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Short communication

Propagation of a Brazilian isolate of *Anaplasma marginale* with appendage in a tick cell line (BME26) derived from *Rhipicephalus* (*Boophilus*) *microplus*

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ABSTRACT

Anaplasma marginale is a tick-borne pathogen of cattle responsible for the disease anaplasmosis. Data suggest that *Rhipicephalus* (*Boophilus*) *microplus* and *R. annulatus* may be the major tick vectors of *A. marginale* in tropical and subtropical regions of the world. In this work we demonstrated the first infection and propagation of a Brazilian isolate of *A. marginale* (UFMG1) in the BME26 cell line derived originally from embryos of *R.* (*Boophilus*) *microplus*. The establishment of *A. marginale* infection in a cell line derived from *R.* (*Boophilus*) *microplus* is relevant for studying the *A. marginale*/tick interface.

1. Introduction

Anaplasma marginale (Rickettsiales:Anaplasmataceae), a tick-borne pathogen of cattle, infects erythrocytes and causes the disease anaplasmosis. The acute phase of anaplasmosis is characterized by anaemia, fever, weight loss, reduced milk production and often death contributing to significant economic loss to the dairy and meat industries (Kocan et al., 2003). Several tick species have been incriminated worldwide as vectors of *A. marginale* (Kocan et al., 2004). In the United States the main vectors are *Dermacentor andersoni, Dermacentor variabilis* and *Dermacentor albipictus*, while in tropical and subtropical regions of the world is *Rhipicephalus* (*Boophilus*) *microplus* and *Rhipicephalus annulatus* (Kocan et al., 2008). In addition, transmission may occur mechanically by hematophagous flies or blood contaminated fomites (Hawkins et al., 1982).

A. marginale is an obligate intracellular organism found exclusively within membrane-bound inclusions in bovine erythrocytes and tick cells. Within the parasitophorous vacuole the pathogen undergoes a developmental cycle that involves reticulated and electron-dense forms. The reticulated or vegetative form is the first form seen within the vacuole that divides by binary fission forming inclusions, and in tick cells, large colonies may contain hundreds of organisms. The reticulated form then transform into the dense form which is the infective form and can survive outside of the cell (Kocan et al., 1992b). Within ticks the developmental cycle of *A. marginale* is complex

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and synchronized with the tick feeding cycle (Kocan et al., 2004) and transmission is effected from the salivary glands. Both cattle and ticks become persistently infected with *A. marginale* and serve as reservoirs of infection (Kocan et al., 1992a,b).

Previously, a major impediment to anaplasmosis research was the lack of a cell culture system. Although cell culture systems were investigated using bovine erythrocytes and other arthropod cell lines (as reviewed by Blouin et al. (2002)), they did not result in continuous propagation of the organism. More recently, cell lines derived from embryos of *Ixodes scapularis* supported the growth of several isolates of *A. marginale* and thus provide new research opportunities for studying the interaction of *A. marginale* and tick cells (Blouin et al., 2002; Blouin and Kocan, 1998; Munderloh et al., 1996a; Zweygarth et al., 2006). Interestingly *I. scapularis* is not a natural vector of *A. marginale*, but these cell lines supported the growth of *A. marginale* and a variety of other rickettsia including *A. phagocytophilum* (Munderloh et al., 1996b, 1999) *E. canis* (Kocan et al., 1998) and *E.* (*Cowdria*) *ruminantium* (Bell-Sakyi et al., 2000).

Development of a cell culture system for propagation of *A. marginale* in a cell line derived from a tick that serves as a natural vector would provide additional opportunities for studying the *A. marginale*/tick interface. In this research we demonstrated the first infection and propagation of a Brazilian isolate of *A. marginale* in the BME26 cell line (Esteves et al., 2008) derived originally from embryos of *R.* (*Boophilus*) *microplus*.

