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Genetic characterization of a novel *Ehrlichia chaffeensis* genotype from an *Amblyomma tenellum* tick from South Texas, USA

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Abstract

Ehrlichia chaffeensis is the causative agent of human monocytotropic ehrlichiosis (HME), a disease that ranges in severity from mild to fatal infection. *Ehrlichia chaffeensis* is maintained in a zoonotic cycle involving white-tailed deer (*Odocoileus virginianus*) as the main vertebrate reservoir and lone star ticks (*Amblyomma americanum*) as its principal vector. Through complete genomic analysis from human ehrlichial isolates and DNA sequences obtained from deer and tick specimens, nine strains of *E. chaffeensis* have been characterized. Few studies have examined the genetic diversity of *E. chaffeensis* in ticks, and some of these investigations have identified that the genetic sequences coincide with the circulating strains reported so far. Here, we report the first evidence of *E. chaffeensis* DNA from an unfed *Amblyomma tenellum* (formerly *Amblyomma imitator*) collected in South Texas. We characterized the genetic variation of this *E. chaffeensis* genotype using conserved gene markers such as *rRNA, dsb*, and *groEL*. We also used gene targets useful to distinguish genotypes, such as the variable length PCR target gene (VLPT) and 120-kDa gene, encoding the tandem-repeat proteins TRP32 and TRP120, respectively. Our results suggest a novel *E. chaffeensis* genotype that exhibited greater variability than other genotypes of *E. chaffeensis* and highlights the role for *A. tenellum* as a potential vector of *E. chaffeensis*.

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Author statement

Esteban Arroyave: Conceptualization, Methodology, Investigation, Formal analysis, Writing- Original draft preparation. **Bethany Quade**: Investigatio, Validation, Data Curation. **Nicole L. Mendell**: Methodology, Investigation, Formal analysis, Writing- Original draft preparation. **Lucas S. Blanton**: Conceptualization, Methodology, Investigation, Formal analysis, Resources, Writing- Reviewing and Editing. **Donald H. Bouyer**: Conceptualization, Investigation, Formal analysis, Writing- Reviewing and Editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2022.101990.

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Keywords

Amblyomma tenellum ; *Ehrlichia chaffeensis* ; Human monocytotropic ehrlichiosis; Variable length PCR target gene; Tandem repeat

1. Introduction

Ehrlichia chaffeensis is the etiologic agent of human monocytotropic ehrlichiosis (HME) – the most frequently diagnosed tick-borne disease in the southern United States. Infection usually results in a mild febrile illness, but severe manifestations and death may occur (Nichols Heitman et al., 2016). *Ehrlichia chaffeensis* is transmitted primarily by the lone star tick (*Amblyomma americanum*) and maintained by the white-tailed deer (*Odocoileus virginianus*), which is recognized as the main reservoir. However, natural infection has been reported in several other vertebrates, such as goats, dogs, red foxes, and coyotes (Paddock and Childs, 2003). *Ehrlichia chaffeensis* DNA has also been detected in other tick species in the USA, such as *Dermacentor variabilis, A. inornatum, A. mixtum*, and *A. maculatum* (Steiert and Gilfoy, 2002; Medlin et al., 2015; Williamson et al., 2010).

HME was discovered in 1986 (Maeda et.al., 1987), but the etiological agent was not isolated until several years later. The first strain of *E. chaffeensis* was isolated in 1991 in a 21-year-old man and designated as Arkansas for its geographic origin (Dawson et al., 1991). Subsequently, multiple isolates from human patients have been obtained from several geographic regions of the USA. At the time of this publication, nine strains of *E. chaffeensis*, all of them with complete genome sequences available from GenBank, have been characterized and include Arkansas str. (Arkansas), West Paces str. (Tennessee), Heartland str. (Nebraska), Jax str. (Florida), Liberty str. (Florida), Osceola str. (Florida), Sapulpa str. (Oklahoma), Saint Vincent str. (Georgia), and Wakulla str. (Florida). Comparative analysis has demonstrated that these *E. chaffeensis* strains exhibit variable pathogenicity in severe combined immunodeficiency (SCID) mice, although genetically they are highly conserved (96% of orthologous genes) (Miura and Rikihisa, 2007).

