Emergence of a Novel *Ehrlichia minasensis* Strain, Harboring the Major Immunogenic Glycoprotein trp36 with Unique Tandem Repeat and C-Terminal Region Sequences, in *Haemaphysalis hystricis* Ticks Removed from Free-Ranging Sheep in Hainan Province, China

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**Abstract:** *Ehrlichia minasensis*, a recently described *Ehrlichia* species that is the most closely related to, but clearly distinct from, *Ehrlichia canis*, has been circulating in not only bovines, cervids, and dogs but also several tick species from Canada, Brazil, France, Pakistan, Ethiopia, and Israel. However, there are no reports of *E. minasensis* in China. The purpose of this study was to explore whether *E. minasensis* is present naturally in ticks in China. Through PCR targeting of the genus-conserved dsb gene, *E. minasensis* DNA was detected in *Haemaphysalis hystricis* ticks removed from free-ranging sheep in Hainan Province, South China in 2017. The partial sequence of the dsb, 16S rRNA, and groEL genes demonstrated that the Hainan strain shared 99% identity with the dsb gene of *E. minasensis* strain UFMG-EV (GenBank: JX629808), with the 16S rRNA of *E. minasensis* isolate E-2650 (MH500005) and with the groEL gene of *E. minasensis* strain UFMG-EV (JX629806), respectively. Moreover, sequence analysis of the major immunogenic tandem repeat protein (trp36) revealed that the Hainan strain harbored a unique tandem repeat sequence (APEAAPVSAPEAAPVSAPVS) and a C-terminal region that differed from those of other known *E. minasensis* strains. Additionally, phylogenetic analysis based on the entire amino acid sequence of trp36 revealed that the Hainan strain was closely related to a recently described *E. minasensis* strain from Brazil, of which the sister clade contained different strains of *E. canis*. The discovery of this novel Hainan strain in *H. hystricis* ticks represents the first known natural presence of *E. minasensis* in South China, highlighting the need for its constant surveillance.

**Keywords:** *Ehrlichia minasensis*; *Haemaphysalis hystricis* tick; free-ranging sheep; South China; trp36
1. Introduction

Ehrlichiosis, which is caused by an obligate, intracellular, gram-negative, tick-borne alphaproteobacterium within the genus *Ehrlichia* (family *Anaplasmataceae*), is an emerging disease in humans, domestic animals, and mice worldwide [1]. The genus *Ehrlichia* consists of five well-described species: *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia canis*, *Ehrlichia ruminantium*, and *Ehrlichia muris* [2]. *Ehrlichia minasensis*, a recently recognized *Ehrlichia* species [3], is closely related to the canine monocytic ehrlichiosis-causing pathogen *E. canis*, with phylogenetic analysis revealing that this new species evolved from highly variable strains of *E. canis* [4]. The geographic distribution of *E. minasensis* is not limited to Canada and Brazil, as was previously reported [5–7], since recent works have discovered this bacterium in Ethiopia [8], France [9], Israel [10], Pakistan [11], and South Africa [12].

*E. minasensis* can be propagated in canine macrophage-like cell lines (e.g., DH82) and *Ixodes scapularis* cell lines (e.g., IDE8) [5,6], and can cause clinical manifestations associated with ehrlichiosis in experimentally infected cattle [5]. *E. minasensis* has been circulating among not only naturally infected dairy cattle, mule deer, and dogs, but also various tick species, including *Hyalomma marginatum*, *Hyalomma anatolicum*, and *Rhipicephalus microplus* [9,11,13]. However, whereas other *Ehrlichia* species (including *E. chaffeensis*) and *Ehrlichia*-like organisms have been detected in *Haemaphysalis hystricis* (*H. hystricis*), *E. minasensis* has not been detected in this tick species so far [14,15]. The hard-bodied *H. hystricis* (also named east Asian mountain haemaphysalid), which is an obligate ectoparasite of mammals, is distributed in China, Japan, Vietnam, India, and Thailand (http://www.catalogueoflife.org/col/details/species/id/4f85d86075bf0ac2ba1e6b55d31d82be).

A very limited number of epidemiologic surveillances works on *E. minasensis* in ticks and domestic animals have been performed. This neglect of *E. minasensis* detection was likely because these agents were considered to have a negligible economic impact on the livestock industry. However, efforts to discover the molecular and antigenic diversity of *E. minasensis* will unquestionably contribute to the development of effective vaccines and reliable immunodiagnostics for this disease as well as to unveiling the microbial factors associated with its disease pathogenesis. Furthermore, very limited information is available on *E. minasensis* in China. Therefore, the purpose of this study was to determine whether *E. minasensis* could be detected in ticks removed from free-ranging sheep in Hainan Province, South China.

