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Master thesis

Cellular and molecular characterization of *Ehrlichia mineirensis* (UFMG-EV), a new organism isolated from *Rhipicephalus (Boophilus) microplus* ticks

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This Master thesis is based on the following publications:

1- **Cabezas-Cruz A**., Zweygarth E., Ribeiro M F B., da Silveira A G J., de la Fuente J., Grubhoffer L., Valdés J J., Passos M F L. **2012**. New species of *Ehrlichia* isolated from *Rhipicephalus (Boophilus) microplus* shows an ortholog of the *E. canis* major immunogenic glycoprotein gp36 with a new sequence of tandem repeats. *Parasites & Vectors*. 5: 291.

2- Zweygarth E., Schöl H., Lis K., **Cabezas-Cruz A**., Thiel C., Silaghi C., Ribeiro M F B., Passos L M F. **2012**. *In vitro* culture of a novel genotype of *Ehrlichia* sp. from Brazil. *Transboundary and Emerging Diseases*. (accepted).

3- **Cabezas-Cruz A**., Vancová M., Zweygarth E., Ribeiro M F B., Grubhoffer L., Passos L M F. Ultrastructure of *Ehrlichia mineirensis*, a new member of *Ehrlichia* genus. **2013**. *Veterinary Microbiology*. (under journal review).

Abstract

Ehrlichia species are the etiological agents of emerging and life-threatening tick-borne zoonoses that inflict serious and fatal infections in companion animals and livestock. The obligately intracellular alpha-proteobacterial genus *Ehrlichia* (Rickettsiales: Anaplasmataceae) is spread all over the world and is comprised of five recognized species that are tick-transmitted, three of them causing human ehrlichiosis (*E. canis*, *E. chaffeensis*, and *E. ewingii*). The agent which causes the heartwater in ruminants (*E. ruminantium*) can potentially infect humans while *Ehrlichia muris* has never been associated with human infections but a closed related organism was involved in human ehrlichiosis cases in Wisconsin and Minnesota, United States. The aim of this work was to characterize a new species of *Ehrlichia* isolated from *Rhipicephalus (Boophilus) microplus* ticks from Minas Gerais, Brazil. We conducted the isolation of five genes generally used for the phylogenetic classification of members of the genus Ehrlichia: *16S rRNA*, *groESL*, *gltA*, *dsb* and *gp36*. The agent was culture in several tick cell lines from hard and soft ticks. Electron microscopy was conducted in *the Ixodes scapularis*–derived IDE8 tick cell line. On the other hand the reactivity of antibodies from *E. canis* and *Anaplasma marginale* naturally infected animals against protein extract of the new agent was assayed. Based on maximum likelihood phylogenetic analyses using *16S rRNA*, *groESL*, *gltA*, *dsb* and *gp36* we concluded that the agent is a new species of the genus Ehrlichia close related to *E. canis* and it was named *E. mineirensis* (UFMG-EV). In correspondence, the ultrastructure of *E. mineirensis* (UFMG-EV) resembles the one of *E. canis*, *E. muris* and *E. chaffeensis* but not the one of *E. ruminantium*. Western blot analyse showed that serum from a naturally infected dog with E. canis crossreacted with *E. mineirensis* (UFMG-EV) protein extract. The *in vitro* infection and propagation of *E. mineirensis* (UFMG-EV) was supported by all tested tick cell lines from hard ticks but not by the soft tick cell line used. Further studies are needed in order to evaluated the pathogenic potential of *E. mineirensis* (UFMG-EV) as well as its putative transmissibility by *R. microplus*.

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1. Introduction

1.1 General

The emergence of multiple *Ehrlichia* species as etiological agents of newly discovered human zoonoses and the previous recognition of these agents as causing serious disease in companion animals and livestock have intensified the interest on these pathogens. *Ehrlichiae* are tick-transmitted obligate intracellular gram-negative bacteria that are maintained in nature by persistent infections of mammalian hosts [1]. They are microorganisms residing within the cytoplasmic vacuoles of monocytes and granulocytes of humans and animals. *Ehrlichia* species elicit illnesses with fever, headache, leukopenia, and thrombocytopenia [2].

The obligately intracellular alpha-proteobacterial genus *Ehrlichia* (Rickettsiales: Anaplasmataceae) is spread all over the world and are comprised of five recognized species that are tick-transmitted, with three of the five causing human ehrlichiosis (*Ehrlicha canis*, *Ehrlichia chaffeensis*, and *Ehrlichia ewingii*) [3]. The agent that causes heartwater in ruminants (*E. ruminantium*) can potentially infect humans [2, 4], however, *Ehrlichia muris* has never been associated with human infection. In addition, numerous candidate entities have been reported (*Ehrlichia walkerii*, *Ehrlichia shimanensis*, *Ixodes ovatus ehrlichia*, *Panola Mountain ehrlichia*), all isolated from hard ticks and mainly characterized by PCR sequencing [3]. To date, only three species of the genus *Ehrlichia* have been reported in Brazil: *E. canis*, *E. ewingii* and *E. chaffeensis* [5].

Different hard ticks species have been associated with transmitting members of the genus *Ehrlichia*: *Rhipicephalus sanguineus* and *Dermacentor variabilis* (*E. canis*), *Amblyomma americanum* [6] and *Dermacentor variabilis* [5] (*E. chaffeensis* and *E. ewingii*), *Haemaphysalis* spp and *Ixodes* spp (*E. muris*) and *Amblyomma* spp (*E. ruminantium*) [6].

1.2 Ehrlichia in vitro culture

The genus *Ehrlichia* consists of five recognized species: *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. muris*, and *E. ruminantium* [7] four of those have been propagated *in vitro*: *E. ruminantium*, the causative agent for heartwater in ruminants [8], *E. canis*, which causes tropical canine pancytopenia [9], *E. chaffeensis*, which causes moderate to severe disease in humans, and *E. muris* [10], isolated from a wild mouse and not yet attributed to a human disease. Recently, an *Ehrlichia*-like agent [10] and a new pathogenic *Ehrlichia* species from the United States [11] were isolated using *in vitro* culture techniques.

New *Ehrlichia* spp. have been isolated from *Rhipicephalus (Boophilus) microplus* ticks in Asia and characterized molecularly [12, 13, 14], but these have not been propagated *in vitro* yet. In Brazil, three *Ehrlichia* spp. have been confirmed, *E. canis* [15] *E. ewingi* [16] and *E. chaffensis* [17] of which *E. canis* was the only species established in cell culture [18].

1.3 Ultrastructure of ehrlichial agents

The ultrastructures of members of the genus *Ehrlichia* have been previously characterized, using mainly DH82 cells [19]. The characterization of *E. ruminantium* was carried out in the IDE8 tick cell line [20] and other studies have addressed the comparison of this agent growing in both systems DH82 and IDE8 cells [21]. There are many common features in ultrastructure uniting these organisms, and, on the other hand, a group of characteristics allows the genogroups to be distinguished ultrastructurally. They are similar in the normal ultrastructure of individual cells and the formation of abnormal, pathological cells of the same type irrespective of the species. The differences are mostly in the structure of their microcolonies (morulae) and their interrelations with the host cells. All *Ehrlichia* spp. studied exist in two morphological forms, reticulate and dense-cored cells. In cells with active phagocytic function, such as DH82 cells, they where not found in phagolysosomes, which strongly suggests that most of the internalised parasites undergo multiplication (morulae) in the parasitophorous vacuoles. Both cell types (reticulate and dense-cored cells) have a cell wall rather loosely connected with the ehrlichial cytoplasmic membrane. Peptidoglycan is not morphologically identified in ultra-thin sections of *Ehrlichia* spp. [19]

1.4 Molecular-taxonomic characterization of Ehrlichia spp.

Polyphasic taxonomy has been advocated to ensure well-balanced determination of taxonomic relationships [22]. Different genes have been proposed to classify ehrlichial agents. The most widely used are *16S rRNA* [23], *groESL* operon [24], *groEL gene* [25], *gltA* [22], *dsb* [26], *gp36* and *gp19* [27]. The *gp36* belong to the group of major immunogenic antigens in *E. canis* and *E. chaffeensis* (*gp47*) and both are orthologs to the mucin-like protein in *E. ruminantium*. These glycoproteins have tandem repeats that contain major B-cell epitopes with carbohydrate determinants, which contribute substantially to the immunoreactivity of these proteins. So far only five types of tandem repeats have been characterized [28]. Of these glycoproteins, *gp36* is the most divergent gene among *E. canis* isolates [29]. Nevertheless, the tandem repeat is highly conserved among different isolates, changing only in the number of repeats [27] and in few amino acids among *E. canis* isolates [29].

1.5 Ehrlichia mineirensis (UFMG-EV)

Recently, an organism has been isolated from the hemolymph of *R. microplus* engorged females which had been collected from a cattle paddock at a farm in Minas Gerais in Brazil (M.F. Ribeiro, personal communication). This organism has been propagated continuously *in vitro*, in a tick cell line (IDE8), bovine aorta endothelial cells (BA886) [30] and in a monocyte-macrophage cell line from a dog (DH82) [31] and has been identified as a new genotype of the genus *Ehrlichia* [32]. This new agent has been named *Ehrlichia mineirensis* (UFMG-EV).

2. Goals of the work

1- Molecular and phylogenetic analyses of the new organism focusing on five genes (*16S rRNA*, *groESL*, *gltA*, *dsb* and *gp36*).

2- *In vitro* propagation of *E. mineirensis* (UFMG-EV) in tick cell lines from different tick species: *Ixodes scapularis* (ISE18), *Ixodes ricinus* (IRE/CTVM20), *Rhiphicephalus (Boophilus) microplus* (BME/CTVM2 and BME/CTVM6), *Boophilus decoloratus* (BDE/CTVM14) and *Ornithodoros moubata* (OME/CTVM 22).

3- Ultrastructural characterization of *E. mineirensis* (UFMG-EV) in IDE8 cells.

4- Preliminary immunoreactive characterization of *E. mineirensis* (UFMG-EV).

3. Materials and methods

3.1 IDE8 tick cell cultures

Uninfected IDE8 cells, originally derived from *I. scapularis* embryos [33] were maintained at 32°C in L-15B medium [34] which was supplemented with 5 % heat-inactivated foetal bovine serum (FBS), 10 % tryptose phosphate broth (TPB), 0.1 % bovine lipoprotein concentrate (MP, Santa Ana, CA, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin. Infected IDE8 cultures were propagated in a modified L-15B medium as outlined above, which was further supplemented with 0.1 % $NaHCO_3$ and 10 mM HEPES. The pH of the medium was adjusted to approximately 7.5. The modified L-15B medium is referred to as complete culture medium (CCM). *E. mineirensis* (UFMG-EV)-infected cultures were propagated at 34°C in 25 cm^2 plastic culture flasks in 5 ml of the CCM.

