIRG09 laptop computer. With the Skanlt for multiskan analysis

software and the raw Optical Density (OD), values were transferred to a protected Excel file E-TE-21 allowing verification of the assay validity criteria and interpretation of the results.

The percentage of relative positivity (PRP), allowing detecting positive samples, was calculated as recommended by manufacturer (CIRAD Guadeloupe):

$$PRP \text{ Value } (\%) = \text{OD of sample/OD of positive control} \times 100.$$

PRP values < 12% were considered negative, those ranging from 12 to 19% doubtful, and PRP values > 19% were considered positive.

2.7. Ehrlichia ruminantium culture

We used bovine aortic endothelial cells (BAEC) to assess the presence or absence of *E. ruminantium* from DMSO-preserved blood samples collected from either experimental (during hyperthermia) or control animals (at the same time). After dilution at 1:1 ratio with complete cell culture media, ~2 ml of diluted blood were added to BAEC cell-culture media. The culture media contained Dulbecco's Modified Eagle Medium (DMEM) High Glucose (4.5 g/l), decomplemented foetal calf serum (5%) and L-Glutamine (2mM). Cell culture plates containing BAEC, diluted blood (infected or not) and media were incubated at 37 °C for two hours and gently shaken each 10 minutes. The plate wells were then washed to remove the blood and 5 ml of culture medium supplemented with antibiotics (100U/ml of penicillin and 100µg/ml of streptomycin) added. The cells were incubated in a 5% CO2 incubator at 37 °C and medium replaced every 24 h. Cells were inspected daily and the proportion (%) of swollen and reflective cells together with the occurrence of morula and lysed cells was noted. Daily observations were continued until the lysis layer became homogenous. This later was then removed and the mixture centrifuged to obtain pellets which were used for DNA extraction followed by PCR or for preservation in liquid nitrogen for future use.

2.8. Nucleotide sequence analysis

Forward and reverse sequences of PCR products targeting the PCS20 gene were used to generate the consensus sequence per sample prior to further analyses. Multiple sequences alignment using ClustalW in MEGA5 (Tamura et al., 2011) was used to check whether the bacterial strains identified in samples of the five groups were the same and correspond to *E. ruminantium*. Pairwise alignments were also performed using Blastn algorithm in GenBank to compare the representative sequences of the groups highlighted by phylogenetic analyses. Phylogenetic relationships were investigated with MEGA5 (Tamura et al., 2011), using 1000 iterations of the Maximum Likelihood method and choosing the Tamura 3-parameter model (Tamura, 1992). Bootstrap supports (i.e., the percentage of trees in which the associated taxa clustered together) is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 10.2365)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 45 nucleotide sequences: 37 from current study and 8 references.

3. Results

3.1. Multiplication of E. ruminantium strain BK242

The two 'amplifier' sheep (group#0) experienced three consecutive days of hyperthermia, 10 to 12 days post-inoculation (Dpi), with peaks at 40.5 °C and 41.2 °C, respectively. The sheep were treated with LAO at Dpi13. Blood collected at the onset of hyperthermia was tested positive by semi-nested PCR. Indirect MAP 1B ELISA assays carried out at the same time were also positive with PRP values of 52% and 48%, respectively. All experimental animals in groups #1 and #3 were inoculated with blood collected from the same sheep on Dpi10.

