Ehrlichia minasensis sp. nov., isolated from the tick Rhipicephalus microplus

Alejandro Cabezas-Cruz, Erich Zweygarth, Marie Vancová, Marzena Broniszewska, Libor Grubhoffer, Lygia Maria Friche Passos, Múcio Flávio Barbosa Ribeiro, Pilar Alberdi and José de la Fuente

1Center for Infection and Immunity of Lille (CIIL), INSERM U1019 – CNRS UMR 8204, Université de Lille, Institut Pasteur de Lille, 1 rue Professeur Calmette, 59019 Lille Cedex, France
2Comparative Tropical Medicine and Parasitology, Ludwig-Maximilians-Universität München, Leopoldstrasse 5, 80802 Munich, Germany
3Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa
4University of South Bohemia, Faculty of Science and Biology Centre of the ASCR, Institute of Parasitology, České Budějovice, Czech Republic
5Departamento de Medicina Veterinária Preventiva, Escola de Veterinária-UFMG, Belo Horizonte, Minas Gerais, Brazil
6Departamento de Parasitologia, ICB-UFMG, Belo Horizonte, Brazil
7SaBio. Instituto de Investigación en Recursos Cinegéticos IREC, CSIC-UCLM-JCCM, Ronda de Toledo s/n, 13005 Ciudad Real, Spain
8Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK 74078, USA

Recently, we obtained a rickettsial isolate (Ehrlichia sp. UFMG-EVT) from the haemolymph of engorged Rhipicephalus microplus tick females. On the basis of maximum-likelihood phylogenetic analysis using 16S rRNA gene, groEL, dsb, gltA and trp36 sequences we showed that Ehrlichia sp. UFMG-EVT belongs to the α-Proteobacteria, family Anaplasmataceae, genus Ehrlichia. Ehrlichia sp. UFMG-EVT is a sister taxon of Ehrlichia canis with 16S rRNA gene, groEL, dsb, gltA and trp36 sequence similarities of 98.3 %, 97.2 %, 94.7 %, 94.3 % and 49.1 %, respectively. Ehrlichia sp. UFMG-EVT has been maintained in the laboratory by continuous passage in the IDE8 tick cell line where the ultrastructure was characterized using electron microscopy and was found to resemble that of E. canis, Ehrlichia muris and Ehrlichia chaffeensis, but not Ehrlichia ruminantium and Ehrlichia ewingii. We propose the name Ehrlichia minasensis sp. nov. for this bacterium to acknowledge the place from where it was initially isolated, Minas Gerais, Brazil. The type strain is strain Ehrlichia sp. UFMG-EVT (=DSM 100393T=TCB-TBB-0018T).

The genus Ehrlichia was reorganized by Dumler et al. (2001) and at the time of writing, comprises five recognized species, Ehrlichia canis, E. muris, E. chaffeensis, E. ruminantium and E. ewingii. Members of this genus are tick-borne bacteria affecting mainly dogs (E. canis, E. chaffeensis and E. ewingii), mice (E. muris) and ruminants (E. ruminantium). However, some species have also been found to infect humans (E. canis, E. ruminantium, E. chaffeensis and E. ewingii) (Allsopp et al., 2005; Perez et al., 2006; Thomas et al., 2009). Transmission of species of the genus Ehrlichia has been associated with different hard tick species such as Rhipicephalus sanguineus and Dermacentor variabilis (E. canis), Amblyomma americanum and Dermacentor variabilis (E. chaffeensis and E. ewingii), Haemaphysalis spp. and Ixodes spp. (E. muris) and Amblyomma spp. (E. ruminantium) (Rar & Golovljova, 2011). We have recently reported the in vitro culture, molecular and morphological characterization of a novel species of Ehrlichia (Cabezas-Cruz et al., 2012, 2013; Zweygarth et al., 2013) which appears to have evolved from highly variable strains of E. canis (Cabezas-Cruz et al., 2014).
The isolation and the in vitro cultivation of *Ehrlichia* sp. UFMG-EVT\(^T\) have been previously described (Cabezas-Cruz et al., 2012; Zweygarth et al., 2013). *Ehrlichia* sp. UFMG-EVT\(^T\) organisms have been cultured in tick cell lines IDE8 and ISE18 derived from *Ixodes scapularis* ticks (Munderloh & Kurtti, 1989), IRE/CTVM20 derived from *Ixodes ricinus* ticks (Bell-Saky et al., 2007), BME/CTVM2 and BME/CTVM6 derived from *Rhipicephalus microplus* ticks (Bell-Saky 2004), BDE/CTVM14 derived from *Rhipicephalus decoloratus* ticks (Lallinger et al. 2010) and OME/CTVM22 derived from Ornithodoros moubata ticks (Bell-Saky et al., 2009). The IDE8 cell line was used to isolate *Ehrlichia* sp. UFMG-EVT\(^T\) and heavily infected culture suspensions were centrifuged at 290 \(g\) for 2 min to remove the majority of the host cells, and 1 ml bacterial suspension was then used to infect the different tick cell lines.