3.2 Infection of other tick cell lines (ISE18, IRE/CTVM20, BME/CTVM2, BME/CTVM6, BDE/CTVM14 and OME/CTVM 22)

IDE8 cell cultures heavily infected with *E. mineirensis* (UFMG-EV) were harvested. The cell suspension was centrifuged (130 x g for 5 min at room temperature) and 1 ml of the supernatant, containing mainly initial bodies, was distributed into culture flasks containing ISE18 [34], IRE/CTVM20 [35], BME/CTVM2, BME/CTVM6 [36], BDE/CTVM14 [37] or OME/CTVM22 [38], respectively. After infection, all these cultures were incubated at 32 °C in CCM. A control flask containing IDE8 cells was treated the same way and used as a positive control. After 3 days the medium was replaced with 5 ml of fresh CCM. Thereafter, 3 ml of CCM were changed twice a week.

3.3 Light Microscopy

Microscopic examinations were carried out to demonstrate *E. mineirensis* (UFMG-EV) in the respective cells. Small samples from the cell layer were removed and smears were prepared. Cytospin® smears were made from cultures when cells were partially in suspension. Smears were allowed to dry before being fixed with methanol and stained with Giemsa.

3.4 Sample processing and electron microscopy

Cells were centrifuged and the pellet was immersed in 20% bovine serum albumin. Cells were immediately frozen using a high pressure freezer (EMPACT2, Leica Microsystems, Vienna, Austria). Freeze substitution was performed in a medium containing 2% OsO_4 in anhydrous

acetone for 96 h at –90°C. Then the temperature was raised to 4°C (4°C/1h). The samples were rinsed three times in acetone, infiltrated at room temperature, embedded in Polybed 812 (Polysciences, Warrington) and finally polymerized at 60°C. Ultrathin sections were contrasted in ethanolic uranyl acetate and lead citrate solutions, and observed in a JEOL 1010 TEM (JEOL Ltd.) at an accelerating voltage of 80 kV. Images were captured using a Mega View III camera (SIS GmbH).

3.5 Genomic DNA isolation

The DNeasy Blood & Tissue Kit (Qiagen Inc. Valencia, Calif.) was used for extraction of DNA from infected IDE8 cells. DNA extraction was performed according to the manufacturer's instructions. The extracted material was eluted from the columns in 100 µl of sterile double distilled H_2O (ddH_2O), and the DNA concentration and purity were determined by measuring the optical density at both 260 and 280 nm with a DNA-RNA calculator (NanoDrop® ND-1000, Peqlab, Erlangen, Germany). Ten-fold dilutions were done with the genomic DNA and separated in aliquot of 10 µl each and kept frozen until their use in a PCR reaction.

3.6 PCR

The primers used in this study are shown in Table 1. The oligonucleotide primers used for the amplification of *dsb* gene and *gltA* gene were designed for this study using primer design software (PrimerSelect; DNAStar, USA) and information from the *E. canis* genome [GenBank: CP000107] [39]. Two independent PCR reactions were performed for each gene. For each PCR amplification, 2µl of extracted DNA was used as the template in a 25 µl reaction mixture containing 20pmol of each primer and 2X PCR Master Mix (Promega, USA). Reaction mixtures without template were used as negative control. In case of the detection of Ehrlichia cell infection by using *16SrRNA,* DNA from non infected cells was used as negative control. The reactions were conducted in an Eppendorf thermocycler (Eppendorf Mastercycler personal AG, 22331 Hamburg, Germany) according to the parameters: 2 min at 94 ^{0}C followed by 40 cycles of 30sec at 94 ^{0}C, 1 min at 45 ^{0}C, and 1.5 min at 72 ^{0}C with a final extension step of 5 min. The PCR products were stained using an ethidium bromide free system, 6X Orange DNA Loading Dye (Thermo Scientific, Germany) and visualized in 0.8% agarose minigels.

Table 1 Primers used in this study for the amplification of the *16S rRNA*, *groESL*, *gltA*, *dsb* and *gp36* genes from *E. mineirensis* (UFMG-EV) genomic DNA

Target	Primers*	Sequence	Expected size (Kb)
16Sr RNA	8 F[9]	5′- AGTTTGATCATGGCTCAG – 3′	1.4
	1448R	5′- CCATGGCGTGACGGGCAGTGTG – 3′	
groEL	HS1[10]	5′- TGGGCTGGTA(A/C)TGAAAT – 3′	1.4
	HS6	5′- CCICCIGGIACIA(C/T)ACCTTC – 3′	
gltA	gltAF1	5′- CTTCTGATAAGATTTGAAGTGTTTG – 3′	1.5
	gltAR1	5′- CTTTACAGTACCTATGCATATCAATCC – 3′	
dsb	dsbF2	5′- CTTAGTAATACTAGTGGCAAGTTTTCCAC – 3′	0.683
	dsbR2	5′- GTTGATATATCAGCTGCACCACCG – 3′	
gp36	EC36-F1[13]	5′- GTATGTTTCTTTTATATCATGGC – 3′	1.0
	EC36-R1	5′- GGTTATATTTCAGTTATCAGAAG – 3′	

*Primers F are forward and R reverse.

3.7 Cloning and sequencing

The resulting PCR products were electrophoresed on a 0.8% agarose gel. The size of the amplified fragments was checked by comparison to a DNA molecular weight marker (100-bp DNA Ladder; Promega, USA). In each case, the single amplified product of the expected size was column purified using the QIAquick PCR Purification Kit (Qiagen, USA) and then ligated into the TOPO TA Cloning Kit (Invitrogen, USA) for subsequent transformation in *Escherichia coli* TOP 10 Chemically Competent cells. For each gene, five individual clones containing the cloned fragment in the TOPO vector were purified using the QIAprep Spin Miniprep Kit (Qiagen, USA) and prepared for sequencing using an ABI 3130 sequencer (Applied Biosystems, USA) and the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with the M13F and M13R vector primer. Both the sense and antisense strands of each PCR-amplified product were sequenced, and the sequences were then manually edited to resolve any ambiguities. A consensus sequence was obtained for each amplified PCR product by comparing both the sense and antisense sequences from the five clones.

3.8 DNA sequence analysis

To find the homology of our sequences we used the database Nucleotide collection (nr/nt) using Megablast (optimize for highly similar sequences) from the BLAST server [40]. Nucleotide sequences were aligned using BLAST [40] and protein sequences were aligned using the multiple-alignment program CLUSTALW [41]. The homologies between sequences were analyzed using MegAlign, DNAStar, USA. Nucleotide sequences were translated to amino acid (aa) sequence by the ExPASy translation tool of the Swiss Institute of Bioinformatics [42].

The phylogenetic analysis was performed as follows: sequences were aligned with MUSCLE (v3.7) configured for highest accuracy [43]. After alignment, ambiguous regions (i.e., containing gaps and/or poorly aligned) were removed with Gblocks (v0.91b) [44]. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT) [45, 46]. Reliability for internal branch was assessed using the bootstrapping method (100 bootstrap replicates). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3) [47]. The nomenclature used in the trees follows the one proposed by Dumler and collaborators [7]. The same analysis of similarity and phylogenetic relationships was performed for the genes *16S rRNA*, *groEL*, *gltA* and *dsb* with the exception that the *dsb* tree is unrooted and the rest are rooted.

3.9 Analysis of the glycoprotein gp36 gene and putative aa sequence

The *gp36* ortholog was tested for the presence of signal peptide sequences with the computational algorithm SignalP trained on gram-negative bacteria [48]. The *gp36* protein sequence was evaluated for potential mucin-type O-linked glycosylation on serines and threonines with the computational algorithm NetOGlyc v3.1 [49] and for N-linked glycosylation was used the NetNGlyc 1.0 Server [50]. The Tandem Repeats Finder database [51] was used to analyze the tandem repeats. The prediction of continuous B cell epitopes was done using the B cells Epitopes Prediction Tool [52] and the 3D structure of the glycoprotein and the predicted epitopes was obtained using the algorithm contained in the ElliPro epitope modeling tool and sequences available in the ElliPro server [53]. As previously reported [27], for the convenience of sequence comparison the *gp36* gene orthologs were divided into three regions: 5' end pre-repeat region, a tandem repeat region, and 3' end post-repeat region**.**

3.10 Protein electrophoresis and Western-blot

When the levels of rickettsemia reached 80%, infected cells were transferred into 15ml tube and centrifuged at 3,320xg for 20min at 4°C and the resulting supernatant was discarded. Proteins were extracted with lysis buffer (150 mM sodium chloride, 1% Triton X-100, 50 mM Tris pH 8) containing protease inhibitors (Complete, Mini, EDTA-free, Roche) and then sonicated (5s/cycle, 5 cycles; 0ºC). After centrifugation at 13 000 x g for 30min at 4°C, the supernatant was collected and concentrated using cut off columns (Millipore, 3K). The protein concentration was determined with DC Protein Assay (Bio-Rad). Uninfected IDE8 cells were processed in the same way. 20µg of proteins from uninfected IDE8 or from *E.mineirensis* infected cells were separated using 12% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were

then blocked with 5% skim milk in TBST (TBS containing 0.1% Tween 20) for 1h at room temperature and probed with either sera from a *E.canis* infected dog or *A.marginale* infected cattle, diluted 1:200 TBST and pre-incubated with 20 µg of IDE8 protein for 1h at 37°C. Immunoactivity was detected either using rabbit anti-dog (Sigma A0793) or anti-bovine (Sigma A0705) IgG alkaline phosphatase conjugate and color developed using BCIP/NBT substrate (Roche)

3.11 Sequences used in this study

The sequences obtained from *E. mineirensis* (UFMG-EV) have been deposited in GeneBank, and their accession numbers are: *16S rRNA* [GenBank: JX629805], *groESL* [[GenBank: JX629806], *dsb* [GenBank: JX629808], *gltA* [GenBank: JX629807] and *gp36* [GenBank: JX629809]. The *16S rRNA*, *groEL*, *gltA*, *dsb* and *gp36* sequences used for the phylogenetic tree or molecular analysis in general were obtained from GenBank and their accession numbers are shown in the Tables and Figures where they have been mentioned.