3.2. Transmission of Ehrlichia ruminantium to naive sheep by Amblyomma variegatum ticks

The onset of hyperthermia (first occurrence ≥ 39.5 °C) was recorded at Dpi13 to Dpi16 for group#1 inoculated animals except for one sheep whose temperature remained < 39.5 °C with no symptom of heartwater until the end of study (Fig. 3A). During the acute hyperthermia phase, the other animals displayed various clinical signs (Table 1). Two of the infected sheep died after three or seven consecutive days of hyperthermia, despite LAO treatment. The post-mortem examination revealed hydropericardium and diffuse pulmonary oedema. *E. ruminantium* was identified in their capillaries by Giemsa-stained brain smear tests and by PCR amplification in their brain. *E. ruminantium* DNA was also detected by PCR in the blood samples of all inoculated sheep and from nymphs (moulted from engorged larvae) that detached from the 5 infected sheep (Table 2). The presence of anti-*E. ruminantium* antibodies was confirmed by ELISA (PRP 38%-70%) in samples collected at the onset of hyperthermia (Table 2). In contrast, *E. ruminantium* DNA was not detected in any of the *A. variegatum* ticks collected from the control animals, which displayed no disease symptom and remained seronegative for *E. ruminantium*.

Two of the animals initially intended for group#2 suffered from serious diarrhoea and had to be withdrawn from the experiment, so that group#2 included only three experimental animals. One of those sheep did not display any symptoms contrary to the other two (Fig. 1B and Table 1). One group#2 sheep died at day 19 post-tick-exposure (Dpte). The autopsy revealed hydrothorax and ascites. The smear test was positive. The diagnostic PCR, ELISA test and cell culture using the blood of the three experimental animals pointed to the presence of the bacterial DNA and anti- *E. ruminantium* antibodies or live bacteria (Table 2). The control animals were negative for the three tests and never showed hyperthermia or symptoms.

3.3. Transmission of Ehrlichia ruminantium to naive sheep by Rhipicephalus microplus ticks

The onset of hyperthermia among sheep of Group#3 occurred at Dpi13-16, except for one sheep whose temperature remained < 39.5 °C. However, they all showed clinical signs (Table 1). As expected, no hyperthermia was recorded in group#3 control animals (Fig. 4A). PCR screening of DNA extract from blood was positive for the five experimental animals (Table 2) but negative for control animals. The MAP 1B indirect ELISA tests were also positive for the sera of experimental animals collected at hyperthermia onset (PRP: 28-72%) and at the end of the experiment (54-84%) (Table 2), but remained negative for the control sheep. For the sheep that did not express hyperthermia, an indirect ELISA positive result was obtained with blood serum collected at Dpi19.

PCR screening was performed on 45 randomly chosen tick-samples collected from group#3 experimental sheep, and on 18 ticks from group#3 controls. All PCRs performed on ticks from controls were negative. Eighty-seven percent of the *R. microplus* samples collected from inoculated sheep were positive (Table 2). The highest rate (100%) of infection by *E. ruminantium* was obtained with the larval pools, whereas 80% of egg pools and females tested before and after egg laying were also positive (Table 2).

Each group#4 animal was infested by larvae from three females tick that had blood-fed on the same group#3 sheep which served to infest the matching group#4 animal (Fig. 2; see matching pairs of sheep in Table 1). The infectious status of these larvae was not fully known. Indeed, three group#4 animals were infested by two tick-families whose siblings were PCR positive as eggs and by a third of unknown infection

