Primary embryonic cells of *Rhipicephalus microplus* and *Amblyomma cajennense* ticks as a substrate for the development of *Borrelia burgdorferi* (strain G39/40)

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Abstract

Borrelia burgdorferi, the agent of Lyme borreliosis, is a spirochetes transmitted by ticks to humans and animals. Its cultivation in vitro in tick cells allows studies of its biology and provides methodology for future research in Brazil, and for the isolation of *Borrelia* spp. We examined in vitro the characteristics of embryonic cells of *Rhipicephalus microplus* and *Amblyomma cajennense* in cell culture and investigated the suitability of embryonic cells as a substrate for cultivation of *B. burgdorferi*. Subcultures were prepared from primary cultures of embrionary cells of *R. microplus* and *A. cajennense* maintained in Leibovitz's (L-15) complete medium at 28 °C and 31 °C, respectively. When a monolayer had formed, the L-15 was replaced with Barbour-Stoener-Kelly medium for experiments to infect cell cultures with *B. burgdorferi*. After 72 hours of cultivation, the spirochetes were counted using an inverted phase contrast microscope and dark-field illumination (400×). Survival, multiplication and the adherence of *B. burgdorferi* for embryonic cells of *R. microplus* and *A. cajennense* were observed. *B. burgdorferi* cultured with embryonic cells of *R. microplus* grew on average to a density (final count) of 2.4×10^7 spirochetes/mL, whereas in cell-free culture, an average of 2.5×10^7 spirochetes/mL were counted. When cultivated with *A. cajennense* cells, the final count of spirochetes was on average 1.7×10^7 spirochetes/mL, while spirochetes cultured under cell-free conditions replicated on average of 2.2×10^7 spirochetes/mL. Similar results were observed in the final count of Spirochetes cultivated in cells of *R. microplus* and *A. cajennense*, when compared with cell-free control. These results demonstrated that cells of *R. microplus* and *A. cajennense* have the potential to be used as growth substrate for *B. burgdorferi* in the study of its interaction with host cells.

Keywords: cell culture, *Borrelia burgdorferi*, tick, *Rhipicephalus microplus*, *Amblyomma cajennense.*

Células primárias embrionárias do carrapato *Rhipicephalus microplus* e *Amblyomma cajennense* como substrato para desenvolvimento de *Borrelia burgdorferi* (Cepa G39/40)

Resumo

Borrelia burgodorferi, o agente da borreliose de Lyme, é uma espiroqueta transmitida por carrapatos aos seres humanos e animais. Seu cultivo in vitro em células de carrapato permite estudos de sua biologia e propicia metodologia para futuras pesquisas no Brasil, para o isolamento de *Borrelia* spp. Nós examinamos in vitro as características de células embrionárias de *Rhipicephalus microplus* e *Amblyomma cajennense*, e a viabilidade de utilização dessas células embrionárias como um substrato para cultivo de *B.burgdorferi*. Subculturas foram preparadas a partir de culturas primárias de células embrionárias de *R. microplus* e *A. cajennense* mantidas em meio Leibovitz's (L-15) completo, a 28 °C e 31 °C, respectivamente. Com a formação da monocamada, o L-15 foi substituído pelo meio Barbour-Stoener-Kelly, para o experimento de infecção com *B. burgdorferi* nas culturas de células. Após 72 horas de cultivo, realizou-se a contagem das espiroquetas, as quais foram avaliadas sob microscópio invertido de contraste de fase e campo escuro (400×). Verificou-se a sobrevivência, a multiplicação e a aderência de *B. burgdorferi* em células embrionárias de *R. microplus* e *A. cajennense*. No estudo da cultura de *B. burgdorferi* com células embrionárias de *R. microplus*, observou-se, na contagem final, média de $2{,}4 \times 10^7$ espiroquetas/mL; no cultivo livre de células, verificou-se média de $2{,}5 \times 10^7$ espiroquetas/mL. No cultivo de *A. cajennense*, a contagem final de espiroquetas foi, em média, $1{,}7 \times 10^7$ espiroquetas/mL, enquanto que, para as cultivadas livres de células, se verificou média de

$2{,}2 \times 10^7$ espiroquetas/mL. Resultado semelhante foi observado na contagem final de espiroquetas cultivadas em células de *R. microplus* e *A. cajennense*, quando comparado com o controle livre de células. Estes resultados demonstraram que células de *R. microplus* e *A. cajennense* têm o potencial para serem utilizadas como substrato para o crescimento de *B. burgdorferi* no estudo da interação com as células do hospedeiro.