Some genes have been defined as markers to determine the genetic differentiation of *E. chaffeensis* genotypes, including the 28-kDa gene expressing the immunodominant p28 protein (Cheng et al., 2003), the variable length PCR target gene (VLPT), and the 120-kDa gene (the latter 2 exhibit tandem variable repeats in the encoded proteins TRP32 and TRP120, respectively) (Sumner et al., 1999; Zambrano et al., 2021). The objective of this study was to determine the genetic variation in a unique genotype of *E. chaffeensis* detected in a potentially undescribed vector, *Amblyomma tenellum.*

2. Material and methods

Questing ticks (unfed) were collected during 2015 from the Laguna Atascosa National Wildlife Refuge, located on the southern tip of coastal Texas (26° 06′ 01.1”N, 97° 19′ 53.1”W) (Fig. 1). The refuge encompasses 97,000 acres of land and provides habitat for migratory birds and a high diversity of wild animals. The tick collection was performed through CO_2 trapping and flagging methods as previously described (Carroll

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and Schmidtmann, 1992; Falco and Fish, 1992). Adult *Amblyomma tenellum* ticks were identified through standard taxonomic keys (Keirans and Durden, 1998; Pratt, 2013), and were confirmed by amplification and sequencing of the mitochondrial gene *16S rRNA* (*rDNA*) (Black and Piesmant, 1994) (Suppl. 1).

Ticks were surface decontaminated by immersing in 5% bleach solution for 5 minutes, then in 70% ethanol for 5 minutes, followed by 3 subsequent 1-minute rinses with sterile phosphate buffered saline (PBS). Adult ticks were pooled in groups of 1 to 5 ticks based on species and sex. Ticks were bisected with a sterile razor blade. Pools of half ticks were stored at −80°C for future isolation. The remaining tick halves were stored in their respective pools at −20°C for eventual DNA extraction. The latter was performed by placing tick pools in 2 mL microcentrifuge tubes with 100 μl of PBS and 2 sterile 4 mm stainless steel grinding balls, which were homogenized with a Retsch MM300 mixer mill (Bio-Rad, Hercules, CA, USA) for 2 min at 30 Hz. DNA from these homogenates were extracted using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) per the manufacturer's instructions.

PCRs were performed using the 5PRIME Hot Master Mix kit (Quantabio, Beverly MA, USA) according to the manufacturer's recommendations. All the PCRs contained 2μl of extracted DNA that was used as a template. The final reagent concentrations were 10 pmol of each primer, 1 U of Taq DNA Polymerase per reaction mixture, 45 mM Cl, 2.5 mM Mg^2+, 200 μM of each dNTP. The PCR was performed in a thermal cycler PCR system 2700 (Applied Biosystems /Thermo Fisher Scientific, Foster City, CA, USA). The primers and thermocycling conditions were performed according to the descriptions by the authors cited in Table 1. The amplified products were separated by 1.5% agarose gel Tris borate-EDTA electrophoresis and visualized by staining with DNA GelRed (Biotium, Fremont, CA, USA).

The PCR products were sequenced at the UTMB Genomics Core Laboratory, and the sequences (forward and reverse) were assembled through the SeqMan program implemented in DNASTAR software (DNASTAR Inc., Madison, WI, USA). Alignments were performed with MUSCLE (**MU**ltiple **S**equence **C**omparison by **L**og-**E**xpectation). The MEGA 7.0.21 program was used for the phylogenetic analysis, the Maximum likelihood method was performed to build the phylogenetic trees. Akaike information criterion from MEGA 7.0.21 was used to determine the best fitting substitution models to construct the ML tree. The bootstrap test with 1000 replications was applied to estimate the confidence of branching patterns.

Tick halves from the −80°C stored PCR positive pool were individually homogenized in 1 mL of cell culture medium (DMEM with 5% FBS and 1% Hepes) using Dounce homogenizers. Four wells of a 24-well plate, which contained confluent layers of DH82 cells, were inoculated with 200 μL of homogenate. Plates were centrifuged for 30 minutes at 800 X *g*. After centrifugation, fresh cell culture medium was added to each of the 4 wells. Plates were incubated at 34°C with 5% of CO_2. The cells were examined for the presence of morulae and by PCR weekly. The infected cells were passed onto 25-cm^2 tissue culture flasks.

