Letter to the Editor

Molecular Detection and Identification of *Candidatus* Ehrlichia Hainanensis, A Novel *Ehrlichia* Species in Rodents from Hainan Province, China*

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Ehrlichia (Anaplasmataceae family) are obligatory intracellular bacteria that infect humans and animals. They are hosted by mammals such as canines, bovines and wild rodents, and are vectored by ticks. In this study, we collected 121 rodent samples comprising 67 *Niviventer fulvescens*, 27 *Rattus tanezumi*, 24 *Chiromyscus* sp., 2 *Rattus nitidus* and 1 *Leopoldamys edwardsi* from Hainan province, which includes the second largest island in China. The presence and genetic diversity of *Ehrlichia* species was evaluated and characterized by amplification and sequencing of 16S rRNA, *groEL* and *gltA* genes. An *Ehrlichia* species was detected in 5 of the 67 *Niviventer fulvescens* samples (7.46%). The 16S rRNA, *groEL* and *gltA* genes showed the highest identity to known *Ehrlichia* sequences (99.20%, 89.87% and 83.86%, respectively). In the phylogenetic trees they formed a cluster distinct from all other species. We propose that this species is a putative novel *Ehrlichia* species, which we suggest be named *Candidatus* Ehrlichia hainanensis. Its pathogenicity to humans remains to be further researched, and molecular surveillance in local populations is needed.

Key words: *Candidatus* Ehrlichia hainanensis; Hainan province; *Niviventer fulvescens*

Ehrlichia is a genus of obligatory intracellular bacteria belonging to the family *Anaplasmataceae*, order *Rickettsiales*. To date, six formally recognized *Ehrlichia* species and some Candidatus species have been reported[1]. They are mainly vectored by ticks, and many are pathogenic to humans and animals. *Ehrlichia canis (E. canis)*, *Ehrlichia ruminantium (E. ruminantium)* and *Ehrlichia muris* are pathogens for canines, ruminants and mice, respectively. *Ehrlichia chaffeensis (E. chaffeensis)*, *Ehrlichia ewingii*, *Ehrlichia muris-like* agent and *E. canis* have been reported to infect humans and to cause symptoms ranging from fever to severe multiple organ failure[2-5]. The common symptoms of human ehrlichiosis are fever, headache, myalgia, malaise, weakness, nausea, leukopenia, vomiting, diarrhea and abdominal pain[6]. These indistinguishable symptoms cause difficulties in diagnosis and may lead to clinical misdiagnosis. In China, multiple *Ehrlichia* species have been identified, including *E. chaffeensis*, *E. canis* and *E. ruminantium*, from ticks, mammals, mosquitoes and leeches[7]. *E. chaffeensis* and *E. canis* are currently the known human *Ehrlichia* pathogens in China. Human monocytic ehrlichiosis disease caused by *E. chaffeensis* has been demonstrated to be widespread in several provinces of China[8]. Rural residents, particularly farmers, are at substantially increased risk of *Ehrlichia* exposure.

Small mammals such as rodents have been demonstrated to be the reservoirs of many *Ehrlichia* species. The *Ehrlichia* sp. HF group has been identified in *Apodemus argenteus*, *A. speciosus*, *Eothenomys smithi* and *Myodes rufocanus bedfordiae*. *Candidatus E. khabarensis* was identified and characterized from *Myodes rutilus*, *M. rufocanus* and *Sorex araneus* in the Russian Far East. *Ehrlichia muris* was first isolated from the tissue of a wild mouse (*Eothenomys kageus*) in Japan, and a human pathogenic *E. muris*-like agent was identified from *Peromyscus leucopus* in the United States. In the host-vector ecosystem, these *Ehrlichia* bacteria can be horizontally transmitted to ticks that infest hosts, and spillover into human populations may also occasionally occur. However, whether they pose a

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threat to public health remains unclear. Evidence of their involvement in the human ehrlichiosis remains to be further determined.

Although human monocytic ehrlichiosis has been common in China, the geographical distribution and genetic diversity of various *Ehrlichia* species have not yet been well studied. In Hainan Province, few investigations and studies have been performed on *Ehrlichia*. Hainan Province contains the second largest island in China, located in the South China Sea. It has an area of 33,900 square kilometers and a population of approximately 9.34 million people. Owing to its tropical climate and landscape, it has become a major tourist attraction receiving tens of millions of visitors each year. To date, more than 20 tick species have been reported in Hainan, including *Ixodes*, *Rhinpicephalus*, *Dermacentor*, *Amblyomma* and *Haemaphysalis*. Vector surveillance has indicated that Hainan Province has the highest rat density in China. Multiple rodent species have been observed, including *Niviventer fulvescens*, *Niviventer niviventer*, *Rattus rattus*, *Rattus norvegicus* and *Rattus flavipectus*. To improve understanding of the *Ehrlichia* distribution in these vectors/hosts and the potential risk to public health in Hainan Province, we examined the molecular evidence of *Ehrlichia* in wild rodents and performed further research in this study.