Palavras-chave: cultura de células, *Borrelia burgdorferi*, carrapato, *Rhipicephalus microplus*, *Amblyomma cajennense*.

1. Introduction

The spirochete *Borrelia burgdorferi* is the etiological agent of Lyme disease (Johnson et al., 1984), a zoonosis transmitted by ticks in the *Ixodes ricinus* complex in the USA, Europe and Asia. Tick cell culture provides a simplified vector system in vitro, mainly for the isolation and propagation of tick-borne pathogens, which may be useful in studies of intracellular and epicellular pathogens, including *B. burgdorferi* (Munderloh and Kurtti, 1995; Bell-Sakyi et al., 2007)**.**

The use of tick tissue in cultures represents a tool for investigation of cell adherence mechanisms, migration within the host, mechanisms of transmission and interaction of the spirochete with host cells (Kurtti et al., 1988). In this context, it has been demonstrated that adherence and invasion of tick cells in cultures by *B. burgdorferi* are analogous to the events that occur in the vector tick (Munderloh and Kurtti, 1995).

A limited number of studies have examined cellular and molecular interactions between pathogens and cultured tick cells in vitro. Kurtti et al. (1988, 1993) reported the cultivation of *B. burgdorferi* in cells derived from a range of tick species and observed optimal development of the spirochete in *Rhipicephalus appendiculatus* embryonic cells (RAE 25). Subsequently, Varela et al. (2007) reported the first isolation of *Borrelia lonestari* (strain LS-1) in embryonic cells of *Ixodes scapularis* (ISE6). These authors emphasized the utility of cultured organisms in the development of assays for accurate diagnosis. *Amblyomma* spp. represent the most common human-biting tick in Brazil and include vectors for other zoonoses. Other tick species could also play a relevant role in the ecology and epidemiology of the Brazilian lyme disease-like or Bagio Yoshinari syndrome (Mantovani et al., 2007; Yparraguirre et al., 2007; Yoshinari et al., 2010).

In Brazil, successful cultivation and the isolation of *Borrelia* spp. have not yet been achieved, although there are indications of the occurrence of this spirochete in humans and animals by serological techniques and molecular diagnostics (Soares et al., 1999; Yoshinari et al., 2003, 2010; Guedes Junior et al., 2008). Therefore, the use of alternative cultivation methods to the standard cultivation in BSK (Barbour, 1984), would open important new possibilities for an attempt to isolate *Borrelia* spp. The main tick species suspected of playing a role in the transmission of *Borrelia* spp. belong to the genera *Rhipicephalus* and *Amblyomma* (Mantovani et al., 2007; Yoshinari et al., 2010). However, there is a report of co-isolation of *Borrelia* spp. during initiation of primary cultures of embryonic cells of *R. microplus* in Brazil (Rezende et al., 2008). This justifies and underscores the importance of research in the interaction of *Borrelia with* this ixodid tick species.

The aim of the research in this article was to identify the characteristics of embryonic cells of *R. microplus* and *A. cajennense* in culture and investigate the suitability of embryonic cells of the ticks *R. microplus* and *A. cajennense* as a substrate for cultivation of *B. burgdorferi*.

2. Material and Methods

Primary embryonic cell cultures of the ticks *R. microplus* and *A. cajennense* - Engorged female ticks of *R. microplus* and *A. cajennense* were collected from cattle pasture and laboratory rabbits *Oryctolagus cuniculus* (New Zealand x Californian) respectively, and were maintained in the laboratory at the Federal Rural University of Rio de Janeiro (UFRRJ). The engorged females were surface sterilized according to the methods of Yunker (1987). The Leibovitz's L-15 medium was supplemented with 10% tryptose phosphate broth, 20% foetal bovine serum, 0.1% bovine albumin (Fraction V) and antibiotics (penicillin G 100 Units/mL, for *R. microplus* cells and gentamicin, 50 mg/mL, for *A. cajennense* cells) adjusted to pH 6.8, and used as the culture medium for the initial growth of tick cells.