4. Results

4.1 In vitro culture and ultrastructural analyses

4.1.1 Infection of tick cell lines

The tick cell lines ISE18, IRE/CTVM20, BME/CTVM2 and BME/CTVM6 were successfully infected with *E. mineirensis* (UFMG-EV) initial bodies originated from infected IDE8 cultures (Figure 1). Only the tick cell line OME/CTVM22, derived from *Ornithodoros moubata* ticks, was refractory to infection with *E. mineirensis* (UFMG-EV). However, it is worth noting that BDE/CTVM14 could not be infected with material derived from IDE8 cell cultures, but became positive when the infectious material derived from an infected BME/CTVM6 culture was used. In addition, *E. mineirensis* (UFMG-EV) infection was lost after 3 passages in BME/CTVM2 cells, whereas in all other tick cell lines (ISE18, IRE/CTVM20, BME/CTVM6) *E. mineirensis* (UFMG-EV) cultures were passaged at least 3 times before being terminated.

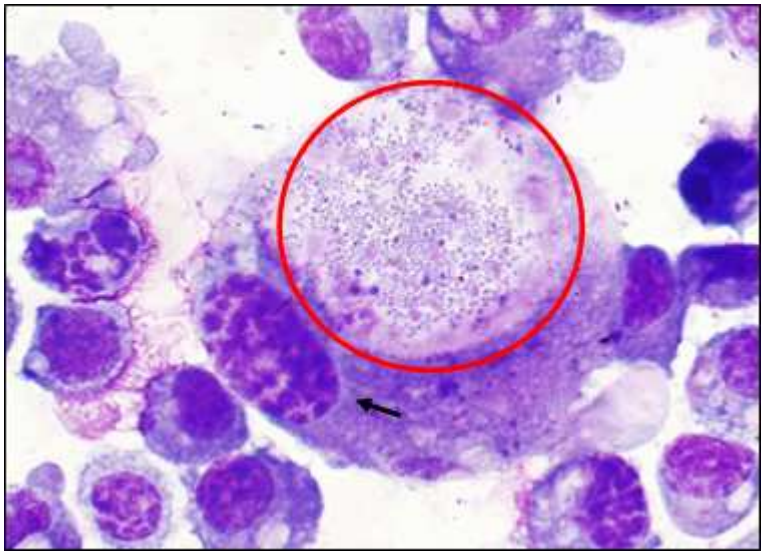

Figure 1. *E. mineirensis* (UFMG-EV)**-infected IDE8 culture.** A heavily infected IDE8 cell showing the morulae (red circle) containing the microorganisms. The black arrow shows the nucleus of the IDE8 cell.

4.1.2 Ultrastructure of E. mineirensis (UFMG-EV)

Infection of IDE8 cultures was confirmed by direct examination of Giemsa-stained cytocentrifuge smears and PCR. Both, microscopic examination and PCR results confirmed that *E. mineirensis* (UFMG-EV) cells (Figure 1) and DNA (Figure 2) were present in the infected IDE8 culture and absent from the uninfected cultures.

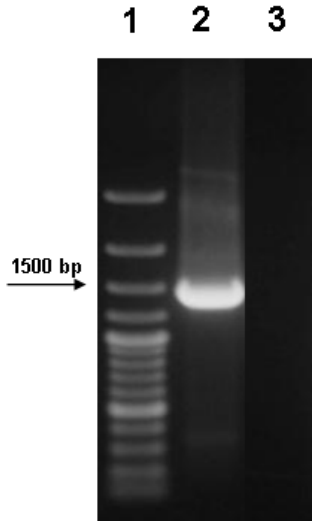

Figure 2. DNA electrophoresis of *E. mineirensis* (UFMG-EV) *16S rRNA*. *100 bp DNA ladder Plus* was used as genetic marker (1). The approximately 1500 bp band of *E. mineirensis* (UFMG-EV) *16SrRNA* fragment is shown (2). No unspecific amplification was observed in the negative control (3).

Uninfected IDE8 cells contained vacuoles and inclusions. The phagolysosomes and secondary lysosomes were present in infected IDE8 cells too (Fig 3A, asterisks), however, they also contained membrane-lined vacuoles containing up to 25 rickettsial organisms 0.4 to 1.5 µm in diameter (Figure 3A). We were able to show the presence of both reticulated (Figure 3A) and electron-dense bodies (Figure 3B). The organisms were round and oval shaped (Figures 3A, B) and they had typical tri-layered cytoplasmic and outer membranes, in some the outer membrane was rippled (Figures 3B inset). We observed numerous reticulate cells of *E. mineirensis* (UFMG-EV) undergoing binary fission (Figure 3C). Parasitophorous vacuoles were surrounded by mitochondria (Figure 3D), and cisterns of rough endoplasmic reticulum (Figure 3E). In several cases, organelles were in tight contact with the vacuole membrane (Figures 3 D, E). Moreover, we observed bundles of microtubules (Figure 3D, inset, white arrows) surrounding the membrane of *morulae* which may be important for the movement of the rickettsia through the cytoplasm. Some rickettsial colonies also contained tiny vesicles visible in the interrickettsial space (Figures 3 B, D, 4, black arrows).

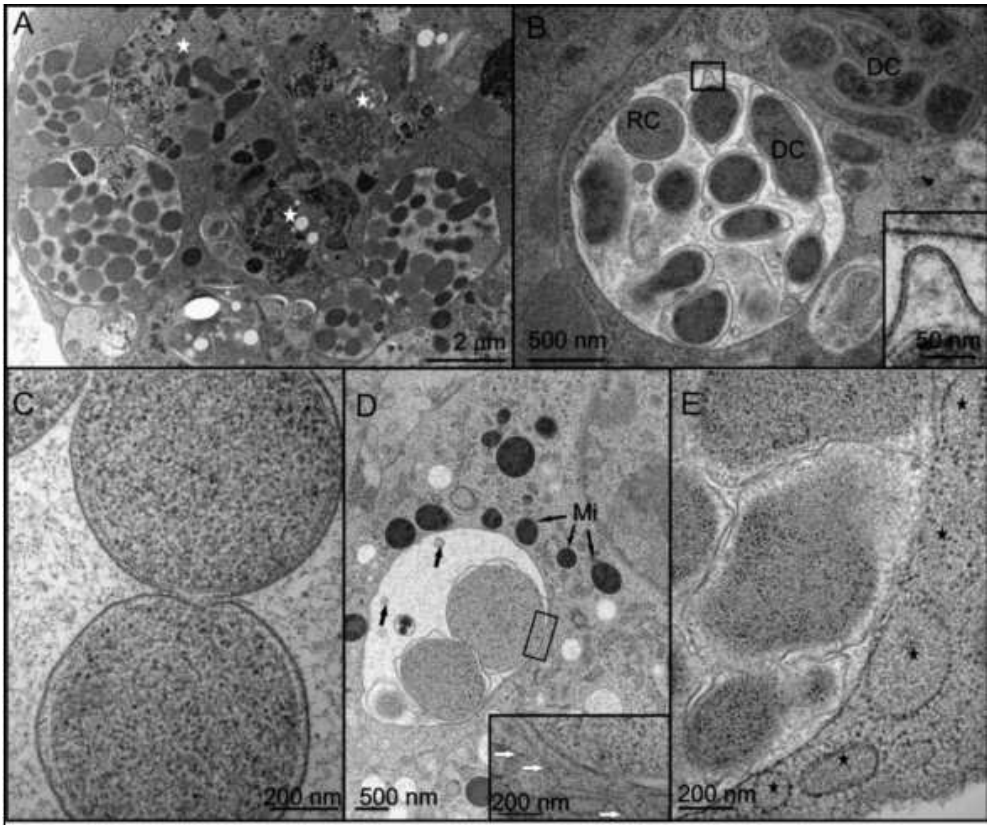

Figure 3. Electron micrograph of *E. mineirensis* (UFMG-EV) - infected IDE8 cells. 3A Cells contain phagolysosomes/secondary lysosomes (white asterisk) and numerous vacuoles with bacteria. 3B Electron- dense bodies (DC), reticulate cells (RC) and small vesicles (black arrows) inside membrane-lined vacuoles. The inset represents a detailed view of the membranes. 3C Reticulate cells undergoing binary fission. 3D The vacuole containing reticulate bodies that have ruffled outer membrane and small vesicles (black arrows) is surrounded with mitochondria (Mi) and microtubules (detail in inset, white arrows) 3E Cisterns of endoplasmic reticulum (black asterisks) in tight contact with the membrane of the *morulae*.

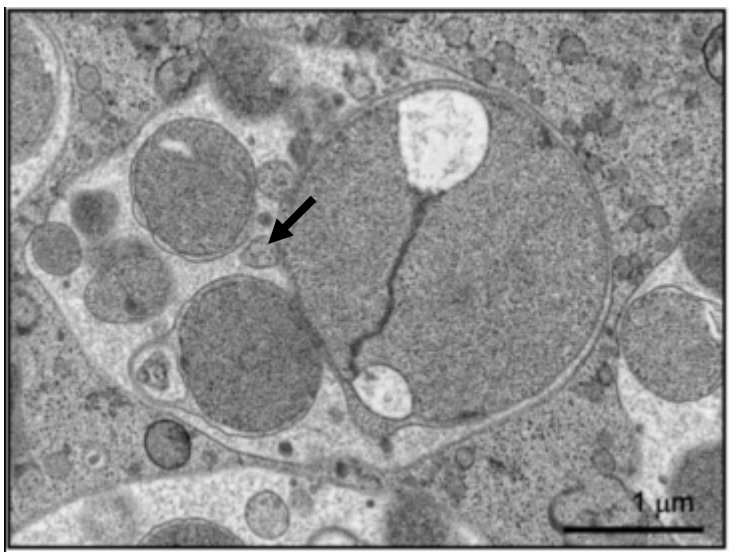

Figure 4. *E. mineirensis* (UFMG-EV) with an unusual morphological structure. The figure shows cell with invagination of the membranes, small vesicles are shown (arrow).