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3. Results

In total, 723 non-fed *A. tenellum* adults were collected. Initial PCR testing of the ticks was achieved to amplify the *16S rRNA* (*Ehrlichia/Anaplasma*) and *dsb* (*Ehrlichia*) genes. Partial DNA fragments from *16S rRNA* and *dsb* were sequenced from one pool containing 5 adult female ticks, for an infection rate of 0.13% (1/723) (assuming only 1 tick in this pool was infected). The sequences obtained for *16S rRNA* exhibited 100% identity with *Ehrlichia chaffeensis* (Suppl. 2). However, the highly conserved *dsb* gene in *Ehrlichia* spp. showed an identity of 97.8% (398/407) in the sequence of this study with 9 nucleotide changes that did not alter the amino acid sequence (Fig. 2)(GenBank accession number of 16S rRNA and *dsb* are MZ457066 and MZ457067, respectively). The conserved *groEL* gene was also amplified, revealing an identity of 98.2% (1347/1371) with known strains of *E. chaffeensis* (Fig. 3), without changes in the encoded amino acid sequence (GenBank accession number of *groEL* sequence: MZ457068).

The VLPT and 120-kDa genes encode the surface-expressed TRP32 and TRP120 antigens, respectively. Both proteins have variable numbers of tandem repeats that are useful in distinguishing *E. chaffeensis* genotypes. The TRP120 encoded protein is nearly identical among the known *E. chaffeensis* strains. This protein contains 80 amino acids in its tandem repeat and varies from two to five repeats. The sequence described in this study revealed 3 tandem repeats which were identical to other *E. chaffeensis* strains (data not shown). In TRP32, five imperfect tandem repeat units containing 30 amino acids have been identified. The amino acid sequence obtained from *A. tenellum* was considerably longer compared to the other reported sequences, including 10 imperfect tandem repeat units (the greatest number of tandem repeats reported so far). Interestingly, the tandem repeat sequences from this study were different from the 5 tandem repeats known from other *E. chaffeensis* genotypes (Fig. 4). Conversely, analysis of the N-terminal domain showed that this region was highly conserved between the strain herein reported and the other strains of *E. chaffeensis*; only one substitution was recognized at position 14 (M>V) (Suppl. 3). The C-terminal domain was considered short in the *E. chaffeensis* genotype from *A. tenellum* (14 aa) compared to other reported strains (61 aa). However, the alignment analysis from the C-terminal region demonstrates homology in 8 amino acids between positions 4 and 14 with a deletion at the first two amino acids, followed by a substitution (D>E) in the third position, and ending with a 5 amino acid sequence (KGEFV). (GenBank accession number of *VLPT* sequence: MZ457069).

Unfortunately, we were unable to obtain an isolate of this new genotype of *E. chaffeensis*. Although the DH82 cell culture medium was consistently PCR positive for 3 months, and morula-like structures were observed on smears made from scrapings of the cell monolayer, we were unable to demonstrate substantial growth and spread of this *A. tenellum* ehrlichial agent to be considered a successful isolation.

4. Discussion

The phylogenetic analysis of *16S rRNA, dsb* and *groEL* genes, along with the analysis of the amino acid sequences of TRP120 and TRP32, indicate that the *Ehrlichia* sp. infecting

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A. tenellum belongs to a unique genotype of *E. chaffeensis*. In the United States, *E. chaffeensis* has been detected mainly in *Amblyomma americanum*, the primary vector of this pathogen, and less frequently in *Dermacentor variabilis*, whose ability to transmit this agent is unclear (Wright et al., 2014). Nine strains of *E. chaffeensis* have been characterized through the polymorphisms of TRP32 and TRP120 (Paddock and Childs, 2003). Differences in virulence among *E. chaffeensis* strains has been reported in severe combined immunodeficiency (SCID) mice (Miura and Rikihisa, 2009) but not in patients with HME due to the limitations of the diagnostic methods to differentiate genotypes from clinical samples. Similar sequences on tandem repeats from TRP32 and TRP120 have been described from human isolates, white-tailed deer (*Odocoileus virginianus*), and *A. americanum*. However, variable numbers of repeat units have been observed depending on the strain, and also differences in the linear order of the individual repeats in TRP32 (Sumner et al., 1999; Yabsley et al., 2003). The sequences obtained in this study show that *16S rRNA* and 120-kDa/TRP120 are consistent with other *E. chaffeensis* genotypes. Conversely, *dsb, groEL*, and the well characterized immunoreactive genes (VLPT/TRP32) exhibited substantial divergence to suggest a novel genotype. Furthermore, TRP32 demonstrates a substantial divergence in the amino acid sequence of the tandem repeats, compared with the other reported strains. Thus, our results support the previous description of the usefulness of TRP32 to differentiate strains of *E. chaffeensis* (Paddock et al., 1997; Seddighzadeh et al., 2009; Zambrano et al., 2021).