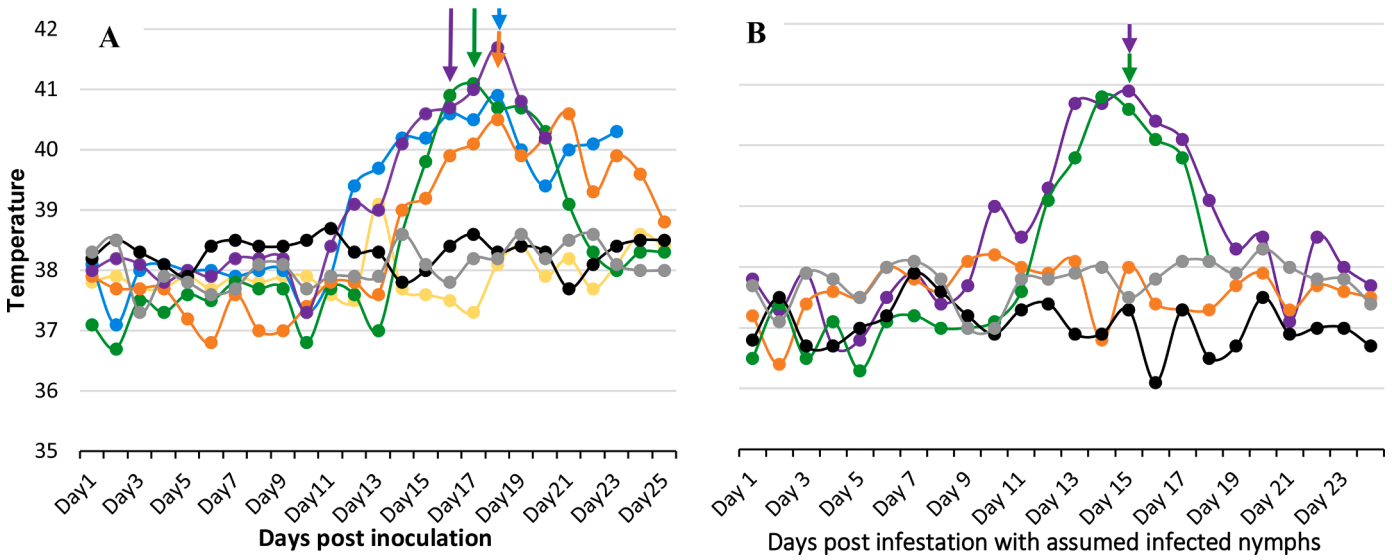

Fig. 3. Rectal temperatures recorded in sheep from group#1, inoculated with blood infected with Ehrlichia ruminantium (A), and group#2, infested by Amblyomma variegatum nymphs moulted from larvae engorged on group#1 animals (B). Colour identity shows the paired sheep from larval to nymph expositions with control pairs in black (A96 in group#1and A3264 in group#2) and grey (A105 in group#1 and A3274 in group#2). Temperature data on experimental pairs of sheep appear in green (A101 in group#1 and A109 in group#2), orange (A108 in group#1 and A106 in group#2) and purple (A3273 in group#1 and A107 in group#2). Yellow (A3263) and blue (A100) in group#1 do not have correspondence in group#2. A100 and A3273 correspond to sheep which died despite long-acting oxytetracycline (LAO) treatment. The arrows indicate the LAO treatment days.

status. Two other animals from group#4 (A3267 and A3270) were infested by one tick-family whose siblings were PCR positive as eggs and two others of unknown infection status.

For group#4 animals, PCR-screening was performed on 8 ticks of various stages collected per sheep. All PCRs performed on the ticks collected from control animals were negative whereas the frequency of *E. ruminantium* detection in ticks ranged from 12.5% to 87.5% across the five experimental sheep (data not shown) and from 20% to 80% (mean: 52.5%) with a maximum for the non-engorged females (Table 2).

Two of the five group#4 experimental animals experienced a weak rise in rectal temperature, mainly from Dpte10 to Dpte14 (Table 1, Fig. 4B). No other clinical sign was observed in group#4 experimental animals (Table 1).

The indirect MAP1-B ELISA tests were all positive (Table 1) with PRP values ranging from 21% to 68% on experimental sheep, showing that these sheep experienced *E. ruminantium* infection. By contrast, PRP-values remained below 12% for group#4 control sheep. The cell culture performed using the blood of each of the five group#4 experimental sheep, collected at the same period, resulted in cell lysis attesting to the presence of the bacteria in the blood (Table 2).

3.4. Genetic relationship

Sequences analyses performed on 37 bacterial isolates, including that used for inoculating group#0 sheep, concluded that the isolated bacteria were *E. ruminantium*. Sequence alignment (Supplementary File 1) showed that most of these sequences (21/36) are identical to that of the BK242 strain. The others differed from the BK242 sequence at either one (2/36) or two nucleotides (13/36) with the coding protein remaining unchanged. Representative sequences of each of the three groups highlighted in the phylogenetic tree have been submitted to GenBank under the accession numbers OM397543; OM397544 and OM397545. The phylogenetic tree did not show any clustering of the mutated sequences that was in accordance with the differences in sheep origin of the bacterial isolates (Fig. 5).