4.2 ***DNA sequence analysis.***

4.2.1 Sequence analysis of 16S rRNA

In order to obtain relevant information from *16S rRNA* at the species level, the primers 8F and 1448R were used to isolate a fragment of ~1.4Kb. Approximately a 1.4Kb amplicon corresponding to the expected size of targeted *16S rRNA* gene fragment was obtained (data not shown). A consensus sequence of 1.384 Kb was obtained from 2 independent PCRs and five clones were sequenced. In total, our sequence had 10 changes of nucleotides when compared with *E. canis* [GenBank: GU810149] with two insertions and three deletions (data not shown). The percent of identities with all the members of the *Ehrlichia* genus are shown in the Table 2 upper triangle. Figure 5 shows the tree build using the maximum likelihood method; it shows that *E. mineirensis* (UFMG-EV) falls in a branch separated from all the previous reported sequences. The tree build with the neighbour joining method using the Kimura 2 parameters substitution model show identical results (data not shown).

Table 2 Identities comparison of *16S rRNA* and *dsb* genes between *E. mineirensis* (UFMG-EV) and other members of the genus *Ehrlichia*

	Percent of nucleotide similarity of *16S rRNA**					
	***Ehrlichia mineirensis* (UFMG-EV)**	***E. canis* [GU810149]**	***E. chaffeensis* [AF147752]**	***E. ewingii* [U96436]**	***E. muris* [AB013008]**	***E. ruminantium* [AF069758]**
***Ehrlichia mineirensis* (UFMG-EV)**	***	98.3 (*16SrRNA*)	96.9 (*16SrRNA*)	96.4 (*16SrRNA*)	94.5 (*16SrRNA*)	95.0 (*16SrRNA*)
***Ehrlichia canis* [AF403710]**	94.7 (*dsb*)	***	98.4 (*16SrRNA*)	97.9 (*16SrRNA*)	97.1 (*16SrRNA*)	97.2 (*16SrRNA*)
***Ehrlichia chaffeensis* [AF403711]**	82.3 (*dsb*)	83.5 (*dsb*)	***	98.1 (*16SrRNA*)	97.6 (*16SrRNA*)	96.9 (*16SrRNA*)
***Ehrlichia ewingii* [AY428950]**	78.6 (*dsb*)	76.9 (*dsb*)	78.0 (*dsb*)	***	97.2 (*16SrRNA*)	97.1 (*16SrRNA*)
***Ehrlichia muris* [AY236484]**	81.1 (*dsb*)	81.1 (*dsb*)	84.5 (*dsb*)	77.2 (*dsb*)	***	96.4 (*16SrRNA*)
***Ehrlichia ruminantium* [AF308669]**	76.9 (*dsb*)	74.6 (*dsb*)	77.1 (*dsb*)	76.6 (*dsb*)	76.4 (*dsb*)	***

Percent of nucleotide similarity of *dsb**.
*The values are % of nucleotide sequence similarity for 1.3Kb (16Sr RNA) and determined from pairwise aligment using DNASTAR software (MegAlign; DNASTAR, Inc., Madison, WI).
Accession Numbers are from GenBank.

The gene *16S rRNA* has a highly variable region located at the 5' end of the gene [54]. This fragment is useful in identifying *Ehrlichia* spp. [23]. Figure 6 shows three changes in nucleotides in *E. mineirensis* (UFMG-EV) in comparison with *E. canis* and seven changes in nucleotides when compared with *Ehrlichia* sp. Tibet which was isolated from *R. microplus* [54].

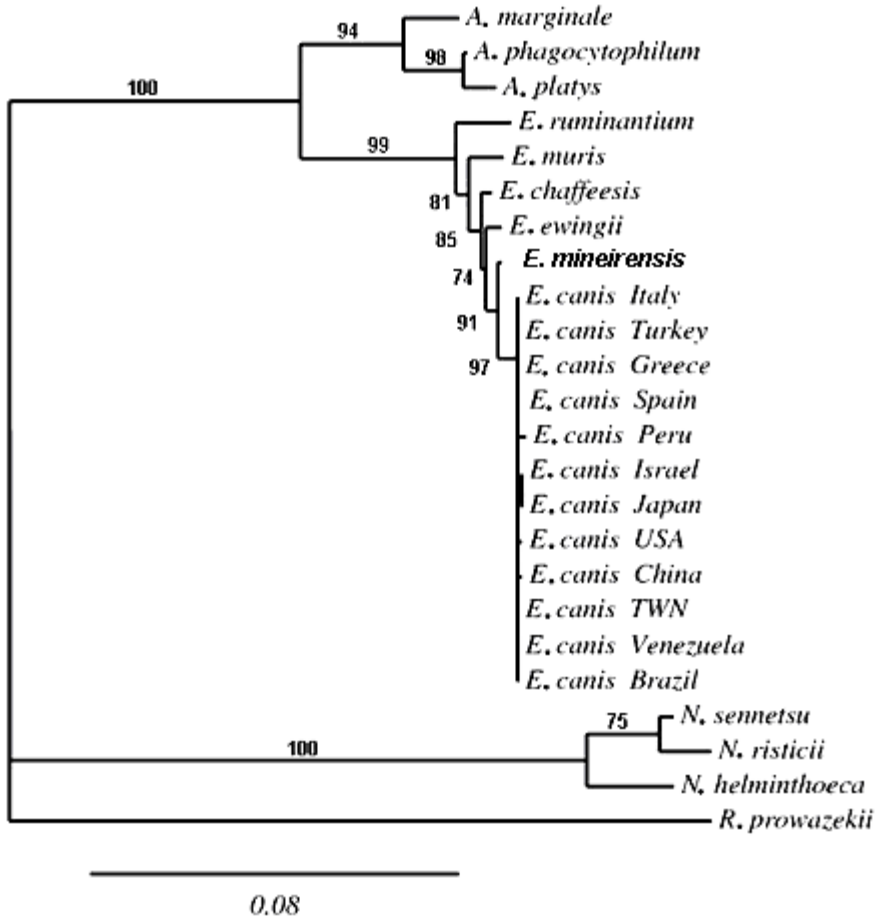

Figure 5. Phylogenetic tree based on the *16S rRNA* gene sequence from members of the family Anaplasmataceae. The tree shows that *E. mineirensis* (UFMG-EV) falls in a branch separated from all the previous reported sequences. Bootstrap values are shown as % in the internal branch. Only bootstrap values equal or higher than 50% are shown. Rickettsia prowazekii 16S rRNA sequence was used to root the tree. The sequences of *16S rRNA* used to build the phylogenetic tree were obtained from GenBank and their accession numbers are as follows: *E. canis*-TWN: GU810149; *E. canis*-China: AF162860; *E. canis*-Japan: AF536827; *E. canis*-USA: M73221; *E. canis*-Venezuela: AF373612; *E. canis*-Brazil: EF195134; *E. canis*-Peru: DQ915970; *E. canis*-Turkey: AY621071; *E. canis*-Israel: U26740; *E. canis*-Spain: AY394465; *E. canis*-Greece: EF011110; *E. canis*-Italy: EU439944; *E. muris*: AB013008; *E. chaffeesis*: AF147752; *E. ruminantium*: AF069758; *E. ewingii*: U96436; *A. marginale*: M60313; *A. phagocytophilum*: M73224; *A. platys*: M82801. *R. prowazekii*: NR044656.

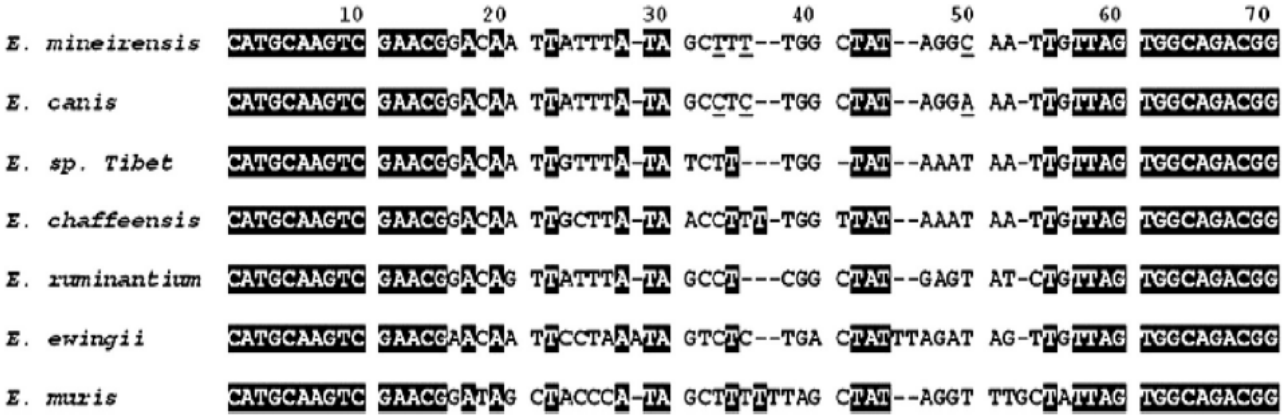

Figure 6. Highly variable region of sequence located at 5' end of the *16S rRNA* gene. Underlined are the nucleotide differences found between *E. canis* and *E. mineirensis* (UFMG-EV). The GenBank accession numbers of the sequences show in the alignment are: *E. muris*, AB013008; *E. chaffeensis*, AF147752; *E. ruminantium*, AF069758; *E. ewingii*, U96436 and *E. canis*, GU810149.

4.2.2 Sequence analysis of dsb

The amplicon obtained from the PCR set up with the primers dsbF2 and dsbR2 gave a band with the expected size of 0.7 Kb. A fragment of 0.683 Kb of the gene *dsb* was obtained and sequenced. *Dsb* gene sequences for available *Ehrlichia* spp. were aligned using clustalW. The alignment shows that *dsb* gene is conserved (76.4% - 94.7%) within the genus (Table 2 lower triangle). The aa sequence shows homology from 72.0% to 95.0% with *E. ruminantium* [GenBank: AF308669, clon 18hw] and *E. canis* [GenBank: AF403710], respectively. When compared with the complete dsb from *E. canis* [AF403710] 10 aa changes are observed (data not shown). The changes are concentrated at the carboxyl-terminus of the protein. Different dsb isolates of *E. canis* share 100% of identity among them (Table 3). The phylogenetic tree shows that *E. mineirensis* (UFMG-EV) *dsb* is separated from its homologs in other species of the *Ehrlichia* genus (Figure 7).