The tick cell lines ISE18, IRE/CTVM20, BME/CTVM2 and BME/CTVM6 were successfully infected with *Ehrlichia* sp. UFMG-EVT\(^T\) initial bodies originating from infected IDE8 cultures, whereas cell line OME/CTVM22 was refractory to infection. In contrast, BDE/CTVM14 cells could not be infected with material derived from IDE8 cell cultures, but could be when the infectious material derived from an infected BME/CTVM6 culture was used. Furthermore, *Ehrlichia* sp. UFMG-EVT\(^T\) infection was lost after three passages in BME/CTVM2 cells, whereas in all other tick cell lines (ISE18, IRE/CTVM20 and BME/CTVM6) *Ehrlichia* sp. UFMG-EVT\(^T\) cultures were passaged at least three times before being terminated.

IDE8 cultures heavily infected with *Ehrlichia* sp. UFMG-EVT\(^T\) were harvested when more than 50 % of the cells had detached from the substrate. The cell culture suspension was then centrifuged at 290 \(g\) for 2 min to remove the majority of the host cells. One millilitre of the cell suspension was distributed into culture flasks containing DH82 medium. Endothelial cells were found to harbour small colonies 16 days after infection, DH82 cells were found to be positive 2 days thereafter. *Ehrlichia* sp. UFMG-EVT\(^T\) was propagated in DH82 cells for more than 1 year, with a mean subculture interval of 26.1 days (range 17–43 days). Endothelial cells were found to harbour small colonies 16 days after infection, DH82 cells were found to be positive 2 days thereafter. *Ehrlichia* sp. UFMG-EVT\(^T\) was propagated in DH82 cells for more than 1 year, with a mean subculture interval of 26.1 days (range 17–43 days). Bovine endothelial cells were propagated for 132 days, with a mean subculture interval of 22 days.

Maximum-likelihood and neighbour-joining phylogenetic analyses were conducted using 16S rRNA gene (GenBank accession no. JX629805), *dsb* (JX629808), trp36 (JX629809), *gltA* (JX629807) and *groEL* (JX629806) sequences of *Ehrlichia* sp. UFMG-EVT\(^T\) (Cabezas-Cruz et al., 2012). High bootstrap values (\(>93\) %) in the phylogenetic trees, using the above genes, support the position of *Ehrlichia* sp. UFMG-EVT\(^T\) as a novel species of the genus *Ehrlichia*, closely related to *E. canis* (Cabezas-Cruz et al., 2012; Aguiar et al., 2014). *Ehrlichia* sp. UFMG-EVT\(^T\) has 98.3 % 16S rRNA gene sequence similarity to *E. canis* TWN (GenBank accession no. GU810149), 96.9 % to *E. chaffeensis* (AF147752), 96.4 % to *E. ewingii* 95E9-TS (U96436), 94.5 % to *E. murs* I268 (AB013008) and 95 % to *E. ruminantium* Mara 87/7 (AF069758) as calculated by pairwise comparisons (Cabezas-Cruz et al., 2012). In addition, the 16S rRNA gene sequence from species of the genus *Ehrlichia* has a highly variable fragment located at the 5′ end of the gene, which is useful in identifying members of the genus *Ehrlichia* (Warner & Dawson, 1996). *Ehrlichia* sp. UFMG-EVT\(^T\) shows three changes in nucleotides in this region when compared to *E. canis* (Cabezas-Cruz et al., 2012). To further support previous analyses (Cabezas-Cruz et al., 2012; Zweygarth et al., 2013; Aguiar et al., 2014), we provide here a revised 16S rRNA gene phylogenetic tree of the genus *Ehrlichia* where *Ehrlichia* sp. UFMG-EVT\(^T\) clearly represents a novel species within this genus (Fig. 1).

We sequenced the genome of *Ehrlichia* sp. UFMG-EVT\(^T\) using DNA extracted from *Ehrlichia* purified from IDE8 cells (Cabezas-Cruz et al., 2015). The genome of *Ehrlichia* sp. UFMG-EVT\(^T\) (GenBank accession nos CDGH01000001–CDGH01000187) consists of 1 414 066 bp, with a G+C content of 30.3 mol%. The origin of replication (oriC), predicted by similarity to the *E. canis* Jake oriC region defined at the DorIC database (http://tubic.tju.edu.cn/ doric/info1.php?ac=OR10030069), seems to be placed in contig ehr000001 in the intergenic space upstream of the divergent genes encoding Uroporphyrinogen decarboxylase (UDP) (EC 4.1.1.37) and Cytochrome c oxidase, subunit III (EC 1.9.3.1), three genes downstream of the *dual* gene. The genome of *Ehrlichia* sp. UFMG-EVT\(^T\) resulted in 944 genes, including protein-coding sequences (CDSs), RNA genes and pseudogenes. Of them, 322 genes encode proteins with enzymic activity, 51 encode membrane proteins, 55 are involved in DNA repair and 144 are connected with oxidoreduction processes (Cabezas-Cruz et al., 2015). Taking in account the scarcity of genomic data from the genus *Ehrlichia* (Fig. 1), the genome of *Ehrlichia* sp. UFMG-EVT\(^T\) will contribute to the study of the genetic diversity of this group of bacteria.