The isolation of rickettsial organisms is usually difficult, particularly ehrlichial agents, because they have a low multiplication rate compared with other pathogens. Our inability to successfully isolate this organism may have been a result of an insufficient number of bacteria, use of a non-optimal cell line (DH82 cells rather than a tick cell line), and freeze storing the ticks, which decreases bacterial viability (Lagier et al., 2015; Standaert et al., 2000).

The current study is the first report of infection by *Ehrlichia* spp. In *A. tenellum*. Previously, *A. tenellum* has been reestablished as a valid species (Nava et al., 2014). *Amblyomma tenellum* is frequently confused with *A. mixtum* due to its morphological similarity (hence its previous name *Amblyomma imitador* by Kohls., 1958), especially in areas such as southern Texas and Central America where both species are sympatric (Keirans and Durden, 1998) however, these two species are easily distinguished by following specific morphological characters (Nava et al., 2014). Although there can be morphological confusion between the two species, *A. tenellum* is genetically more similar to *A. americanum* than to the *A. cajennense* complex to which *A. mixtum* belongs (Nava et al., 2014; Beati et al., 2013). However, *A. tenellum* and *A. americanum* not only share their genetic closeness, these two species are also sympatric and feed on a variety of shared vertebrates. These ecological characteristics could lead to the occurrence of spillover from one tick species to another (Guglielmone et al., 2006; Paddock and Childs, 2003). It is important to highlight that *A. tenellum* has been reported parasitizing humans (Guzman-Cornejo et al., 2011), and it is also a potential vector of *R. rickettsii*, the agent of Rocky Mountain spotted fever (Oliveira et al., 2010).

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Although *E. chaffeensis* has been isolated exclusively in the United States, DNA sequences closely related to *E. chaffeensis* have been detected from ticks in other areas of the world. In South America, DNA of an *Ehrlichia* sp. closely related to *E. chaffeensis*, has been detected in other *Amblyomma* species (i.e., *A. tigrinum, A. triste*, and *A. parvum*) (Cicuttin et al., 2017; Tomassone et al., 2008). Unfortunately, there are no available sequences of the VLPT gene for those ehrlichieae, limiting the phylogenetic analysis and the evolutionary origin of these genotypes. Although the above tick species have been shown to feed on humans, no cases of human ehrlichiosis have been demonstrated in this part of the continent.