Table 3 Unique aa changes in the carboxyl terminal of *Ehrlichia mineirensis* (UFMG-EV) dsb differ from *E. canis* dsb available in the GenBank

Isolates	aa position[1]						
	Identity %[1]	160	162	168	184	185	204
***Ehrlichia canis* [AF403710]**	100	V	Q	H	H	Y	**T**
***Ehrlichia canis* Uberlandia [GU586135]**	100	.	.	.	.	.	.
***Ehrlichia canis* Sao Paulo [DQ460715]**	100	.	.	.	.	.	.
***Ehrlichia canis* Jaboticabal [DQ460716]**	100	.	.	.	.	.	.
***Ehrlichia mineirensis* (UFMG-EV)**	94.0	A	K	Y	N	H	**A**

1- Positions and % of identities are based on the sequence of *E. canis* [GenBank: AF403710]. The dots below the aa letters mean conserved positions. Accession Numbers are from GenBank.

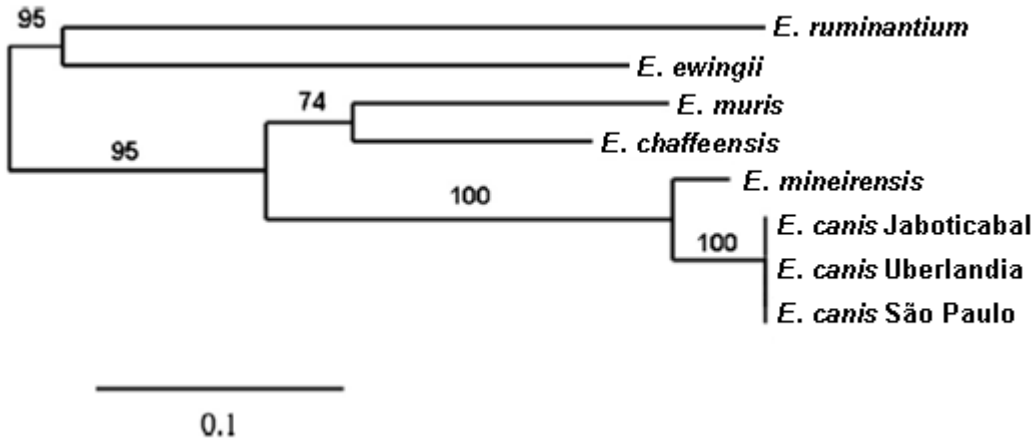

Figure 7. Phylogenetic unrooted tree based on the *dsb* gene sequences from members of the family Anaplasmataceae. The tree shows that *E. mineirensis* (UFMG-EV) falls in a clade separated from all the previous reported sequences and the previously reported *E. canis* dsb sequences. Bootstrap values are show as% in the internal branch. Only bootstrap values equal or higher than 50% are shown. The GenBank accession numbers of the dsb sequences used to build the tree are: *E. canis*, AF403710; *E. canis* Uberlandia, GU586135; *E. canis* Jaboticabal, DQ460716; *E. canis* Sao Paulo, DQ460715; *E. muris*, AY236484; *E. chaffeensis*, AF403711; *E. ruminantium*, AF308669, clon 18hw; *E. ewingii*, AY428950.

4.2.3 Sequence analysis of groESL operon

The amplification with primers HS1-HS6 produced a PCR product in the expected size 1.4Kb. The nucleotide sequences of the PCR products amplified from *E. mineirensis* (UFMG-EV) contained a reading frame corresponding to the 26 aa carboxyl-terminus of *groES*, 416 aa of the amino-terminal end of *groEL*, and the spacer between them. The length of the nucleotide sequence of the spacer region in the sequence reported here were 95 bases. Sequence homology analyses were done for each of the nucleotide sequences and the deduced aa sequences from the partial GroES and GroEL reading frames. Nucleotide and aa sequence homologies with other members of the *Ehrlichia* genus are presented in Table 4. A phylogenetic tree based on multiple sequence alignment of the 1.249 Kb corresponding to *groEL* is presented in Figure 8.

Table 4 Identities comparison of *groEL* gene and putative aa sequence between *Ehrlichia mineirensis* (UFMG-EV) and other members of *Ehrlichia* genus

	Percent of nucleotide (nt) similarity*					
	***E. mineirensis* (UFMG-EV)**	***E. canis***	***E. chaffeensis***	***E. ewingii***	***E. muris***	***E. ruminantium***
***Ehrlichia mineirensis* (UFMG-EV)**	***	97.2 (nt)	92.3 (nt)	91.0 (nt)	92.0 (nt)	87.3 (nt)
Ehrlichia canis [U96731]	99.0 (aa)	***	92.5 (nt)	90.9 (nt)	92.4 (nt)	87.6 (nt)
Ehrlichia chaffeensis [L10917]	97.0 (aa)	97.0 (aa)	***	91.7 (nt)	94.3 (nt)	87.8 (nt)
Ehrlichia ewingii [AF195273]	95.0 (aa)	95.0 (aa)	96.0 (aa)	***	91.5 (nt)	88.0 (nt)
Ehrlichia muris [AF210459]	97.0 (aa)	97.0 (aa)	99.0 (aa)	97.0 (aa)	***	87.3 (nt)
Ehrlichia ruminantium [U13638]	92.0 (aa)	92.0 (aa)	93.0 (aa)	92.0 (aa)	93.0 (aa)	***

Percent of amino acid (aa) similarity*.
*The values showed are % of nucleotide and aa sequence similarity of 1.249 Kb determined from pairwise aligment using DNASTAR software (MegAlign; DNASTAR, Inc., Madison, WI) and 416 aa of the amino terminal determined from ClustalW.
Accession Numbers are from GenBank.

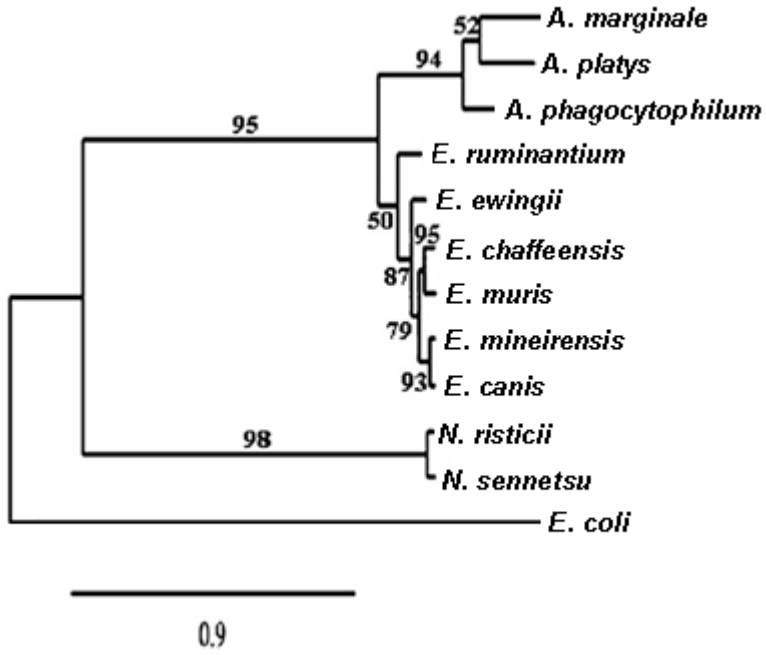

Figure 8. Phylogenetic tree based on the *groEL* gene sequence from members of the family Anaplasmataceae. The tree shows that *E. mineirensis* (UFMG-EV) falls in a branch separated from all the previous reported sequences. Bootstrap values are shown as % in the internal branch. Only bootstrap values equal or higher than 50% are shown. *E.coli groEL* gene (accession number X07850) was used to root the tree. The GenBank accession numbers of the sequences used to build the tree are: *E. muris*, AF210459; *E. chaffeensis*, L10917; *E. ruminantium*, U13638 ; *E. ewingii*, AF195273; *A. marginale*, AF165812; *A. phagocytophilum*, U96729; *A. platys*, AY008300; *N. sennetsu*, U88092; *N. risticii*, U96732; *E. canis*, U96731.

4.2.4 Sequence analysis of gltA gene

Primers gltAF1 and gltAR1 were designed in this study using information from *E. canis* genome [GenBank: CP000107] and *E. chaffeensis gltA* gene sequence [GenBank: AF304142]. The full length of *gltA* gene of *E. mineirensis* (UFMG-EV) was isolated. A single band of ~1.5Kb was

obtained from the PCR reaction (data not shown). The full length gene of 1.251 Kb was obtained after sequencing and consensus analysis. The putative citrate synthase protein predicted using the *E. mineirensis* (UFMG-EV) *gltA* gene was 416 aa. Table 5 shows the nucleotide and the aa similarities with other members of the *Ehrlichia* genus. The *gltA* gene has been proposed as an alternative tool for the phylogenetic analysis of the genus *Ehrlichia* [22]. Using the maximum likelihood method we built a phylogenetic tree showing that *E. mineirensis* (UFMG-EV) falls in a brach apart from any previously reported *gltA* genes in the family Anaplasmataceae (Figure 9).

Table 5 Identities comparison of *gltA* gene and putative aa sequence between *E. mineirensis* (UFMG-EV) and other members of *Ehrlichia* genus

	Percent of nucleotide (nt) similarity*					
	***E. mineirensis* (UFMG-EV)**	***E. canis***	***E. chaffeensis***	***E. ewingii***	***E. muris***	***E. ruminantium***
***Ehrlichia mineirensis* (UFMG-EV)**	***	94.3 (nt)	84.6 (nt)	80.9 (nt)	84.8 (nt)	77.6 (nt)
***Ehrlichia canis* [AF304143]**	94.0 (aa)	***	85.0 (nt)	82.2 (nt)	85.4 (nt)	79.0 (nt)
***Ehrlichia chaffeensis* [AF304142]**	82.0 (aa)	84.0 (aa)	***	82.0 (nt)	87.0 (nt)	78.9 (nt)
***Ehrlichia ewingii* [DQ365879]**	79.0 (aa)	80.0 (aa)	77.0 (aa)	***	82.5 (nt)	79.4 (nt)
***Ehrlichia muris* [AF304144]**	82.0 (aa)	84.0 (aa)	85.0 (aa)	78.0 (aa)	***	79.6 (nt)
***Ehrlichia ruminantium* [AF304146]**	74.0 (aa)	77.0 (aa)	75.0 (aa)	75.0 (aa)	77.0 (aa)	***

Percent of aa similarity*.
*The values showed are % of nucleotide and aa sequence similarity of the full length determined from pairwise aligment using DNASTAR software (MegAlign; DNASTAR, Inc., Madison, WI) and the putative encoded aa determinated from ClustalW.
Accession Numbers are from GenBank.