From November to December of 2019, rodents were trapped in cages by using bait. In total, 121 rodents were captured in Qiongzhong autonomous county in the middle of Hainan Province. All animals were captured alive and then anesthetized to minimize suffering. The rodents were sacrificed by cervical dislocation. The tissue samples were collected and stored in RNAlater. After being washed twice with phosphate buffer, the liver samples were subjected to total DNA extraction with a DNA/RNA isolation kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. The extracted DNA was stored at −20 °C before species identification and *Ehrlichia* detection. The rodent species were identified by experienced field biologists and then confirmed by sequencing of the mt-cyt b gene.

Ehrlichia was detected with hemi-nested PCR primers targeting a conserved region of 16S rRNA as previously described[7]. The length of PCR fragments was 546 bp. The amplification parameters for both the first and second PCR were 94 °C for 3 min, followed by 40 cycles of: 94 °C for 30 s, 49 °C for 40 s, 72 °C for 45 s and an additional 10 min at 72 °C. For better phylogenetic analysis, a 16S rRNA fragment of 1,256 bp was obtained from the positive samples by using previously reported primers[7]. Heat shock protein gene (*groEL*) fragments 1,116 bp in length were amplified by conventional PCR with hemi-nested PCR primers[7]. The 970 bp citrate synthase gene (*gltA*) fragment was amplified with primers as previously described[7]. The PCR reactions were performed with a Sensoquest PCR System LabCycler Standard P (Germany). Amplification parameters for the first and second PCR included 94 °C for 3 min, followed by 40 cycles of: 94 °C for 30 s, 47 °C for 70 s, 72 °C for 45 s and an additional 10 min at 72 °C. PCR amplicons were analyzed by electrophoresis on 1.0% agarose gels. The PCR products shorter than 800 bp were subjected to Sanger sequencing by Sangon Biotechnology Company (Shanghai, China). The amplicons longer than 800 bp were cloned into the pMD19-T cloning vector (TaKaRa), transformed into *E. coli* and plated onto culture dishes. The obtained clones were collected and sent for Sanger sequencing.

Ehrlichia sequences (16S rRNA, *groEL* and *gltA* sequences) obtained in this study, as well as those retrieved from GenBank (Supplementary Table S1 available in www.besjournal.com), were aligned with the Clustal W method implemented in the MEGA program, version 6.0[9]. Nucleotide sequence identities were calculated with the MegAlign program available in the DNASTAR Lasergene package, version 7.0 (DNASTAR, Inc., Madison, WI, USA). Phylogenetic trees based on these three genes were constructed with the maximum likelihood method in the PhyML v3.0 package (http://www.atgc-montpellier.fr/phyml/binaries.php) with the best-fit GTR+I+Γ4 model of nucleotide substitution, as determined by jModeltest. Bootstrap values higher than 70% were considered significant.

In this study, a total of 121 rodents were captured in Qiongzhong autonomous county, Hainan Province, Southern China. These rodents represented five species: 67 *Niviventer fulvescens*, 27 *Rattus tanezumi*, 24 *Chiromyscus* sp., 2 *Rattus nitidus* and 1 *Leopoldamys edwardsi*. Hemi-nested PCR targeting the 16S rRNA gene was performed to screen the *Ehrlichia* DNA in 121 liver tissue samples. Consequently, PCR products of the expected size (546 nt) were recovered from five *N. fulvescens* (5/67, 7.46%). The results were confirmed by DNA sequencing. No positive results were detected from *R. tanezumi* and other rodent samples.

Partial 16S rRNA (1,256 bp), *groEL* (1,116 bp) and *gltA* (970 bp) sequences were successfully obtained from three randomly selected samples. Through

BLAST and MegAlign analysis, the 16S gene sequences of the three strains were found to have 99.92%–100% nucleotide identity, and the highest nucleotide identity (99.20%) was observed with the *Ehrlichia* sp. EHt224 characterized from *Hyalomma truncatum* in Niger (AF311968.1) and *Ehrlichia* sp. EBm52 from *Boophilus microplus* in Thailand (AF497581.1). The *gltA* gene showed the highest nucleotide identity to *Ehrlichia* sp. TC248–16 reported in Xinjiang (83.86%), whereas the highest nucleotide identity with *Ehrlichia* sp. TC248–16 (89.87%) was identified for the *groEL* gene. In all phylogenetic trees, the sequences obtained in this study formed a distinct cluster far from any other *Ehrlichia* species (Figure 1). We propose that this is a novel species, which we suggest be named *Candidatus* E. hainanensis. All sequences have been submitted to GenBank (MT875365–MT875373).