Egg masses were aseptically collected 12 days after the onset of oviposition in the case of *R. microplus* and after 22 days for *A. cajannense*. The eggs were transferred to a beaker, sterilized with acetone and washed eight times with sterilized distilled water. Then, 2 mL of Leibovitz's L-15 culture medium supplemented were added to the eggs of each tick species, and the eggshells were broken by applying pressure with the piston of a 20 mL hypodermic glass syringe. Following disruption of most of the eggshells, the suspended material was strained through a pore 1 glass filter to remove intact eggs and shell fragments.

After straining, the material was centrifuged at 73× *g* for 8 minutes in a benchtop centrifuge. The pellet obtained from the *R. microplus* cell suspensions was resuspended in L-15 culture medium and distributed as 4-mL aliquots into three individual 25-cm^2 flasks. All the cell suspensions were incubated at 28 °C in a bacteriological incubator. The pellet obtained from *A. cajannense* eggs was resuspended in the same way, but it was divided into 4-mL aliquots and transferred into two 25-cm^2 flasks. The cell suspensions were incubated at 31 °C in a bacteriological incubator. Cultures were examined daily using a phase-contrast inverted microscope and the culture medium was replaced weekly.

Maintenance and subculture of the *R. microplus* cells - Once the cells had formed a monolayer, which occurred after 23 days of growth of *R. microplus* cells, the first subcultures were established. After the third passage, cells were detached from the bottom of the flask by mechanical agitation and then transferred to 15-mL centrifuge tubes, with centrifugation at 73× *g* for 8 minutes. The culture supernatant was discarded and the cell pellet was resuspended in 6 mL of supplemented L-15 medium, pH 6.8, without antibiotics. Three aliquots of the suspension cell (2 mL each) were distributed into LTs (Leighton tubes) capped with coverslips and incubated at 31 °C.

Maintenance and subculture of the *A. cajennense* cells - The *A. cajennense* cells of one 25-cm^2 flask of the first subculture made after 30 days of incubation, were detached with trypsin-EDTA (Sigma) and incubated at 37 °C for 10 minutes. Afterwards, the cells were transferred to a 15 mL centrifuge tube in 5 mL of Leibovitz's L-15 and centrifuged at 73× g for 8 minutes, the cell pellet was resuspended in 3 mL of supplemented L-15 medium, and washed once with medium. The cell pellet was resuspended in 8 mL of L-15 medium, with antibiotic. Four aliquots of the cell suspension (2 mL) were transferred into LTs with coverslips and incubated at 31 °C.

Maintenance of *Borrelia burgdorferi* - The North American strain G39/40 of *B. burgdorferi,* kindly provided by Dr. Natalino Yoshinari, the School of Medicine, University of São Paulo, Brazil, was used in all experiments. These spirochetes are currently maintained at –196 °C in liquid nitrogen in the Laboratory of Parasitic Diseases, UFRRJ, for over 100 passages. Aliquots of 2 mL were thawed, and added to Barbour-Stoenner-Kelly (BSK-H - Sigma®), pH 7.2 and centrifuged at 73× *g* for 10 minutes. The pellet was resuspended in 3 mL of BSK-H, transferred to 15-mL Falcon tubes and incubated at 34 °C. Spirochetes were evaluated using an inverted microscope fitted for dark field and phase contrast microscopy.

Development of B. burgdorferi in embryonic R. microplus cells - Cell development was detected by inverted phase-contrast microscopy one week after the embryonic cells had been subcultured into 3 Leighton Tubes (LTs) by inverted phase contrast microscopy. At that point, the L-15 culture medium in 2 LTs was replaced with BSK-H medium at the same time, and 1 LT with cells were maintained only in L-15 medium (CC). Spirochetes were counted in a Neubauer chamber under a phase-contrast microscope. The inocula of 3×10^6 Spirochetes in 300 µL BSK-H were introduced into 2 tests LTs with cells (LTC) and into 2 tubes with 2 mL of BSK-H alone (CFT). All tubes were incubated at 34 °C, and the first inspection of the cultures was undertaken after 24 hours using an inverted phase contrast microscope. Spirochetes were observed and counted using an inverted phase contrast microscope (400×), 72 hours after the introduction of the inoculum. Subsequently, embryonic cells were stained using Giemsa's stain.