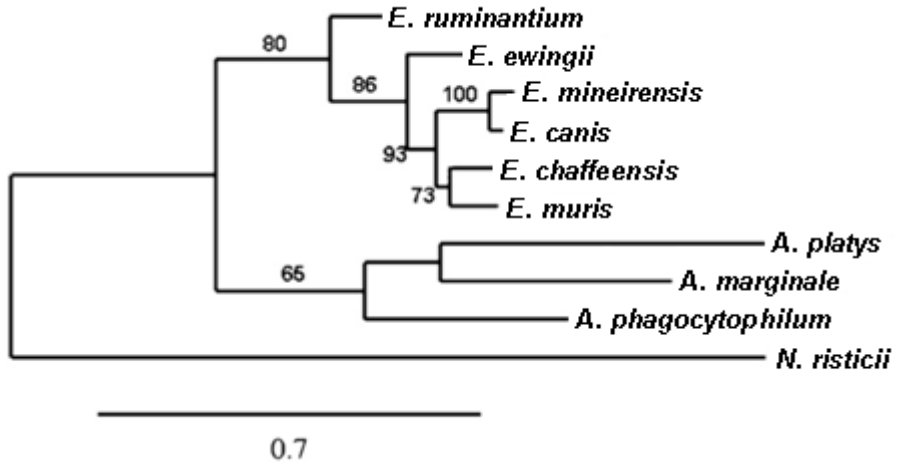

Figure 9. Phylogenetic tree based on the citrate synthase (gltA) gene sequences from members of the family Anaplasmataceae. The tree shows that *E. mineirensis* (UFMG-EV) falls in a clade separated from all the previously reported sequences. Bootstrap values are show as % in the internal branch. Only are showed bootstrap values equal or higher than 50%. *N. risticii gltA* sequence was used to root the tree. The GenBank accession numbers of the *gltA* sequences used to build the tree are as follow: *E. canis*, AF304143; *E. muris*, AF304144; *E. chaffeensis*, AF304142; *E. ruminantium*, AF304146; *E. ewingii*, DQ365879; *A. marginale*, AF304140; *A. phagocytophilum*, AF304138; *A. platys*, AY077620.

4.2.5 Sequence analysis of the gp36 gene and the putative encoded protein sequence

The *gp36* based PCR products derived from the isolate reported here had a molecular size of 1000 base pairs (bp) (data not shown). Subsequent cloning of the PCR amplicons followed by sequencing showed that our gene was 0.948 Kb encoding a predicted protein with 315 aa and a molecular mass of 31.51 KDa (28.89 KDa without the predicted 23-aa signal peptide). We found that the gp36 protein isolated in our study is a putative glycoprotein. The aa sequence of gp36 in our study has five potential sites of O-glycosylation and two of N-glycosylation. The O-carbohydrates were predicted to be linked to three serines (S) of the tandem repeat region at position 155, 164 and 173 and two threonines (T) present in the post-repeat region at position 286 and 289. We explored as well the possibility to find N-glycosylation on putative glycosylated asparagines (N). Two sequons of N-glycosylation (N-Xaa-T/S) at the pre-repeat region were found: **NRS** (at position 81) and **NFS** (at position 106).

4.2.5.1 Differences found in the Region I (The 5' end pre-repeat region)

Alignment of the *gp36* ortholog obtained in this study revealed that our sequence was 422 nucleotides in length encoding for 141 aa (Table 6). The nucleotide and predicted aa sequences exhibited relatively low identities, ranging from 54.9% to 91.2%, and from 38.0% to 82.0%, respectively, in comparison with related genes previously published for the *gp36* orthologs in *E. canis*, *E. chaffeensis* and *E. ruminantium* [28] (Table 6).

Table 6 Length and percent of nucleotide and aa homology of the 5' end pre-repeat region between the orthologs of *gp36* in *Ehrlichia mineirensis* (UFMG-EV) and related genes

		Nucleotide		aa	
Source	Strain	Length[1]	Homology[2]	Length[3]	Homology[4]
Ehrlichia mineirensis	(UFMG-EV)	422	-	141	-
Ehrlichia canis gp36	TWN1 [EF551366]	425	91.2	142	82
	Louisiana [DQ146151]	428	88.2	143	78
	Sao Paulo [DQ146154]	428	88.4	143	78
	Cameroon [DQ146155]	428	88.6	143	79
Ehrlichia chaffeensis gp47	Arkansas [DQ085430]	471	61.8	157	52
	Sapulpa [DQ085431]	461	62.1	154	53
	Jax [DQ146156]	461	60.7	154	51
	St Vincent [DQ146157]	461	62.1	154	53
***Ehrlichia ruminantium* mucin-like protein**	Highway [AF308673]	410	54.9	137	38

1 - The length were determinate using the Tandem Repeats Finder database [30].
2 - Percent of nucleotide homology were calculated with MegAlign, DNAStar, USA. Comparing with *E. mineirensis* (UFMG-EV).
3 - The length was determined using ClustalW [20] in comparison with *Ehrlichia mineirensis* (UFMG-EV).
4 - Percent of aa homology were calculated with ClustalW [20]. Comparing with *E. mineirensis* (UFMG-EV).
Accession Numbers are from GenBank.

4.2.5.2 Region II (the tandem repeat region)

Region II in *E. mineirensis* (UFMG-EV) contains 16 tandem repeats of 27 bp, each encoding nine aa. The single tandem repeat had the sequence VPAASGDAQ and was completely different to the sequences reported for glycoprotein orthologs of *gp36 E. canis*, *gp47 E. chaffeensis* and *E. ruminantium* mucin-like protein (Table 7). The tandem repeat of *E. mineirensis* (UFMG-EV) is a serine enriched area of the total protein sequence but does not contain threonine. Its glycoprotein gene shows a high C + G percent in the whole gene (42.0%) and in the tandem repeat region (52.1%).

Table 7 Summary of *Ehrlichia* tandem repeats present in gp36 glycoprotein orthologs

Source	Strain	Repeat			Consensus tandem repeat sequence (aa)[2]
		Length (bp)[1]	No.[1]	Homology% (bp)[1]	
Ehrlichia mineirensis	(UFMG-EV)	27	16.0	100	VPAASGDAQ
Ehrlichia canis gp36	TWN1 [EF551366]	27	13.2	100	TEDSVSAPA
	Louisiana [DQ146151]	27	5.2	99	.....
	Sao Paulo [DQ146154]	27	18.2	100	.....
	Cameroon [DQ146155]	27	16.2	100	.....
	IS [EF636663]	27	11.2	99	TEDPVSATA
Ehrlichia chaffeensis gp47	Arkansas [DQ085430]	57	7.0	99	ASVSEGDAVVNAVSQETPA
	Sapulpa [DQ085431]	99	4.5	99	EGNASEPVVSQEAAPVSESGDAANPVSSSENAS
	Jax [DQ146156]	99	4.5	98	.....
	St Vincent [DQ146157]	99	3.4	98	.....
Ehrlichia ruminantium **mucin-like protein**	Highway [AF308673]	27	21.7	99	VTSSPEGSV
	Welgevonden [CR767821]	27	56.0	95	.....
	Gardel [CR925677]	66	16.9	99	SSEVTESNQGSSASVVGDAGVQ

1 – The length (bp), No of nucleotide repeats and the % of Homology were determinate using the Tandem Repeats Finder database [21].
2 – The dots below the tandems mean conserved aa sequence.
Accession Numbers are from GenBank.

4.2.5.3 Region III (the 3' end post-repeat region)

The comparison of region III among the orthologs shows that it is a quite variable region, presenting differences in length, nucleotide and aa sequence. It has been widely revised by [28] and [29]. Our sequence was 94-bp length, which differ from any previously reported (data not shown). The percent identities of nucleotide and aa sequence in this region when compare with *E. mineirensis* (UFMG-EV) go from 12.2% (*E. chaffeensis* St Vincent, DQ146157) to 75% *(E. canis* TWN1, EF551366*)* and from 10% (*E. chaffeensis* St Vincent) to 32% (*E. canis* TWN1), respectively. *E. ruminantium* Highway mucin-like protein has 37.3% (bp) and 21% (aa) of homology with *E. mineirensis* (UFMG-EV).

4.2.5.4 B cell epitopes analysis

The presence of B cell epitopes in the putative gp36 protein was predicted. The presence of one continuous B cell epitope was predicted in a highly hydrophobic repeat tandem region of our protein (197-212). Considering that gp36 (*E. canis*) and gp47 (*E. chaffeensis*) were the closest orthologs, we attempted to find B cell epitope in the tandem repeat of these species using the same algorithm employed for *E. mineirensis* (UFMG-EV). We found the presence of continuous B cell epitopes in the tandem repeat of *E. canis* gp36 [GenBank: EF560599] and *E. chaffeensis* gp47 [strain Arkansas, DQ085430 and strain St. Vincent, DQ146157]. The continuous epitopes found in these last three sequences were localized between the aa position 139-158, 195-225 and 203-218, respectively. The corresponding primary structures of the epitopes are shown in Figure 5A-E. We then compared the predicted 3D structures of the epitopes found in the gp36 orthologs in *E. mineirensis* (UFMG-EV), *E. canis* and the two from different strains of *E. chaffeensis*. We found that all epitopes were exposed on the surface of the predicted 3D structure of each protein. The superposition analysis of the epitopes 3D structure showed that they were structurally dissimilar with a root mean square deviation (rmsd) of 5-6Å between the epitope of *E. mineirensis* (UFMG-EV) and others three Figure 10A-E. A linear correlation between the rmsd and % (dis)similarities among structure and sequences, respectively, is a valid interpretation for the evolution of homolog proteins [55]. Correlation for the epitopes of *E. mineirensis* (UFMG-EV) when compared with the other three orthologs gives an $R^2 = 0.77$.