Rodents are the main reservoirs of many human pathogens, and they play key roles in the transmission of zoonotic diseases, such as hemorrhagic fever with renal syndrome, Lassa fever and rickettsiosis. In this study, rodents including *R. tanezumi*, *N. fulvescens*, *Chiromyscus* sp., *R. nitidus* and *L. edwardsi* captured from Hainan Province were detected for the presence of *Ehrlichia* through hemi-nested PCR targeting the 16S rRNA genes. Subsequently, a novel *Ehrlichia* species genetically most closely associated with *Ehrlichia* sp. strain Tibet was identified from *N. fulvescens* samples belonging to the genus *Niviventer*. The family *Muridae* is widespread in mountain and forest areas in Southern China. Although rodents have been well recognized as the reservoir of Rickettsiales bacteria, very few studies on Rickettsiales bacterial pathogens from *Niviventer* have been performed to date. This is one of the few *Ehrlichia* species identified and characterized from *Niviventer* rodents. Although *N. fulvescens* are mainly distributed in mountain and forest areas, ongoing urbanization and deforestation provide increasing opportunities for rodents to contact humans. The high *N. fulvescens* positivity rate (7.46%) may suggest the potential risk of human infection. Furthermore, as a reservoir of *Ehrlichia* bacteria, *N. fulvescens* might be involved in transmission cycles of *Candidatus* E. hainanensis in nature, because free-living rodents are common hosts of ticks and have the potential to transmit *Ehrlichia* bacteria to ticks. Whether ticks act as vectors in the cycle remains to be determined; if so, the exposure risk to humans may be further increased.

In this study, we obtained the partial sequence of the 16S rRNA gene (1,256 bp), the *groEL* gene (1,116 bp) and a *gltA* gene fragment (970 bp) from three *Ehrlichia* strains. In all three phylogenetic trees, the *Candidatus* E. hainanensis significantly differed from other *Ehrlichia* bacteria and formed a distinct clade.

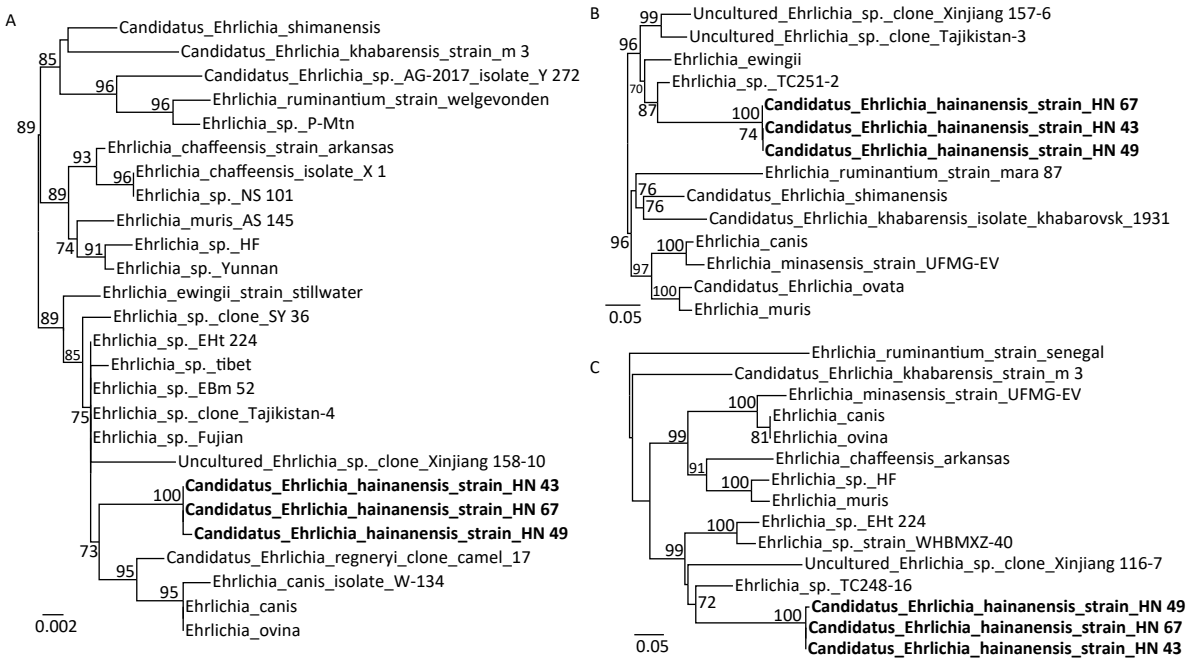

Figure 1. Phylogenetic tree based on the nucleotide sequences of *Candidatus* Ehrlichia hainanensis 16S rRNA (A), *groEL* (B) and *gltA* (C) genes, as well as those obtained from GenBank.

Genetic analysis indicated that the highest similarity of nucleotide sequences between *Candidatus* E. hainanensis and other *Ehrlichia* species was 99.20% for 16S rRNA, 89.87% for the *groEL* gene and 83.86% for the *gltA* gene. These results of molecular-genetic analysis sufficiently supported that *Candidatus* E. hainanensis is a putative new *Ehrlichia* species.

This study provides a better understanding of the genetic diversity of *Ehrlichia* species in rodents from Southern China. *Ehrlichia* is a group of endosymbiotic bacteria that infect humans and animals. *E. chaffeensis* is currently the most prevalent *Ehrlichia* infecting humans. