

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

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Recently, we obtained a rickettsial isolate (*Ehrlichia* sp. UFMG-EV^T) 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-EV^T belongs to the α -Proteobacteria, family Anaplasmataceae, genus *Ehrlichia*. *Ehrlichia* sp. UFMG-EV^T 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-EV^T 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-EV^T (=DSM 100393^T=TCB-TBB-0018^T).

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; Zwegarth *et al.*, 2013) which appears to have evolved from highly variable strains of *E. canis* (Cabezas-Cruz *et al.*, 2014).

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The isolation and the *in vitro* cultivation of *Ehrlichia* sp. UFMG-EV^T have been previously described (Cabezas-Cruz *et al.*, 2012; Zweygarth *et al.*, 2013). *Ehrlichia* sp. UFMG-EV^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-Sakyi *et al.*, 2007), BME/CTVM2 and BME/CTVM6 derived from *Rhipicephalus microplus* ticks (Bell-Sakyi 2004), BDE/CTVM14 derived from *Rhipicephalus decoloratus* ticks (Lallinger *et al.* 2010) and OME/CTVM22 derived from *Ornithodoros moubata* ticks (Bell-Sakyi *et al.*, 2009). The IDE8 cell line was used to isolate *Ehrlichia* sp. UFMG-EV^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-EV^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-EV^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-EV^T cultures were passaged at least three times before being terminated.

IDE8 cultures heavily infected with *Ehrlichia* sp. UFMG-EV^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 canine macrophages (Wellman *et al.*, 1988) or BA886 bovine aorta endothelial cells (Yunker *et al.*, 1988) together with 4 ml modified L-15B medium and incubated at 34 °C. After 3 days, the medium was replaced with 5 ml fresh 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-EV^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-EV^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-EV^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-EV^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. muris* 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-EV^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-EV^T clearly represents a novel species within this genus (Fig. 1).

We sequenced the genome of *Ehrlichia* sp. UFMG-EV^T using DNA extracted from *Ehrlichia* purified from IDE8 cells (Cabezas-Cruz *et al.*, 2015). The genome of *Ehrlichia* sp. UFMG-EV^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=ORI10030069>), seems to be placed in contig ehr000001 in the intergenic space upstream of the divergent genes encoding Uroporphyrinogen decarboxylase (UPD) (URO-D) (EC 4.1.1.37) and Cytochrome *c* oxidase, subunit III (EC 1.9.3.1), three genes downstream of the *dnaJ* gene. The genome of *Ehrlichia* sp. UFMG-EV^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-EV^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-EV^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-EV^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-EV^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).

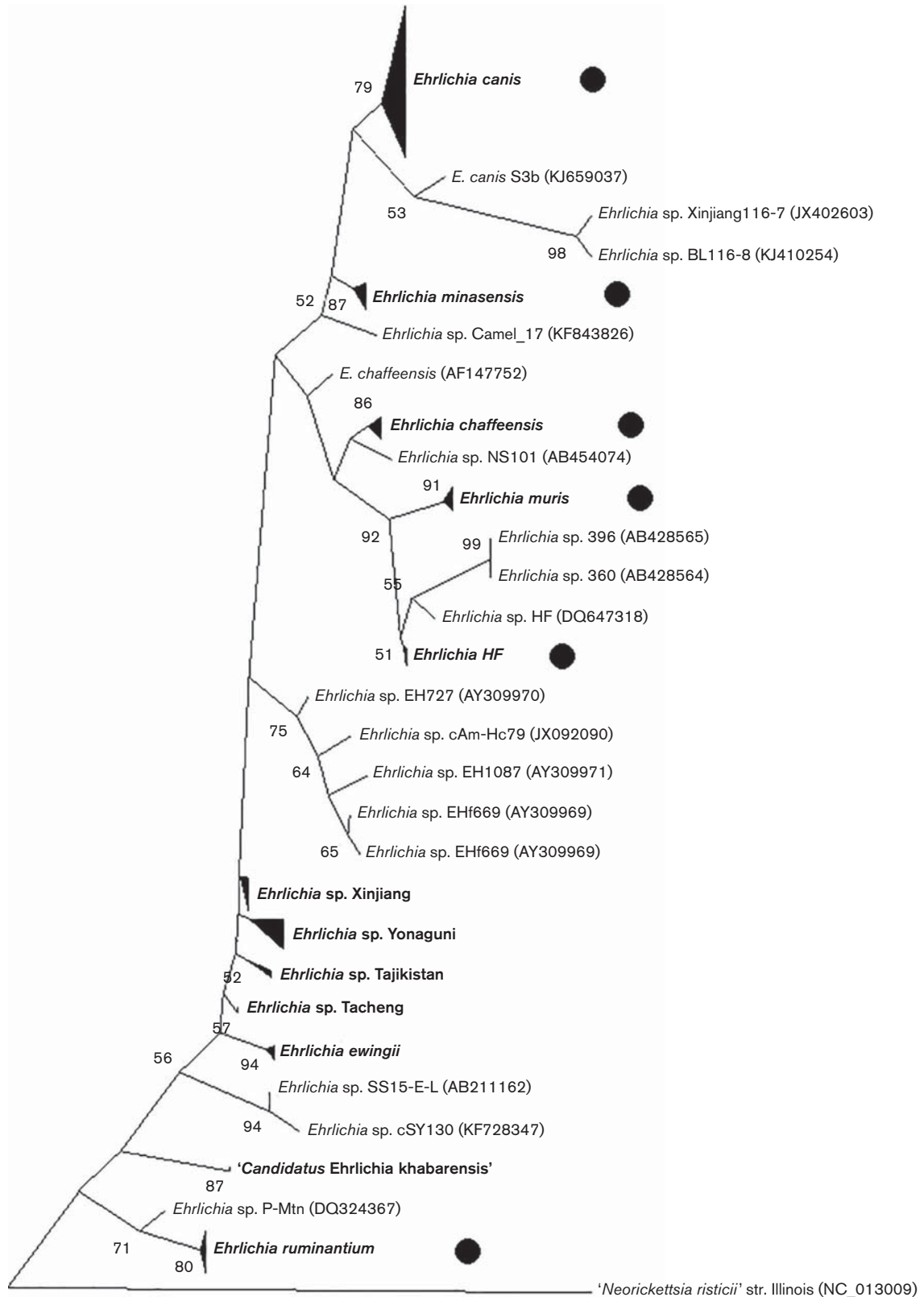


Fig. 1. Neighbour-joining phylogenetic tree of the genus *Ehrlichia* based on 16S rRNA gene sequences from 153 members of the genus *Ehrlichia* collected from the GenBank database. Species of the genus *Ehrlichia* for which at least one genome is published are shown (filled circles). Clusters for which more than one isolate has been reported were collapsed (bold names). GenBank accession numbers of unique isolates are shown in parentheses.

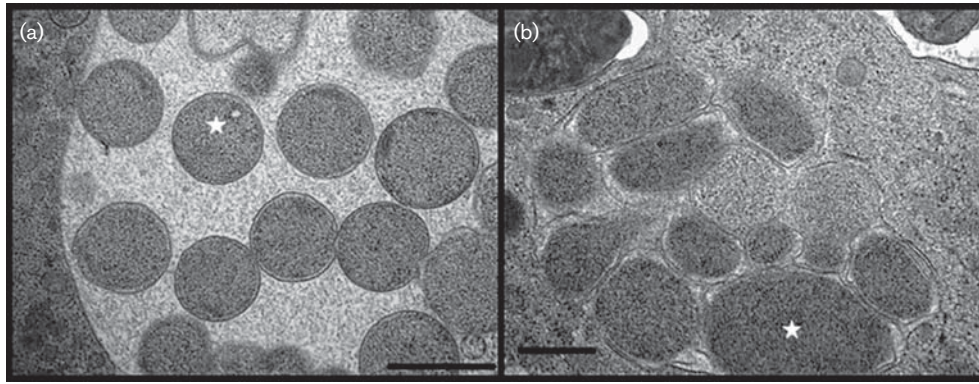


Fig. 2. Morphology of *Ehrlichia minasensis* sp. nov. showing reticulate cells (a) and electron-dense bodies (b); stars indicate an example of each structure. Bars, 1 μm (a) and 500 nm (b). Further information regarding the ultrastructure of this organism can be found in Cabezas-Cruz *et al.* (2013).

For the morphological characterization of *Ehrlichia* sp. UFMG-EV^T 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-EV^T 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-EV^T to *E. canis*-*E. chaffeensis* strains (Popov *et al.*, 1998).

Taken together, these data indicate that *Ehrlichia* sp. UFMG-EV^T 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-EV^T (= 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).

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