4. Discussion

The chief objective of this study was to investigate whether or not the maternal inheritance of *E. ruminantium* observed in West African *R. microplus* ticks (Biguezoton et al., 2016b) can be followed by the transmission of the bacterium by the infected larvae feeding on sheep. To ensure the robustness of the study design, a layered set of controls was implemented on three levels. Firstly, we verified and confirmed that all the Bali-Bali sheep involved in the study were naïve for *E. ruminantium* before their arrival at the experimental station. Second, we included a comparative experiment showing transmission of *E. ruminantium* by nymphs of *A. variegatum,* its biological vector, as a result of transstadial maintenance of the bacterium from larvae to nymphs. Thirdly, we included in the *R. microplus* and *A. variegatum* experiments, several control animals to guard against potential *E. ruminantium* contamination in the course of the study. Repeated PCR-screening and ELISA-testing performed on the control animals or their ticks did not show any spill-over of *E. ruminantium* from experimental to control animals in any experiment.

The virulence of the BK242 bacterial strain was consistent when delivered to the animals either as infected blood or through transmission by *A. variegatum* ticks with severe disease characterised by the onset of hyperthermia at 10-16 days post blood inoculation or tick infestation and lasting 3-7 days. However, one infected sheep of each groups#1 to #3 did not display hyperthermia. The range of incubation periods highlighted in this study is longer than that observed in the same Bali-Bali sheep by Adakal et al. (2004) but consistent with other past studies: incubation periods in sheep ranged from 9 (Mulinge, 1990) to 14 days (Allsopp, 2015). Such results allow confirmation of the virulence of the presently used BK242 bacterial strain but might also indicate, at least for groups #1 and #3 experimental animals, a slightly lower bacteraemia than observed in another study (Adakal et al., 2004). In contrast, *E. ruminantium* transmission by *R. microplus* resulted in a mild infection: two animals displayed transient fever with rectal temperature above 39.5 °C in only one on days 12-13 after tick infestation.

The presence of the bacteria in the blood of experimental sheep exposed to *E. ruminantium*-infected *A. variegatum* or *R. microplus* (group#2 and group#4, respectively) was confirmed by several

Table 1
Clinical signs recorded among monitored sheep. Indications in parentheses detail the matching sheep pairs across experiments (group#1 and #2 for the *A. variegatum* experiment and group#3 and #4 for the *R. microplus* experiment). 'Days post exposure' referred to either the time delay since inoculation of infected blood (dpi for group#1 and #3 sheep) or exposure to presumably infected ticks (dpte for group#2 and #4 sheep).

Groups	Sheep (infection source if any)	Highest Body temperature	Days post exposure	Clinical Signs
Group#1	A96 (control)	none	none	none
	A105 (control)	none	none	none
	A3263 (blood)	39.1	13	none
	A100 (blood)	40.9	18	General body weakness, dyspnea, lateral recumbency with paddling, tremors, death
	A101 (blood)	41.1	17	General body weakness, anorexia, lateral recumbency with paddling, tremors
	A108 (blood)	40.6	21	weight loss, tremors, anorexia
	A3273 (blood)	41.7	18	Anorexia, dyspnea, paresis, decubitus, lateral recumbency with paddling, death
Group#2	A3264 (none, ticks from A96)	none	none	none
	A3274 (none, ticks from A105)	none	none	none
	A109 (ticks from A101)	40.8	14	General body weakness, dyspnea, tremors, lateral recumbency with paddling, death
	A106 (ticks from A108)	38.2	10	none
	A107 (ticks from A3273)	40.9	15	Anorexia, tremors,
Group#3	A98 (control)	none	none	none
	A3268 (control)	none	none	none
	A3267 (blood)	41.2	16	Tremors, anorexia
	A3270 (blood)	39	22	Tremors, anorexia
	A97 (blood)	40.8	17	Tremors, anorexia
	A104 (blood)	40.5	16	weight loss, tremors, anorexia
	A3265 (blood)	41.1	16	Tremors, anorexia
Group#4	A98 (none, ticks from A98)	none	none	none
	A3268 (none, ticks from A3268)	none	none	none
	A3266 (ticks from A3267)	39.7	12	none
	A3272 (tick from A3270)	38.5	17	none
	A3262 (ticks from 104)	39.3	11	none
	A94 (ticks from A97)	38.7	16	none
	A45 (ticks from A3265)	38.7	20	none