Development of *B. burgdorferi* in embryonic *A. cajennense* cells - After two subcultures in LTs and, once a monolayer of *A. cajennense* cells was observed, the L-15 culture medium in 4 LTs was replaced with 2 mL BSK-H, and the other 2 LTs with cells were maintained only with medium. Then, inocula of 2.35×10^6 Spirochetes in 300 µL BSK-H were introduced into 2 tests LTs with cells (LTC) and into 2 tubes with 2 mL of BSK-H alone (CFT). The cultures were incubated at 34 °C. Growth was observed using a phase contrast microscope and by observing the BSK-H . After 72 hours, acidification of the BSK-H medium and multiplication of *B. burgdorferi* were observed and spirochetes counted in a Neubauer chamber under a phase-contrast microscope. The *A. cajennense* cells were Giemsa-stained.

The viability of the spirochetes grown in tick cells was determined after 72 hours by transferring 300 µL of the cultured sample to centrifuge tubes containing 3 mL BSK-H, and incubating them at 34 °C. Motility of Spirochetes sub-cultured twice was visualized by dark field microscopy (400×), and at the end of the cultivation period, *B. burgdorferi* aliquots were frozen in 80% glycerol at –196°.

3. Results

Characteristics of Embryonic cells of Rhipicephalus microplus - The embryonic cells presented round epithelioid, polyhedral and globular morphologies, as well as fibroblastoid and/or elongated shapes. Some cells presented vacuolized cytoplasm and harboured eccentric nuclei. These cells remained viable in vitro for six months and during four subcultures.

Development of Borrelia burgdorferi in Rhipicephalus microplus cells - It was possible to observe the development of *B. burgdorferi* in *R. microplus* cells after 72 hours (Figure 1). It was observed that, from an initial inoculum of 3×10^6 spirochetes/mL in the cultivation of embryonic cells of *R. microplus*, each LTC tube had a concentration of 2.5×10^7 and 2.3×10^7 spirochetes/mL (average 2.4×10^7). In the two CFT tubes that only contained BSK-H, the number was 2.5×10^7 spirochetes/mL in each tube (Figure 2 and 3). Giemsa staining at 72 hours, revealed the adherence of *B. burgdorferi* to the embryonic cells of *R. microplus*. The embryonic cells remained firmly adhered to the coverslip even when they grew in BSK-H culture medium.

Characteristics of Embryonic cells of A. cajennense – It could be observed that the majority of the *A. cajennense* tick cells presented a fibroblastoid shape and were always elongated, with some being star-shaped. Moreover, the nuclei were eccentric and strongly stained. These cells survived for four subcultures, and after seven months degeneration was observed. The embryonic *A. cajennense* cells remained adhered to the coverslip of LTs even when grown in BSK-H.

Development of B. burgdorferi in embryonic A. cajennense cells – In embryonic *A. cajennense* cells, development of *B. burgdorferi* stopped within 72 hours (Figure 4). The cultures grown in LTC tubes contained a 1.8×10^7 and 1.6×10^7 spirochetes/mL in each tube

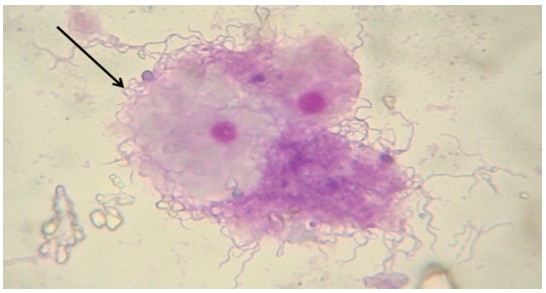

Figure 1. Adherence of *Borrelia Burgdorferi* in primary embryonic cells epithelioids of *Rhipicephalus microplus* (arrow), Giemsa stain, Magnification, ×1,000.