Recently, the same species (strain *Ehrlichia* sp. UFMT-BV) was isolated by another group in Brazil and was shown to be pathogenic to cattle (Aguiar et al., 2014). Phylogenetic and evolutionary analyses using the trp36 gene showed that both *Ehrlichia* sp. UFMG-EVT\(^T\) and *Ehrlichia* sp. UFMT-BV evolved recently from a highly variable clade of *E. canis* under adaptive diversifying selection (Cabezas-Cruz et al., 2014). This suggested that *Ehrlichia* sp. UFMG-EVT\(^T\) represents an example of recent host-shift within the genus *Ehrlichia*. In agreement with this, firstly, while the common tick vector for *E. canis* is *Rhipicephalus sanguineus*, *Ehrlichia* sp. UFMG-EVT\(^T\) was isolated from *Rhipicephalus microplus* haemolymph (Cabezas-Cruz et al., 2012) and secondly, while *E. canis* is mainly pathogenic for dog, *Ehrlichia* sp. UFMT-BV was found to be pathogenic for cattle (Aguiar et al., 2014).
Fig. 1. Neighbour-joining phylogenetic tree of the genus *Ehrlichia* based on 16S rRNA gene sequences from 153 members of the genus *Ehrlichia* collected from the GenBank database. Species of the genus *Ehrlichia* for which at least one genome is published are shown (filled circles). Clusters for which more than one isolate has been reported were collapsed (bold names). GenBank accession numbers of unique isolates are shown in parentheses.
For the morphological characterization of *Ehrlichia* sp. UFMG-EVT we used electron microscopy. Infected IDE8 cells were prepared by high-pressure freezing and freeze substitution method, and were embedded into epoxide resin as described previously (Cabezas-Cruz et al., 2013). We found micro-colonies of *Ehrlichia* sp. UFMG-EVT located inside membrane-bound vacuole(s) in the cytoplasm of host cells. Vacuoles were surrounded by mitochondria and cisterns of rough endoplasmic reticulum and contained fibrillar materials. Vacuoles contained round- or oval-shaped bacteria of 0.4–1.5 μm diameter with a Gram-negative cell-wall structure. We observed binary fission only in the reticulate forms. Reticulated and electron-dense forms were present either simultaneously in one vacuole, or one vacuole contained only one morphological form (Fig. 2). Similarly, non-synchronized maturation was observed in *E. chaffeensis* replicating in the tick cell line ISE6 (Dedonder et al., 2012). Rarely, vacuoles were opened to the extracellular space. These results indicate morphological similarity of *Ehrlichia* sp. UFMG-EVT to *E. canis*-*E. chaffeensis* strains (Popov et al., 1998).

Taken together, these data indicate that *Ehrlichia* sp. UFMG-EVT represents a novel species of the genus *Ehrlichia*, for which the name *Ehrlichia minasensis* sp. nov. is proposed.

**Description of *Ehrlichia minasensis* sp. nov.**

*Ehrlichia minasensis* (mi.nas.en’sis. N.L. fem. adj. minasensis pertaining to Minas Gerais).

An obligate intracellular bacterium found in haemolymph of the cattle tick *Rhipicephalus microplus* and pathogenic for cattle. Can be grown in *Ixodes scapularis* cell lines (IDE8) and dog macrophages (DH82) at 32 °C and 37 °C, respectively. The ultrastructural appearance of the culture isolate is typical for the genus *Ehrlichia* (Fig. 2). The organism is located inside membrane-bound vacuoles in the cytoplasm of host cells (Fig. 2). Bacterial cells are round or oval, 0.4–1.5 μm diameter and can be observed inside host cells using Giemsa stain. To date, the species has been isolated from *Rhipicephalus microplus* and naturally infected cattle from Brazil and Canada. Phylogenetic analyses (Fig. 1) and the draft 1.41 Mb genome sequence indicates that the species is a sister taxa of *E. canis*.

The type strain, *Ehrlichia* sp. UFMG-EVT (= DSM 100393^T=TCB-TBB-0018^T), was isolated from a partially engorged *Rhipicephalus microplus* female collected in Minas Gerais, Brazil in 2010. The G+C content of the type strain is 30.3 mol% (genome sequencing).

**Acknowledgements**

This research was supported by the EU FP7 ANTIGONE project number 278976. The authors thank Dr Ulrike G. Munderloh (University of Minnesota, USA) for permission to use the IDE8 cell line, and Dr Lesley Bell-Sakyi and the Tick Cell Biobank at The Pirbright Institute, UK. ACC was supported by a grant from the Ministère de l’Éducation Supérieure et de la Recherche de France.

**References**