Table 2
***Ehrlichia ruminantium* detected by PCR, cell culture and ELISA test.** Only the results relating to the experimental animals are highlighted, as the results of the control animals were all negatives.

Experiments	Groups	Samples	PCR	ELISA-test	Cell culture screening
Amblyomma variegatum	**Group#1**	Blood	5/5 (100%)	5/5 (100%)	n.a
		Nymphs	10/10 (100%)	n.a	n.a
	Group#2	Blood	3/3 (100%)	3/3 (100%)	3/3 (100%)
		Cell culture isolates	3/3 (100%)	n.a	n.a
Rhipicephalus microplus	**Group#3**	Blood	5/5 (100%)	5/5 (100%)	n.a
		Engorged females (2/sheep)	8/10 (80%)	n.a	n.a
		Females after laying (2/sheep)	8/10 (80%)	n.a	n.a
		Egg pools (2/sheep)	8/10 (80%)	n.a	n.a
		Larvae pools (3/sheep)	15/15 (100%)	n.a	n.a
	Group#4	Blood	5/5 (100%)	5/5 (100%)	5/5 (100%)
		Cell culture isolates	4/5 (80%)	n.a	n.a
		Nymphs pool (2/sheep)	7/10 (70%)	n.a	n.a
		Males (2/sheep)	4/10 (40%)	n.a	n.a
		Females (non-engorged) (2/sheep)	8/10 (80%)	n.a	n.a
		Engorged females (2/sheep)	2/10 (20%)	n.a	n.a

complementary methods, including successful isolation of *E. ruminantium* in cell cultures from the sheep blood and serology with the MAP 1B indirect ELISA. The same results were also acquired, more indirectly, using pCS20 semi-nested PCR that were performed on sheep blood or on ticks feeding on these sheep. By contrast, the ticks fed on control sheep always tested negative, and the control sheep never harboured antibodies against *E. ruminantium*. Sequences analysis confirmed that the PCR products obtained from samples of the groups #0 to #4 are that of *E. ruminantium*. Likewise, sequencing of PCR products confirms that the *E. ruminantium*-BK242 strain inoculated into the initial sheep (group#0) was also that isolated afterwards in cell culture, ticks and blood of sheep infected by infected-tick bites. The sites of mutations observed for 15 of the 36 sequences of samples compared to that of BK242 in this study probably stem from intrinsic mechanisms of the bacterium's functioning. Interestingly, these mutations were synonymous (see sequences OM397543; OM397544 and OM397545 in supplementary file S1).

Several functional hypotheses may explain the absence of high hyperthermia or other symptoms in animals infected by infected *R. microplus* larvae in comparison with other experimental sheep. The simpler explanatory hypothesis would be that they experienced too low bacteraemias to display severe disease symptoms. The experimental sheep that received *E. ruminantium* via the inoculation of infected sheep-blood are likely to be infected by a higher number of bacterial cells than those exposed to the bite of *E. ruminantium*-infected *R. microplus* larvae. Previous experiments using the Gardel strain showed that reproducibility in symptoms requires a minimum of 21 000 to 94 000 bacterial