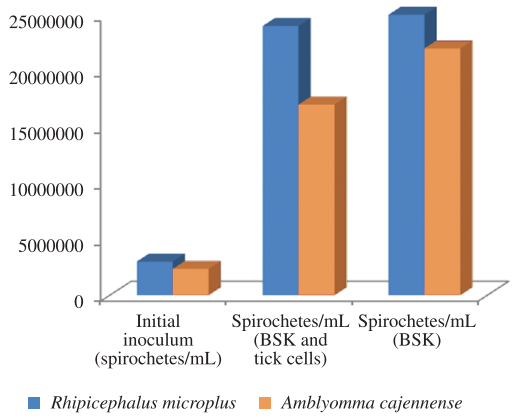

Figure 2. Evaluation of growth of *Borrelia burgdorferi* in tick cells *Rhipicephalus microplus* and *Amblyomma cajennense* in Barbour Stonner Kelly (BSK-H) medium and tubes cell-free.

(average 1.7×10^7) and CFT tubes contained 1.9×10^7 and 2.4×10^7 spirochetes/mL (average 2.2×10^7) (Figure 2 and 3). However, with cells from *R. microplus*, Giemsa staining revealed the adherence of spirochetes to the embryonic *A. cajennense* cells, which presented a degenerated appearance.

The viability test demonstrated that the subcultured spirochetes had grown and were motile within 3 days of transfer to fresh BSK-H.

4. Discussion

Yunker (1987) reported that tick cells can proliferate in a few days and that even sub-confluent monolayers of cells can be subcultured. In this study, there was formation of a monolayer of embryonic cells of *Rhipicephalus microplus* and *Amblyomma cajennense* on day 23 and 30 cultures, respectively, at which time they were subcultured. Eide and Caldwell (1973) reported the growth of *A. americanum* cells for a period of 30 days but without subculture. So far, there have been no reports of growth of embryonic cells of *A. cajennense* with subcultures. The cells observed in this study are initially fibroblastoid and epithelioid globose

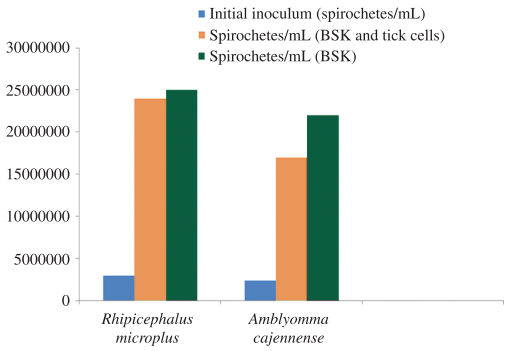

Figure 3. Evaluation of growth of *B. burgdorferi* from the initial inoculation and after growth in tick cell cultures and in BSK-H cell-free medium.

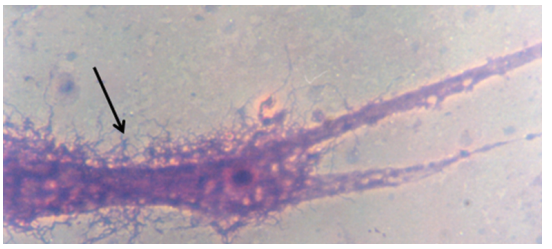

Figure 4. Adherence of *Borrelia Burgdorferi* in primary embryonic cells and/or elongated fibroblast-like cells of *Amblyomma cajennense* (arrow), Giemsa stain, Magnification ×1,000.

cells, as well as polyhedral and stellate cells, while some cells with eccentric nuclei and vacuolated cytoplasm appear later, according to Yunker (1987) and Rezende et al. (2003).

Borrelia burgdorferi invades and colonizes tissues within vertebrate and tick hosts, offering quite divergent environments and defense responses. To elucidate the mechanisms of cellular adherence and migration within the host, the interaction of spirochetes with host cells has been examined using cultured tick (Kurtti et al. 1988, 1993). These events as adherence and interaction of spirochetes were presented in this study and reported from the initial inoculum, 3×10^6 and 2.35×10^6 spirochetes in *R. microplus* and *A. cajennense* cell cultures, respectively, we observed an increase of three generations of *B. burgdorferi* after the final count in both tick cell cultures. It was found that in both cell cultures the values observed indicated that multiplication in LTC and CFT with *R. microplus* and *A. cajennense* cells were similar. Although spirochete numbers were higher in *R. microplus* cultures, the number of spirochetes inoculated initially was also greater than that added to *A. cajennense* cultures. This result shows that it is possible for spirochetes to develop in tick cells, similar to those seen in BSK-H medium.