Further study of wildlife from Laguna Atascosa National Wildlife Refuge, and their association with *A. tenellum*, should be considered through serological and molecular approaches to determine the potential reservoirs that comprise the enzootic cycle of this *E. chaffeensis* genotype. Considering that *A. tenellum* has been involved parasitizing humans and domestic animals (e.g., dogs, goats, horses, and cattle) (Keirans and Durden, 1998; Düttmann et al., 2016), the human and veterinary significance of the *Ehrlichia* genotype described in this study should be elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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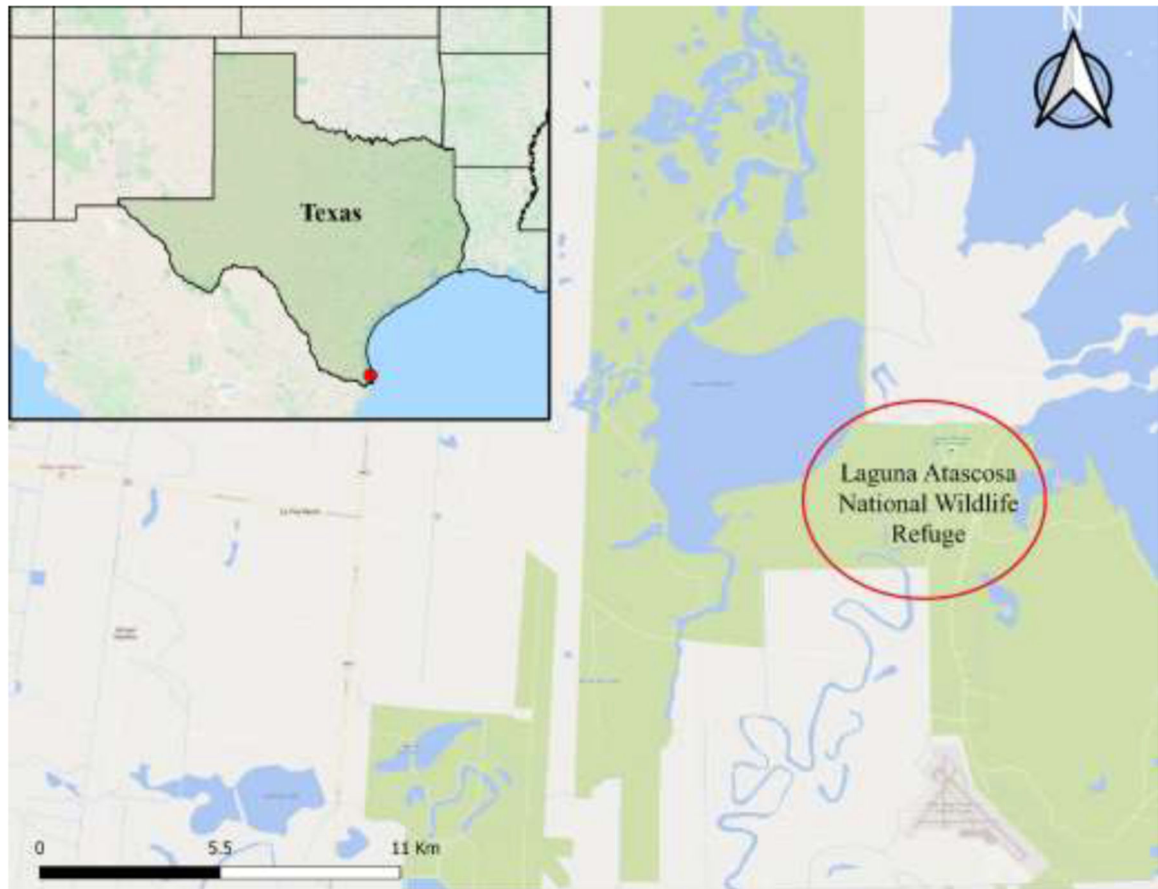

Fig. 1.
Location of the sampling site where the *A. tenellum* ticks were collected.

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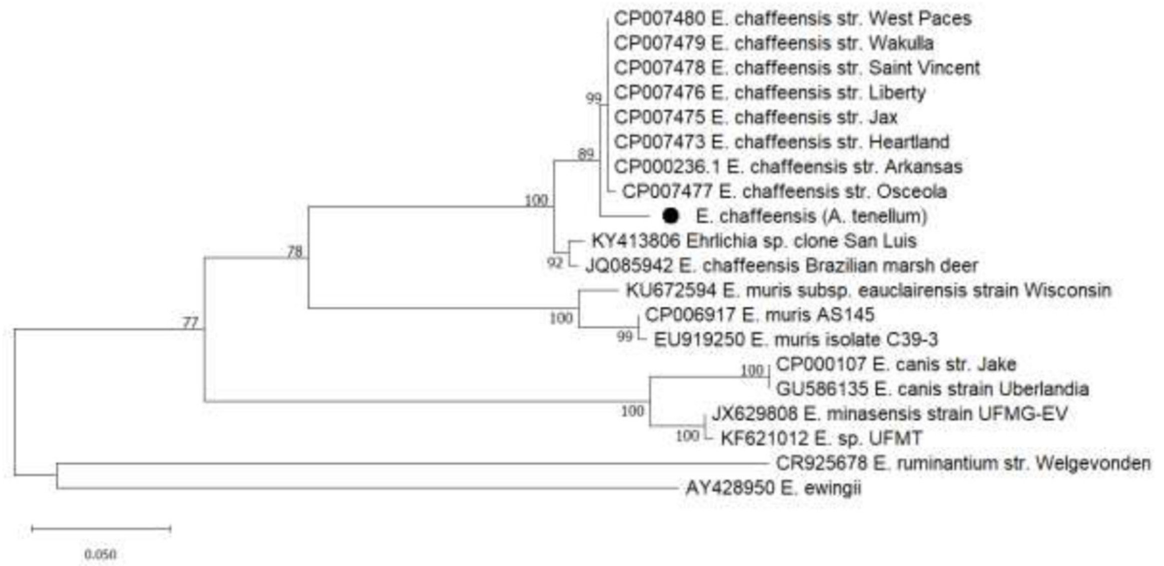

Fig. 2.