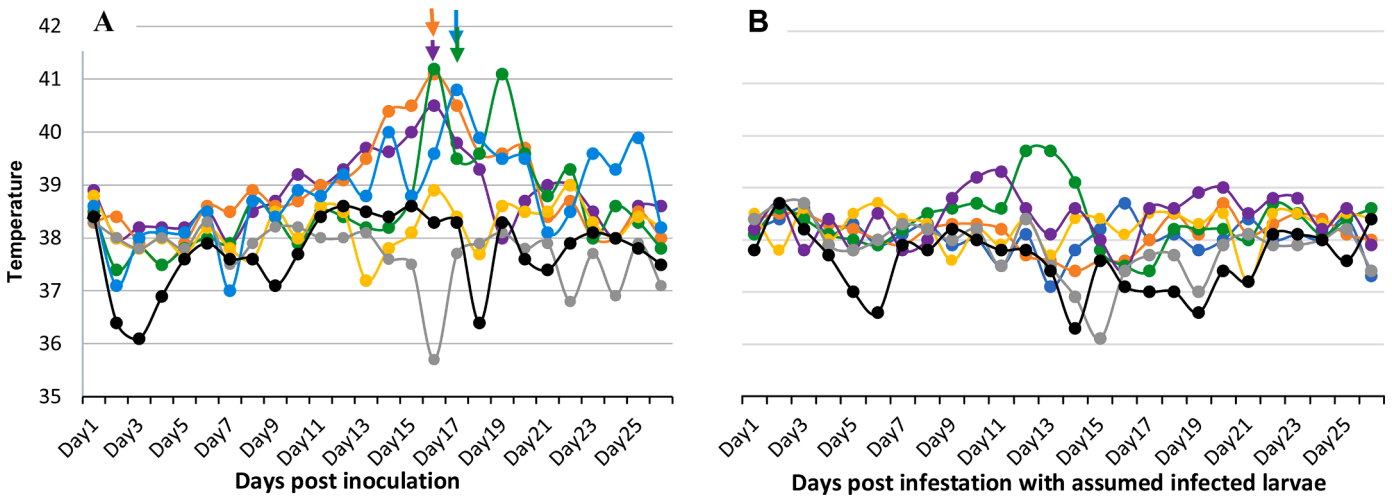

Fig.: 4. Rectal temperatures recorded in sheep from group#3, inoculated with blood infected with *Ehrlichia ruminantium* (A), and group#4, infested by *Rhipicephalus microplus* larvae descending from females fed on group#3 animals (B). Control animals' temperatures appear as black (A98) and grey (A3268) lines while those of experimental animals appear as coloured lines. The colour marks the paired sheep across the two-step experiment (orange: animal A3265 in group#3 and A45 in group#4; green: animals A3267 in group#3 and A3266 in group#4; yellow: animals A3270 in group#3 and A3272 in group#4; purple: animals A104 in group#3 and A3262 in group#4; blue: A97 in group#3 and A94 in group#4). The arrows indicate the long-acting oxytetracycline (LAO) treatment days.

cells (elementary bodies) (Vachiéry et al., 2006). It is not yet possible to confirm this hypothesis, nonetheless it is noteworthy that subclinical forms of heartwater had been recurrently described by previous studies (Kasari et al., 2010; Pascucci et al., 2014; Mapham and Vorster, 2017). Complementarily, the differences in vertebrate versus tick origins of the bacteria that had infected the inoculated versus tick-infested sheep may also have played a role in the expression pattern of bacterial genes in *R. microplus* as reported in related bacteria (Ganta et al., 2007). The present experiments were not designed for testing whether the sheep infested by infected *A. variegatum* or *R. microplus* ticks were exposed to different numbers of infectious bacteria. The knowledge acquired from the experiments does not allow of testing such a dose hypothesis. First, although a fixed number of *A. variegatum* nymphs (N=65 per sheep) infested group#2 sheep, we did not control the exact number of *R. microplus* larvae deposited on the back group#4 sheep and did not know precisely the percentage of these larvae that were actually infected. Second, one may expect a difference in the bacterial multiplication in the salivary glands among tick stages. Third and finally, a potential difference in mechanism of multiplication of *E. ruminantium* in salivary glands of *R. microplus* (not yet known) and in its natural vector *A. variegatum* could also lead to the difference observed among our experiments. It is also noteworthy that further molecular investigations are required for settling whether the mechanisms involved in the transstadial maintenance of the bacterium in *A. variegatum* differ from those allowing transovarial transmission in *R. microplus*.