2. Materials and Methods

2.1. Tick Collection and DNA Extraction

In June 2017, 82 adult ticks were removed from free-ranging sheep (*n* = 16) bred on one farm located in Haikou, Hainan Province (longitude 110.53, latitude 19.81), South China. The ticks were collected according to standardized sampling procedures [16] and were stored at −80 °C until tested. Total DNA was extracted directly from pooled tick samples (5 ticks per pool, same tick species and same host) using the Wizard® Genomic DNA Purification Kit (Promega, Shanghai, China) according to the manufacturer’s instructions. The collected ticks were identified to the species level by PCR amplification targeting the 16S rRNA gene fragment and the cytochrome coxidase subunit 1 (*cox1*) gene [17,18] (primer sequences and PCR conditions as shown in Table 1).
Table 1. Primers used in this study.

| Species         | Target | Primer Name          | Sequence                                      | PCR Condition                                                                 | Length | References |
|-----------------|--------|----------------------|-----------------------------------------------|------------------------------------------------------------------------------|--------|------------|
| ticks           | 16S rDNA | 16S+1                | CCGTCTGAACCTACAGATCAAG                        | 95 °C 5 min, 35 × (95 °C 30 s, 57 °C 30 s, 72 °C 40 s), 72 °C 10 min       | 460 bp | [17]       |
|                 |        | 16S-1                | CTGCTCAATGATTTTTTTAAATTGCTGTGG                |                                                                 |        |            |
|                 |        | LCO1490              | GGTCAACAAAATCATAAGATATTGG                     | 95 °C 5 min, 35 × (95 °C 30 s, 57 °C 30 s, 72 °C 40 s), 72 °C 10 min       | 650 bp | [18]       |
|                 |        | HCO2198              | TAAACTTCAGGTCACCAAAAAATCA                     |                                                                 |        |            |
| Ehrlichia       | cox1   | LCO1490              | GGTCAACAAAATCATAAGATATTGG                     | 95 °C 5 min, 35 × (95 °C 30 s, 57 °C 30 s, 72 °C 40 s), 72 °C 10 min       | 460 bp | [17]       |
| minasensis      |        | HCO2198              | TAAACTTCAGGTCACCAAAAAATCA                     |                                                                 |        |            |
| Ehrlichia       | dsb    | dsb-330              | GATGATGTCTGAAAGATGAACAAAT                   | 94 °C 5 min, 35 × (94 °C 30 s, 50.5 °C 60 s, 72 °C 60 s), 72 °C 10 min     | 400 bp | [19]       |
| minasensis      |        | dsb-728              | CTGCTCGTCTATTITTTACTCTTAAAGT                 |                                                                 |        |            |
|                 |        | Ehr-16S-D            | GGTACCCACAGAAGATCC                           | 94 °C 5 min, 35 × (94 °C 30 s, 54 °C 60 s, 72 °C 60 s), 72 °C 10 min       | 345 bp | [9]        |
|                 |        | Ehr-16S-R            | TAGCCTACTCGTTTACAGC                         |                                                                 |        |            |
|                 | 16S rDNA | Ehr-groel-F          | GTTGAARACTGATGGTAGTC                      | 94 °C 5 min, 35 × (94 °C 30 s, 55 °C 60 s, 72 °C 60 s), 72 °C 10 min       | 590 bp | [9]        |
|                 |        | Ehr-groel-R          | ACGCGTTCCTACGYTCYTAAC                      |                                                                 |        |            |
|                 | groEL  | TRP36-F2             | TTAAAACAAATAATCACACTA                      | 94 °C 5 min, 35 × (94 °C 30 s, 46 °C 60 s, 72 °C 60 s), 72 °C 10 min       | 800–1000 bp | [17] |
|                 |        | TRP36-R1             | AAGATTAACCTAATACCTAAATTACT                  |                                                                 |        |            |
2.2. PCR Amplification and DNA Sequencing of the dsb, 16S rRNA, groEL, and trp36 Genes of E. minasensis

The purified DNA was tested in four individual PCR amplifications using primers targeting a portion of the disulfide bond formation protein (dsb) gene, the 16S rRNA gene, the heat shock protein (groEL) gene and the glycoprotein trp36 (trp36) gene. The reactions (20 µL) contained 2 µL of template DNA, 0.5 mM of each primer, and 10 µL of 2 × EasyTaq PCR SuperMix (TransGen, Beijing, China). Detailed information about the primers and PCR condition is shown in Table 1. The positive PCR product was subjected to DNA sequencing (ABI PRISM 377 DNA sequencer). The full sequence for both strands of each DNA template was determined to ensure maximum accuracy of the data.