Phylogenetic tree of ***dsb*** nucleotide sequence obtained in this study (●) and others available in GenBank. Maximum-likelihood tree were used to build the phylogenetic tree using Tamura 3-parameter model plus gamma distribution. A total of 340 nucleotides were analyzed.

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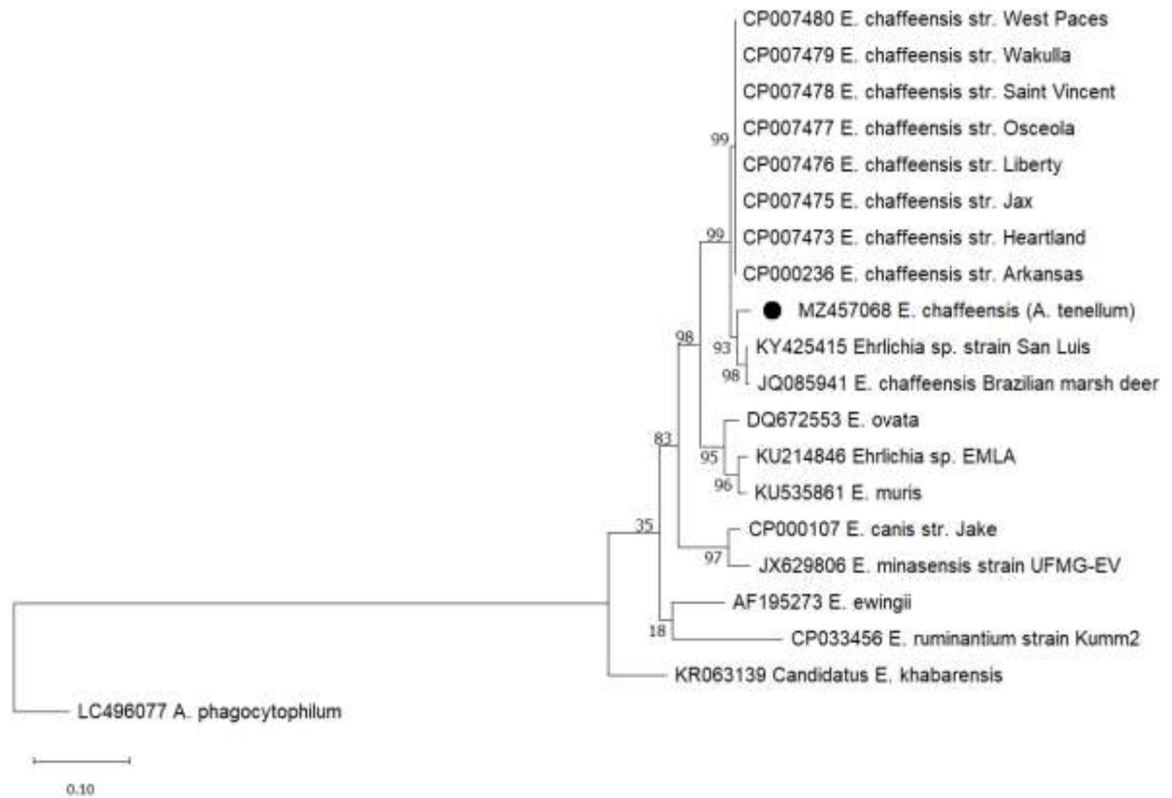

Fig. 3.

Phylogenetic tree of ***groEL*** gene from *Ehrlichia* spp., inferred using the Maximum Likelihood method based on the HKY+G model with a discrete Gamma distribution (1.0). The bootstrap consensus tree inferred from 1000 replicates. We used the orthologous sequence *groEL* of *Anaplasma phagocytophilum* as an outgroup. There were a total of 1151 positions in the final dataset analyzed. The circle ● corresponds to the sequence obtained in this study.