The growth of *B. burgdorferi* using embryonic cells from the *R. microplus* and *A. cajennense* was successful in spite of the fact that neither of the tick species is known

to be specific vectors of this spirochete. Adherence of *B. burgdorferi* to cultured cells does not appear to be highly specific, although differences in their affinities for different tick cells types have been observed. Indeed, the capacity of *B. burgdorferi* to interact with a diversity of tick cells may be an important factor that contributes to its infectivity in several divergent hosts (Kurtti et al., 1993).

Martins et al. (1996), examining haemolymph of the female *B. microplus* identified *Borrelia theileri*, considering it the vector tick and the morphological characteristics of the organism. *Borrelia theileri* multiplies in the vertebrate and invertebrate, probably being endemic in populations of one or two species of ticks (Smith et al., 1978). There are reports in Brazil that indicate the circulation of this species (Madureira et al., 2004; Madureira, 2007). However, the spirochete of *Borrelia* spp. has not been isolated yet, even after the attempts in samples of human material, ticks and wild animals (Costa et al., 2002; Mantovani et al., 2007)

The tick *Amblyomma americanum* has been identified as the host of the *Borrelia lonestari* in southern United State (Barbour and Hayes, 1996) and Varela et al. (2007) isolated *B. lonestari* from *A. americanum* and cultivated it in embryonic cells of *Ixodes scapularis* (ISE6). Munderloh and Kurtti (1995) observed that the adherence of *B. burgdorferi* to the cells of *R. appendiculatus* correlated with infectivity and viability. The present study found adherence and development of spirochetes to the cells of *R. microplus* and *A. cajennense*. The development of this type of study is important because these ticks are involved in the transmission of spirochetes of the genus *Borrelia* in Brazil (Fonseca et al., 2005; Mantovani et al., 2007; Yoshinari et al., 2010).

Rezende et al. (2008) demonstrated the occurrence of *Borrelia* spp. in primary embryonic cells of *R. microplus* ticks in Brazil, reporting that during the first days of tick cell cultivation it was possible to identify spirochetes in the cellular environment that appeared to be the result of a natural infection of the tick. Other studies have been conducted in Brazil. Yparraguirre et al. (2007) have identified a spirochete denoted "*Borrelia* sp. BR", a group of spirochetes aligned with *B. lonestari* and *B. theileri*. It has been shown that these species of spirochetes and *B. burgdorferi* present common ancestors. In the present study, the use of tick cells for cultivation of *B. burgdorferi* as a model was chosen, observing its development 72 hours after the inoculation, inferring that spirochetes from this genus have a potential to develop in tick cell cultures that live in a Brazilian environment.

In the present study, embryonic cells of *R. microplus* e *A. cajennense* degenerated following the introduction of *B. burgdorferi*, but normal cells were also observed. In agreement with our observations, Kurtti et al. (1988) reported that samples of tick embryonic cells grown with *B. burgdorferi* and stained with Giemsa's stain showed both normal and degenerated embryonic cells. Zung et al. (1989), Burgdorfer (1989) and Benach et al. (1987) reported damage to the chorion when *B. burgdorferi* adhered to the surface of tick oocytes. Apparently, the same degeneration also occurs in embryonic tick cells that grow in vitro with *B. burgdorferi*.

In this study, morphological characteristics of primary embryonic cells of *R. microplus* and *A. cajennense* agreed with those reported in the literature. Besides, we have shown that embryonic cell cultures of *R. microplus* and *A. cajennense* can serve as a substrate for *B. Burgdorferi*. These results were satisfactory and showed a new perspective for growing *Borrelia* spp. So far, in Brazil, this agent is uncultivable in standard culture media for this agent.

More studies on the growth of tick cells and pathogenic organisms are necessary since reports on this subject are scarce. The growth of *Borrelia* spp. isolated from ticks, animals and humans in Brazil may be relevant to the improvement of the diagnosis of borreliosis.

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