2.3. DNA Sequence Analysis and Phylogenetic Analysis

All sequences obtained in this study were assembled and compared with sequences available in the GenBank database, using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences were translated to their corresponding amino acid (aa) sequences using the EMBOSS Transeq tool (https://www.ebi.ac.uk/Tools/st/emboss_transeq). The nucleic acid and aa alignments were performed with the ClustalW multiple sequence alignment application that is included in the BioEdit software package. Phylogenetic analyses based on the partial coding sequence (CDS) of the dsb, 16S rRNA and groEL genes and the aa sequence of trp36 were conducted in MEGA X [20]. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model (dsb, 16S rRNA and groEL genes) and the Jones–Taylor–Thornton (JTT) matrix-based model (trp36), respectively. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining (NJ) and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model (trp36) and a maximum composite-likelihood approach (dsb, 16S rRNA and groEL genes), and then selecting the topology with a superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 19 (dsb), 18 (16S rRNA), and 12 (groEL) nucleotide sequences and 10 amino acid sequences (1D). All positions containing gaps and missing data were eliminated. In total, 223 (dsb), 279(16S rRNA), and 530 (groEL) positions were in the final dataset.

3. Results and Discussion

3.1. Identification of Tick Species

The nucleic acid sequences of the 16S rRNA and cox1 genes indicated that all ticks collected in this study were of the H. hystricis species.

3.2. Sequence Analysis of the dsb, 16S rRNA and groEL Genes of E. minasensis

In this study, only one of the 16 sample pools (6.25%, 5 ticks from the same sheep) was PCR positive for the genus-conserved dsb, 16S rRNA, groEL genes of E. minasensis and E. canis. Upon comparison with sequences available from the GenBank database, the dsb, 16S rRNA, and groEL genes of the E. minasensis Hainan strain identified in this study (GenBank MN463729) were found to have 99% partial CDS similarity to the dsb genes from E. minasensis strain UFMG-EV (JX629808; 363/365), isolate E-2650 (MH500007; 343/344), strain 1E (KM015219; 325/329) and to the 16S rRNA of E. minasensis isolate E-2650 (MH500005; 342/345) as well as to the groEL gene of E. minasensis strain UFMG-EV (JX629806; 624/626), respectively. In addition, the phylogenetic tree based on the partial CDS of dsb, 16S rRNA, and groEL genes revealed that the Hainan strain grouped together with E. minasensis into a clade of which the sister clade had different strains of E. canis (Figure 1A–C).
Figure 1. Cont.
Figure 1. Phylogenetic tree based on the gene sequences of dsb (A), 16S rRNA (B), groEL (C), and amino acid sequences of trp36 (D) from geographically dispersed Ehrlichia minasensis and Ehrlichia canis strains, as inferred by the maximum-likelihood method using other species of Ehrlichia as a genus outgroup and other strains of Anaplasma as a genuine outgroup. The tree with the highest log likelihood of −925.65, −449.26, −2007.15, and −2916.24 (A–D) are shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

3.3. Sequence Analysis of the trp36 Gene

The gene that encodes trp36 has been widely used as a target for molecular investigations of E. canis and E. minasensis and for distinguishing between the two species [4,5,21]; therefore, the complete trp36 gene of the Hainan strain was amplified and sequenced. Sequencing of the PCR amplicon revealed that the trp36 gene was 891 bp in size, encoding a predicted protein of 296 aa. According to the nucleotide sequence analysis, the trp36 gene of the Hainan strain shared 97% identity with the trp36 gene sequence of E. minasensis strain UFMT (KF870578; 395/406), 97% with the trp36 gene of strain UFMT-BV (KT970785; 380/391), and 93% with the trp36 gene of strain UFMG-EV (JX629809; 406/435), as well as 92% with the trp36 genes of E. canis strain Bloemfontein (KC935387; 400/433), strain 222 (KC479021; 400/433), and strain 171 (KC479020; 400/433). The deduced aa sequence of the Hainan strain is shown in Figure 2.
3.4. Sequence Analysis of the N-Terminal Region and the Upstream Tandem Repeat Region of the trp36 Gene