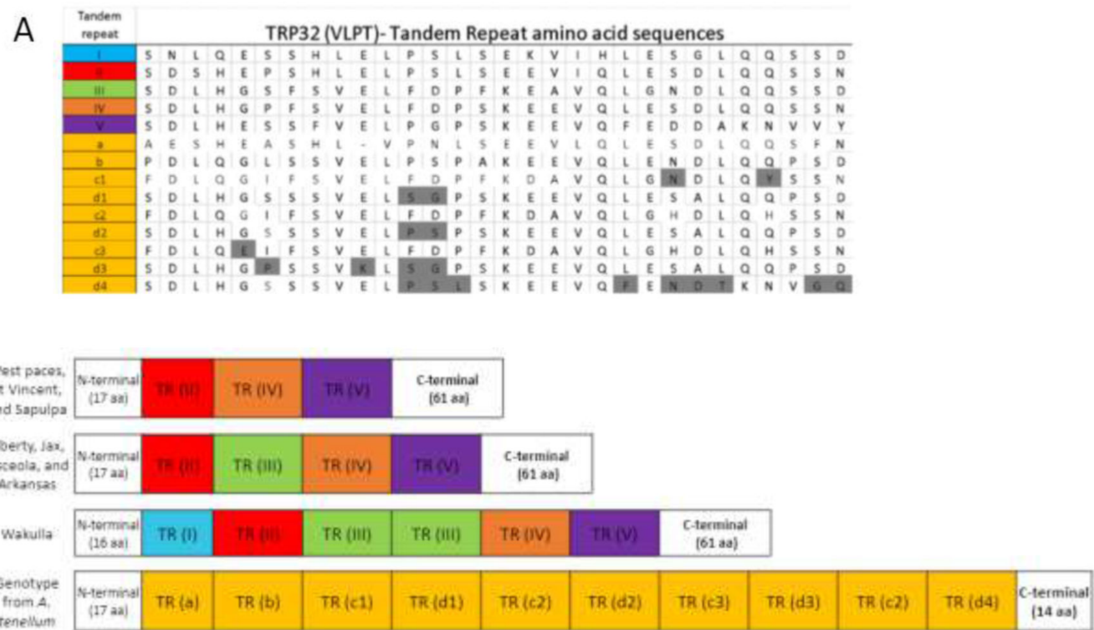

Fig. 4.

Multiple alignment of TRP32 (VLPT gene) amino acid sequences among *E. chaffeensis* strains compared with the novel *E. chaffeensis* genotype obtained from *A. tenellum*.

(A) Tandem repeat units from the different *E. chaffeensis* genotypes. The amino acids highlighted in gray represent residues divergent from the genotype identified in *A. tenellum* tick from this study. **(B)** Order in which the repeat units occur in the different *E. chaffeensis* strains. The yellow boxes represent the tandem repeat units from the genotype described in this study.

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Table 1

Primers used for the amplification different targets from *E. chaffeensis* genotype.

Target	Primer sequence (5` - 3`)	Amplicon size	Reference
16s rRNA	EHR16SD - GGT ACC YAC AGA AGA AGT CC	350pb	Parola et al., 2000
	EHR16SR - TAG CAC TCA TCG TTT ACA GC		
dsb	Dsb-330 - GAT GAT GTC TGA AGA TAT GAA ACA AAT	400 pb	Doyle et al., 2005
	Dsb-728 - CTG CTC GTC TAT TTT ACT TCT TAA AGT		
groEL	groESL HS1a - AIT GGG CTG GTA ITG AAA T	1300 pb	Liz et al., 2000
	groESL HS6a - CCI CCI GGI ACI AIA CCT TC		
	groESL HS43 - ATW GCW AAR GAA GCA TAG TC		
	groESL HSVR - CTC AAC AGCA GCT CTAG TAGC		
VLPT	TRP32 FB3 - GCC TAA TTC AGA TAA ACT AAC	370 – 780 pb	Paddock et al.,1997
	TRP32 FB5 - AAA TAG GGT ATA AAT ATG TCA C		
120-kDa	TRP120 F1 - GAG AAT TGA TTG TGG AGT TGG	1100 – 1500 bp	Paddock et al., 1997
	TRP120 R2 - ACA TAA CAT TCC ACT TTC AAA		