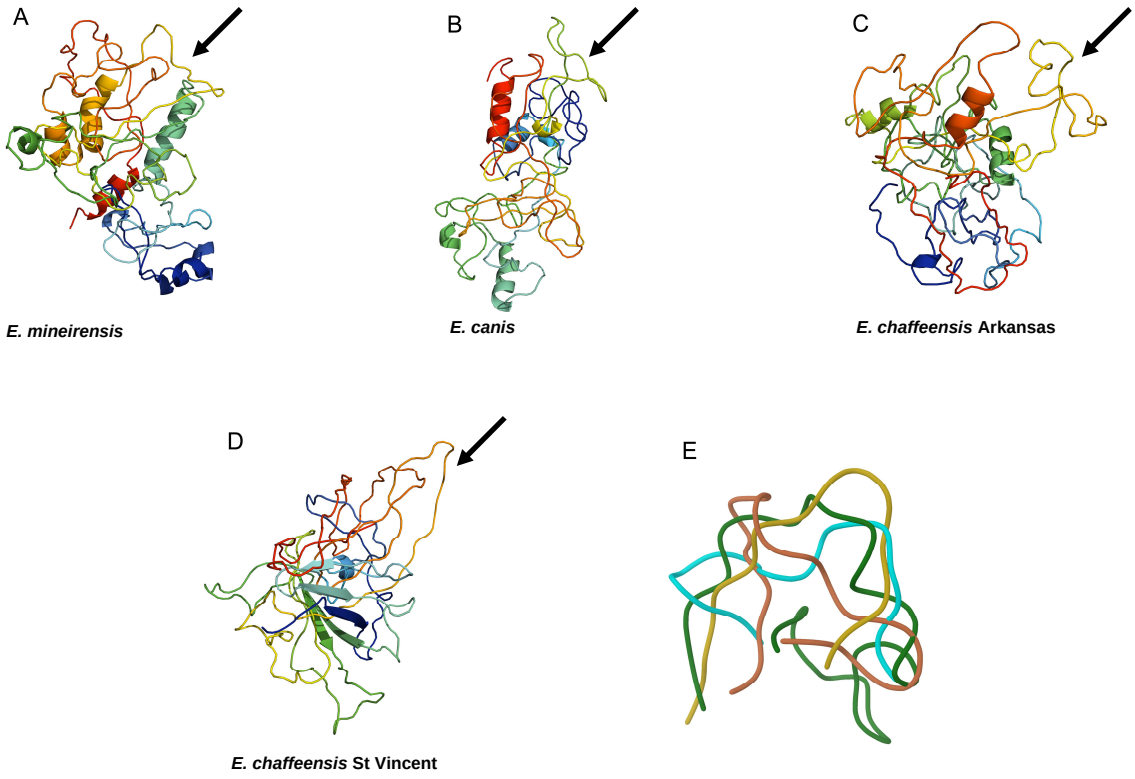

Figure 10 A-E Epitope identification. The modeled 3D structures for *E. mineirensis* (UFMG-EV) (A), *E. canis* (B; GenBank: EF560599), and *E. chaffeensis* (C and D; GenBank: DQ085430, DQ146157, respectively) depict the position of the predicted epitope (→). Protein structures are colored from blue (N-terminus) to red (C-terminus) according to the residue position. An epitope Cα superimposition (E) of *E. mineirensis* (UFMG-EV) (cyan), *E. canis* (brown), *E. chaffeensis* (GenBank: DQ085430; green) and *E. chaffeensis* (GenBank: DQ146157; yellow) depicting the differences in their overall structures, *E. mineirensis* (UFMG-EV) having a 5-6 Å difference compared with the other epitopes).

4.3 Protein electrophoresis and Western blot

The Western blot shows cross reactivity of anti-*E. canis* and anti-*A. marginale* serum against *E. mineirensis* (UFMG-EV) proteins extract (Figure 11AB). Some differences in immune recognition were found about 75-95 kDa between *E. canis* and for *E. mineirensis* (UFMG-EV). Anti-*A. marginale* serum basically recognized proteins at the same size in both *E. canis* and *E. mineirensis* (UFMG-EV) protein extracts.

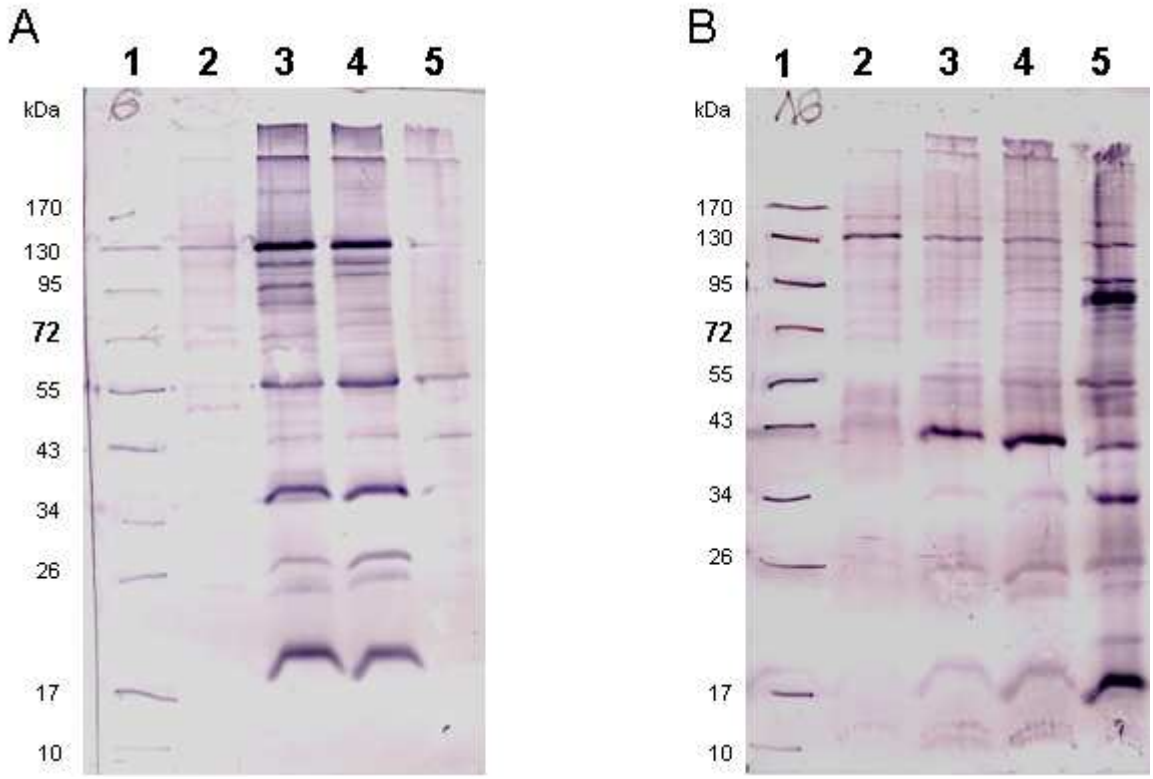

Figure 11. Westerm Blot of *E. mineirensis* (UFMG-EV) proteins extract. Serum from both *E. canis* naturaly infected dog (A) and *A. marginale* naturaly infected cow (B). Numbers on columns: 1. Molecular weight marker; 2. IDE8 uninfected cells; 3. *E. canis* infected IDE8 cells; 4. *E. mineirensis* (UFMG-EV) infected IDE8 cells and 5. *A. marginale* infected IDE8 cells. Numbers on lines are the molecular weight of the marker.

5. Discussion

5.1 In vitro cultures and ultrastructure of E. mineirensis (UFMG-EV).

Here we report the identification of a novel *Ehrlichia* sp. isolated from *R. microplus* engorged females which were collected from a cattle paddock in Belo Horizonte, Brazil. The organisms gave rise to a continuous culture in *I. scapularis* IDE8 tick cells. Initially it was thought that the cultures grow *A. marginale,* firstly because the organisms were isolated from cattle ticks, and secondly, because some of the cattle near-by were carrier animals of *A. marginale*. For further characterization a PCR was conducted, specific for *A. marginale,* however, it did not amplify the DNA isolated from the culture (data not shown). These results strongly suggested that another agent was isolated. At the same time, infection experiments using culture-generated elementary bodies showed that they were able to infect DH82 cells. This cell type supports the growth of *E. canis* [9], the *Ehrlichia* species which is closely related to *E. mineirensis* (UFMG-EV). The DH82 infection experiment and an additional PCR test [56] (data not shown) then clearly indicated the new agent as an *Ehrlichia* species.

In addition *E. mineirensis* (UFMG-EV) elementary bodies did infect bovine endothelial cells (Zweygarth, personal communication). Similar results were reported for *E. ruminantium*, an agent which however targets primarily endothelial cells of diseased ruminants [57]. However, from our culture results it seems unlikely that *E. mineirensis* (UFMG-EV) prefers endothelial cells as targets, because its development in endothelial cells took quite some time, on average 22 days, whereas *E. ruminantium* can finish its developmental cycle in cultured endothelial cells within 3 days [58]. On the other hand, *E. canis* was previously established in a human microvascular endothelial cell line but it fails to grow in the bovine endothelial cell line which was used in the present experiments (Zweygarth, personal communication).

E. mineirensis (UFMG-EV) was isolated from the hemolymph of *R. microplus* which is commonly known as the cattle tick. It is a one-host tick, and from its life cycle, all three feedings stages of any individual tick occur on the same individual host [59], therefore, if *R. microplus* is the vector of the newly identified *Ehrlichia* sp., then its transmission has to be transovarial. In contrast, *E. canis* is transmitted very frequently by the three-host tick *R. sanguineus* [60]. In transmission experiments it was found that *E. canis* was not transmitted transovarially [61], similarly, *E. ruminantium,* which is transmitted by another 3-host tick of the genus *Amblyomma* [62], is also not transmitted transovarielly [62]. *Ehrlichia* spp. have never been found in cattle in Brazil. Nevertheless, it is assumed that the novel *Ehrlichia* sp. originates from cattle, although there is

only circumstantial evidence as outlined above. The only *Ehrlichia* sp. diagnosed in Brazilian herbivores is *E. chaffensis,* which was found in the marsh deer (*Blastocerus dichotomus*) [63] and the brown brocket deer (*Mazama gouazoubira*) [64]. In the Americas, only *E. ruminantium* has been described in ruminants in the Caribbean [65] and a tick-transmitted *Ehrlichia* from Georgia, USA, that was closely related to *E. ruminantium* [66]. Very recently, a novel *Ehrlichia* genotype was detected in naturally infected cattle in Canada [67]. These organisms were isolated from host animals, on the other hand, several *Ehrlichia* ssp. have been isolated from *R. microplus* ticks in Asia, collected from infested cattle, and characterized molecularly [54, 13, 14].