The N-terminal region of trp36, which contained 141 aa, was 98% identical to that of *E. minasensis* strains UFMT (AHI42992; 123/126) and MFMT-BV (AMW87052; 118/121), whereas it shared 89% identity with that of *E. canis* strain 171 (AGQ51636; 115/129), 87% with that of strain TWN4 (ABX71625; 112/129), and 86% with that of strain Pocone C6 (KY522826; 360/419), when compared against aa sequences available from the GenBank database. In line with reported studies [5,6], the predicted aa sequence of the N-terminal region of trp36 from *E. minasensis* exhibited the highest identity (93–98%) with that from all reported *E. minasensis* strains. This minor diversity was also observed within *E. canis* (Figure 2), since there are no antibody epitopes in the N-terminal region and thus potentially less immune-driven adaptations [5,22]. Interestingly, the sequence upstream of the tandem repeat (TR) region (IVSQAQ5VLSSI) of the Hainan strain was partially identical to that of *E. canis* strains from China and Thailand (ABS82573, ABU44524, ABV26011, CP025749, and MF771084: IVSQAQ), and completely different to that of *E. minasensis* strains from Brazil and Canada (AMW87052 and AHI42992: LVNQAQ; and AFV15304: LVNQAQVLLPSG) and of *E. canis* strains from Costa Rica, Peru, and Turkey (KU194227, MF095619, and MG905718: IVNQAQAILSSAT). However, the potential roles of the upstream TR region of the trp36 genes from *E. minasensis* and *E. canis* are still unknown.

3.5. Sequence Analysis of the Tandem Repeat Region and C-Terminal Region of the trp36 Gene

The TR region of the trp36 gene of the Hainan strain contained six TRs of 60 bp in length, each encoding 20 aa. The single TR had the sequence APEAAPVSAPEAAPVSAPVS and was completely different to the TR sequences reported for glycoprotein orthologs of trp36 from *E. canis* and *E. minasensis* (Figure 2). In addition, the C-terminal sequence of the gene from the Hainan strain was 105 bp in length, encoding 35 aa, which also differed from any previously reported *E. canis* and *E. minasensis* C-terminal sequences (Figure 2). Taken together, the results suggested that the TR aa sequence of trp36 was the most divergent region between this Hainan strain and *E. minasensis* and *E. canis*, suggesting that a recent TR diversification was likely driven by immune pressure, since the major antibody epitope is located in this region [5,6,22].
3.6. Phylogenetic Relationship Analysis Based on trp36

To further elucidate the genetic characteristics of this novel E. minasensis Hainan strain, a phylogenetic tree was generated on the basis of the entire aa sequence of trp36. According to the phylogenetic tree, which was built using the maximum-likelihood method based on the Jones–Taylor–Thornton (JTT) matrix-based model, this Hainan strain isolated from the H. hystricis tick clustered into the same clade as other E. minasensis strains from Brazil, of which the sister clade had different strains of E. canis [5,23]. The isolation of such highly similar clade strains from distinct animal and tick species in different regions of the Americas and Asia indicates that the E. minasensis strains could be intercontinental and interdispersed transmitted by some specific way, such as via migrating birds [24,25]. However, no similar E. minasensis strain was detected in ticks from other regions of China, although 1060 adult ticks were collected from free-ranging livestock and pets in one province in East China (Zhejiang, Haemaphysalis longicornis, n = 18), two provinces in Northeast China (Jilin, H. longicornis, n = 282; and Heilongjiang, Haemaphysalis japonica, n = 349, Dermacentor nuttalli, n = 131, and Ixodes persulcatus, n = 73), and one province in North China (Inner Mongolia, Dermacentor nuttalli, n = 207) during the years 2016 to 2018. Therefore, our current data suggest that no E. minasensis strain was introduced into mainland China from Hainan Island of South China and that circulation of the strain was limited.

4. Conclusions

Our current data indicate that a novel E. minasensis strain, which harbors the major immunogenic glycoprotein trp36 with unique TR and C-terminal region sequences, existed in H. hystricis ticks removed from free-ranging sheep in South China but not in other regions of the country. However, further studies are needed to address the question of whether H. hystricis is a competent tick vector for this E. minasensis strain and whether this new bacterial strain is an emerging pathogen of sheep, goats or other ruminants, including dairy and beef cattle. In addition, our findings suggest a need for the constant epidemiologic surveillance for E. minasensis strains in domestic animals and wildlife in China in order to stay abreast of the potential introduction of novel variants from other ticks and hosts.

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