Whatever the precise mechanistic basis involved in the mild infection outcome in the present *R. microplus* experiments compared to the *A. variegatum* experiments, present results could have significant implications for the epidemiology of heartwater in the West Africa since such subclinical infections are believed to confer protection against severe disease following re-infection (Camus et al., 1996; Adakal et al., 2004). Unfortunately, it was not possible to verify during this experiment whether the group#4 sheep were immune/protected against challenge with infected natural vector or infected blood. The presence of antibodies detected by MAP1B-ELISA does not guarantee protection since immunity against heartwater is primarily cellular rather than humoral (Stewart, 1987). Thus, overall, a potential generalisation of the present results to tick and bacterial strains circulating in other places where *R. microplus* co-occur in high abundance with *Amblyomma* spp. vectors of *E. ruminantium* might suggest that the *R. microplus* transmission of *E. ruminantium* could contribute to improve the protection of the most susceptible sheep and goats from severe forms of the disease.

5. Conclusion

We have hereby demonstrated that maternal inheritance of *E. ruminantium* by *R. microplus* progeny can result in competent vector transmission causing a mild infection in naïve ovine hosts. We speculate that such subclinical disease may provide immunity against severe disease in the field, and future studies will attempt to test this hypothesis. Moreover, there is need to reproduce the present studies with different tick and pathogen isolates to investigate the implications for the epidemiology of heartwater where the disease occurs. The results of the *A. variegatum* experiment recall the harmfulness of this tick species in transmission of *E. ruminantium* to hosts. Hence, *A. variegatum* tick control programs need to be maintained and/or improved mainly in susceptible animal herds in areas where *E. ruminantium* occurs.

Besides, the difference between infections with *E. ruminantium* transmitted by *A. variegatum* or *R. microplus* pointed out interesting research questions which have not been explored within this study. Thus, this paper can be considered as the first step in a long process of investigations which will provide accurate scientific responses related to our finding's implication in heartwater epidemiology in West Africa.

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Ethics

This study was conducted under approval number No. 002-2018/EC-CIRDES issued by the ethics committee of CIRDES (www.cirdes.org). Despite the measures taken to avoid the death of the animals during the experiment, some died suddenly. This illustrates how heartwater can cause the sudden death of sensitive animals in the field.