As far as electron microscopy is concerned, uninfected IDE8 cells contained vacuoles and inclusions similar to those previously described as phagolysosomes [68] (data not shown). The phagolysosomes and secondary lysosomes were present in infected IDE8 cells too (Fig 3A, asterisks), however, they also contained membrane-lined vacuoles containing up to 25 rickettsial organisms 0.4 to 1.5 µm in diameter (Figure 3A). We were able to show the presence of both reticulated (Figure 3A) and electron-dense bodies (Figure 3B) which have been described previously for other members of the genus *Ehrlichia* [19, 20]. The organisms were round and oval shaped (Figures 3A, B) and they had typical tri-layered cytoplasmic and outer membranes, in some the outer membrane was rippled (Figures 3B inset). It is noteworthy to mention that we did not find high rickettsial polymorphism as previously reported for *E. ruminantium* [20]. We observed numerous reticulate cells of *E. mineirensis* (UFMG-EV) undergoing binary fission (Figure 3C). Parasitophorous vacuoles were surrounded by mitochondria (Figure 3D), and cisterns of rough endoplasmic reticulum (Figure 3E). In several cases, organelles were in tight contact with the vacuole membrane (Figures 3 D, E) similarly to observations made by others [21]. Moreover, we observed bundles of microtubules (Figure 3D, inset, white arrows) surrounding the membrane of *morulae* which may be important for the movement of the rickettsia through the cytoplasm. Some rickettsial colonies also contained tiny vesicles visible in the interrickettsial space (Figures 3 B, D, 4, black arrows), as has been described for *A. marginale* in IDE8 cells [68].

The establishment of *E. mineirensis* (UFMG-EV) in tick cell culture provides a source of material for the study of this pathogen [32]. The use of this culture system will lead to an increase of our knowledge and understanding of *E. mineirensis* (UFMG-EV) development in ticks. Here we showed the successful ultrastructural characterization of this new agent in IDE8 cells using high resolution techniques of electron microscopy. Even thought further studies are

needed to clarify the pathogenic potential of this agent, our recent [69] and present studies provide new insides in the biology of this new species of the genus *Ehrlichia*.

5.2 Molecular and phylogenetic characterization

Polyphasic taxonomy has been advocated to ensure well-balanced determinations of taxonomic relationships [22]. Different genes have been proposed to classify ehrlichial agents, however, the most widely used are *16S rRNA* [23, 54], *groESL* operon [24], *groEL* gene [25], *gltA* [22], *dsb* [26], *gp36,* and *gp19* [27].

Sequence comparison of the *16S rRNA* gene is recognized as one of the most powerful and precise methods for determining the phylogenetic relationships of bacteria [70, 23, 25]. Our results were consistent with previous phylogenetic analysis of *Ehrlichia* spp using the *16S rRNA* gene sequences [13, 54]. In this study, our analysis of a relevant fragment of *16S rRNA* sequences revealed that the novel agent found in Brazilian *R. microplus* ticks was closely related to *E. canis* [GenBank: GU810149], but was also closely related to *E. chaffeensis* [GenBank: AF147752] showing 98.3% and 96.9% of homology, respectively. It is worth noting that the hypervariable region *16S rRNA* is well conserved in members of the same species (data not shown) and are different among members of the *Ehrlichia* genus [23, 54]. However, our hypervariable region of *16S rRNA* was different when compared with other members of the *Ehrlichia* genus.

Since the *16S rRNA* gene is known to exhibit a high level of structural conservation with a low evolutionary rate, levels of sequence divergence greater than 0.5% in comparisons with nearly complete *16S rRNA* gene sequences of members of the genus *Ehrlichia* have been considered sufficient to classify organisms as different species [23, 54, 70]. The levels of divergence of the *16S rRNA* sequence between this novel Brazilian ehrlichial agent and the closest member of the Anaplasmataceae, *E. canis* was 1.7% in pairwise comparisons of 1384 base sequences (data not shown), and this level of difference should be sufficient to classify the novel ehrlichial agent as a new species of the genus *Ehrlichia*. Furthermore, the *16S rRNA* phylogenetic tree constructed with a maximum likelihood method show that *E. mineirensis* (UFMG-EV) falls in a different clade separated from any previously reported *Ehrlichia* spp.

The genes *groEL* [25] and *gltA* [22] have been proposed as an alternative to *16S rRNA* for the phylogenetic analysis of the Anaplasmatacaea family as they are less conserved than *16S rRNA* among the family members [22] and *dsb* gene has been previously used to classified members of the *Ehrlichia* genus [26]. It is important to note that the spacer of the *groESL* operon was 95 bp

in *E. mineirensis* (UFMG-EV), which differs from the reported for *E. canis*, *E. chaffeensis*, *E. ruminantium* with 93, 100 and 96 bp, respectively [24]. The *gp36* orthologs are a divergent gene in *E. canis*, *E. chaffeensis* and *E. ruminantium* due to their high evolutionary pressure [28, 29]. This gene has been used to differentiate new isolates of *E. canis* where *16S rRNA* was not well suited to discriminate between *E. canis* isolates [27].

In our study the level of similarity among ehrlichial *gltA* and *dsb* were lower than that of *16S rRNA* and *groEL* gene sequences in the genus *Ehrlichia*. *E. canis* was the closest *Ehrlichia* sp. to *E. mineirensis* (UFMG-EV) in all the studied genes. Similar phylogenetic relationships are observed between other members of the *Ehrlichia* genus – i.e., *E. chaffeensis/E. muris*, *N. risticii/N. sennetsu* and *A. marginale/A. platys*.

The architecture of *gltA*, *groEL* and *dsb* based phylogenetic trees were similar to that of the tree derived from the *16S rRNA* gene sequences. However, the trees constructed from *gltA* and *dsb* show more divergence than that from the *16S rRNA* and *groEL* gene. The difference of *E. canis* and *E. mineirensis* (UFMG-EV) was well established in all the four trees based on nucleotide sequences. *E. mineirensis* (UFMG-EV) was well defined, with higher bootstrap values in the *gltA* (100) and *dsb* (100) based trees than for those of the *16S rRNA* (97) and *groEL* (93) based tree.

Based on aa homology and genomic synteny analyses, it has been determined that the mucin-like protein of *E. ruminantium*, *gp36* of *E. canis* and *gp47* of *E. chaffeensis* are orthologs [28]. Identity of 87.2% has been found in the pre-repeat region among geographically distant *E. canis* isolates [27]. The single tandem repeat was highly conserved among isolates (TEDSVSAPA) with variations in the number of repeats [27, 28, 29] and few conservative changes in amino acid sequences [29]. The tandem repeat genetic unit varies in length (from 27bp – 99bp) among the different orthologs, number of repeats (from 3.4 - 56) and the homology of the nucleotide and the aa sequence encoded in the repeat (Table 7). Our sequence contains a tandem repeat that shares an extremely low homology with the gp36 orthologs reported until now ranging from 22% (*E. ruminantium* and *E. canis)* to 33% (*E. chaffeensis*). Doyle *et al.* [28] describes *gp36* and *gp47* as glycoprotein sharing O-glycosylation predicted sites in the serines and threonines of the tandem repeat. It is noteworthy that the tandem repeat of our sequence does not contain threonine; nevertheless, we predicted three sites of O-glycosylation in the serines of the tandem repeat and two in threonines of the post-repeat region. Two N-glycosylation sites were found in our aa sequence. The analysis for N-glycosylation was done for *E. ruminantium*, *E. canis* and for

E. chaffeensis ortholog sequences (data not shown) and potential sites of N-glycosylation were found as well for these sequences. Glycosylation plays a crucial role in the immunogenicity of these glycoproteins [28, 29]. Deglycosylation of the *gp36* tandem repeat drastically reduces its immunogenicity [28]. Both *gp36* and *gp47* are described as the major immunoreactive protein of *E. canis* and *E. chaffeensis* and the tandem repeats contain the major antibody epitope [28, 29]. It was found that the tandem repeat of gp36 from *E. mineirensis* (UFMG-EV) contain the major B cell epitope previously reported for the glycoprotein orthologs. The prediction of the 3D structure of the B cell epitopes present in the tandem repeat shows a high structural divergence among the closest gp36 orthologs in *E. mineirensis* (UFMG-EV), *E. canis* and *E. chaffeensis*. These structural differences may explain the results obtained by Doyle *et al.* [28] in which neither gp36 nor gp47 reacted with heterologous antisera.

The C + G content of the *gp36* gene of *E. mineirensis* (UFMG-EV) is higher than the rest of the orthologs previously reported (data not shown). The C + G content in specific genes have been used in systematics as support for the classification of organisms [22].

Although it is well known that *Babesia bovis*, *B. bigemina* and *Anaplasma marginale* are the most common etiological agents transmitted by *R. microplus* ticks [71], the detection of any species of *Ehrlichia* in *R. microplus* ticks has been infrequently reported. The first two reports were in China in the Guangxi Autonomous Region in 1999 [72] and Tibet in 2002 [54]; the second in Thailand in 2003 [13] and the latest one in Xiamen, China in 2011 [14]. Except the isolate from Guangxi, *E. canis* [72], the rest share, based on *16S rRNA*, a 99.9% of homology [13, 14] and differ from the ehrlichial species previously reported and classified as *Ehrlichia* sp. strain Tibet [12]. In the present study, determined by pairwise alignment, the *E. mineirensis* (UFMG-EV) isolated from *R. microplus* shares 97% of similarity with the *16S rRNA* sequences of the referred species (data not shown). This is the second report of a new *Ehrlichia* sp isolated from *R. microplus*, but the first to be reported on the American continent. The identification of *E. mineirensis* (UFMG-EV) in *R. microplus* ticks suggests a potential of infection and transmission of this agent to cattle in the area where infected ticks are present. Further studies are needed to test whether this agent is a pathogenic strain of Ehrlichia genus or merely a symbiont of *R. microplus*.

6. Conclusions

1. After polyphasic molecular taxonomy analysis we concluded that *E. mineirensis* (UFMG-EV) constitute a new species of the genus Ehrlichia close related to *E. canis*.
2. Both Prostriata and Metastriata tick cell lines are able to support the *in vitro* infection and propagation of this organism.
3. Soft tick (Argasidae) cell line OME/CTVM 22, from *O. moubata*, is not able to support the *in vitro* infection and propagation of *E. mineirensis* (UFMG-EV).
4. The ultrastructure of *E. mineirensis* (UFMG-EV) resembles the one from some members of the genus Ehrlichia (*E. muris*, *E. canis* and *E. chaffeensis*) but no from others (*E. ruminantium*).
5. Polyclonal antibodies present in serum of *E. canis* and *A. marginale* naturally infected animals crossreact with protein extract from *E. mineiresis* (UFMG-EV).

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