2. Materials and methods

2.1. Tick cell line

The BME26 cells, originally isolated from embryos of *R.* (*Boophilus*) *microplus* (Esteves et al., 2008), were maintained in L-15B300 medium (Munderloh et al., 1999) and supplemented with 5% heat-inactivated FBS (Gibco), 10% TPB (Difco), penicillin (100 units ml^{-1}) and streptomycin (100 $\mu g\ ml^{-1}$) (Gibco) and 0.1% bovine lipoprotein concentrate (ICN), pH 7.2. Cultures were grown at 34 °C in 25 cm^2 plastic flasks (Nunc) in 5 ml of the medium, and the medium was replaced once a week.

2.2. Preparation of inoculum and infection of BME26 cells

A Brazilian strain of *A. marginale* with an inclusion appendage (UFMG1) (Ribeiro et al., 1997), which was recently established and propagated in IDE8 tick cells (Bastos et al., 2008) was used to infect BME26 cells. Five milliliters of culture suspension were removed from a 20-day-old culture and were transferred to plastic tube. The tube was immersed in liquid nitrogen for 5 min for cell disruption and rickettsia releasing, followed by incubation in a water bath at 37 °C until thawing was complete. Approximately 500 μl of the culture suspension was inoculated immediately onto BME26 cell monolayers in flask containing 4.5 ml of culture medium. The BME26 cells infected with *A. marginale* were maintained in *Anaplasma* medium (Munderloh et al., 1996a) at 34 °C. The organisms were subcultured through 10 passages by adding 20-day-old infected cells and uninfected cells at a ratio of 1:10.

2.3. Light microscopy

Eight, 14 and 20 days post-infection, cells were collected by scraping and centrifuged onto microscope slides at $1{,}000 \times g$ for 5 min using a cytocentrifuge (Fanem). Cells were air-dried, stained with Panoptico (Newprov) stain and observed under oil immersion in an Axiofhot microscope (Zeiss).

2.4. Polymerase reaction chain (PCR) and nucleotide sequence

DNA was extracted from 10^7 infected cells as described previously (Medina-Acosta and Cross, 1993). PCR conditions and primer design (PER1 And PER2) from *Anaplasma and Ehrlichia-wide* for the amplification of a fragment of the 16S rDNA gene were also described earlier (Goodman et al., 1996). The PCR product was separated on 1.5% agarose gel electrophoresis, stained with ethidium bromide, purified using the Wizard SV Gel kit (Promega) and cloned into a pGEM®-T Easy Vector System (Promega). Sequencing was performed using the Big Dye Primer Cycle Terminator System and analyzed on a PRISM® 310 Genetic Analyzer (reagents and equipment from Applied Biosystems).

2.5. Transcriptional levels of MSP4 gene from A. marginale

Total RNA was isolated from 10^6 BME26 cells at 3, 7 and 14 days post-*A. marginale* infection ($n = 3$) in its eighth passage using Trizol reagent (Gibco), as recommended by the manufacturer. The mRNA levels of the major surface protein 4 (*msp*4) gene (Oberle and Barbet, 1993) were determined in BME26-infected cells by quantitative real-Time PCR (qRT-PCR). Reactions were performed using the primers MSP45 and MSP43 (de la Fuente et al., 2001) and Script One-Step™ RT-PCR Kit with SYBR® Green (Bio-Rad), Bio-Rad iQ5 Thermal Cycler (Bio-Rad) equipment. Amplification was normalized against β-actin3 using the comparative Ct method. Differences among the transcriptional levels, which were represented by arbitrary units, at different days post-infection were evaluated using Student's *t*-test.

3. Results and discussion

Small intracellular colonies of *A. marginale* were observed in Panoptico-stained smears 8 days post-infection during the second passage in BME26 cells (Fig. 1A, arrow). With continued cultivation (fourth passage) 14 days post-infection we detected cells harboring parasitophorous vacuoles densely filled with tiny organisms (Fig. 1B, arrow). Approximately 20 days post-infection, during the same passage, large colonies with many rickettsiae were observed into cytoplasm of the infected cells (Fig. 1C, arrows), as well as bacterial being released from cells (Fig. 1D, arrows). At this time the level of infection was approximately 15–20%. The infected BME26 cells exhibited a cytophatic effect by detaching from the