CRediT authorship contribution statement

Mireille V. Some: Conceptualization, Investigation, Formal

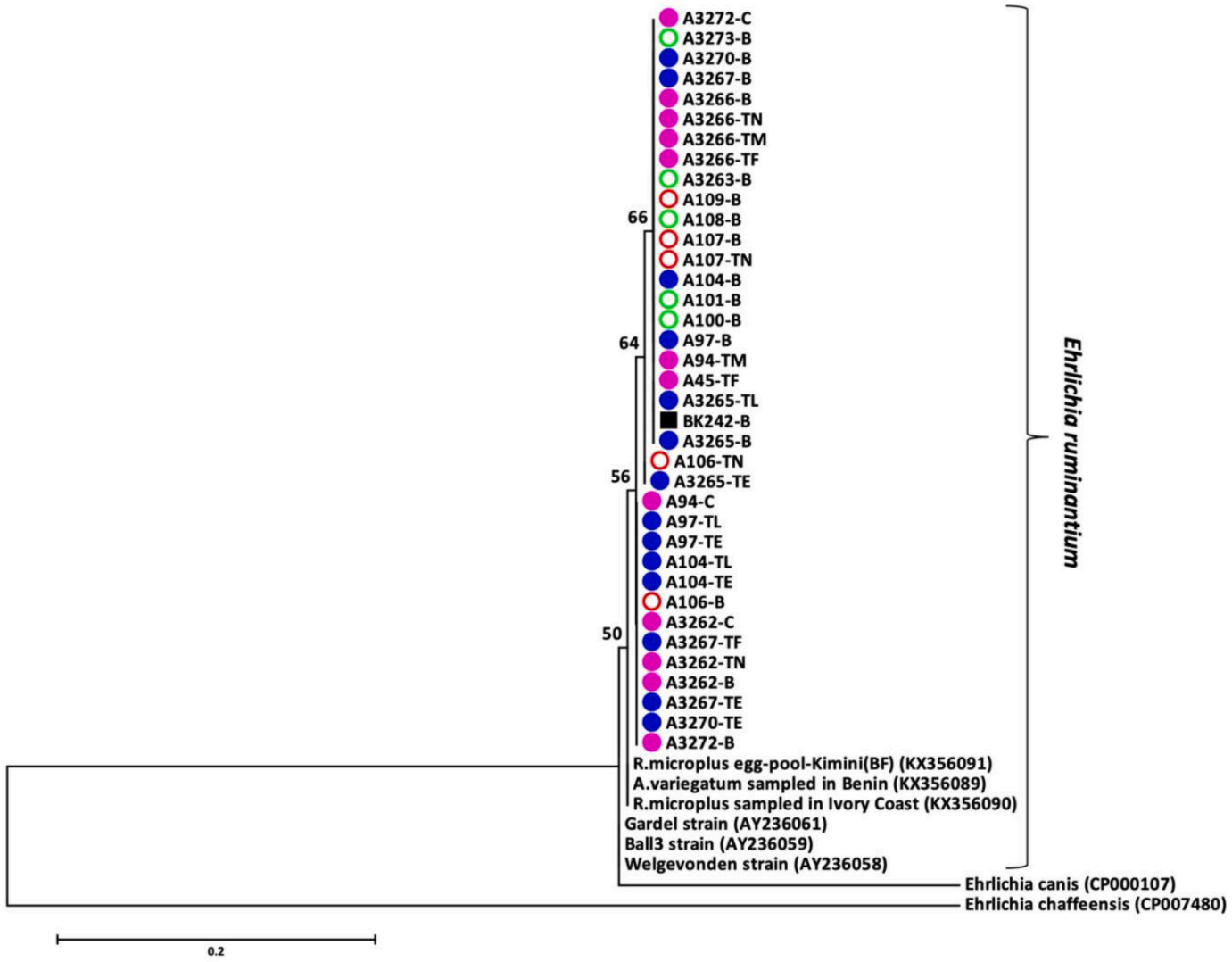

Fig. 5. Phylogenetic relationships among bacterial isolates of diverse origins. Filled circle refer to bacterial isolates collected along the *R. microplus* experiment and empty circles to those from the *A variegatum* experiment. Circle colours indicate to which sheep group the sequenced bacterial isolates originated: green for group#1, red for group#2, blue for group#3 and purple for group#4. The black filled square refers to a bacterial sample from the A95 sheep (group#0). For current study sequences, names are composed by the name of the corresponding animal followed by the reference of type of sample: i.e., B=Blood; C=(cell) Culture; TE= Ticks Eggs; TL= Ticks Larvae; TN= Nymph Ticks; TM= Male Ticks; TF= Female Ticks. Among reference sequences (GenBank accession codes in parentheses), the indication BF refers to a sample collected in Burkina Faso.

analysis, Writing – original draft, Writing – review & editing. **Abel S. Biguezoton:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **Naftaly Githaka:** Conceptualization, Writing – review & editing. **Hassane Adakal:** Writing – review & editing. **Guiguigbaza-Kossigan Dayo:** Writing – review & editing. **Adrien Belem:** Writing – review & editing. **Sébastien Zoungrana:** Investigation, Writing – review & editing. **Frédéric Stachurski:** Conceptualization, Writing – original draft, Writing – review & editing. **Christine Chevillon:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2022.102117.

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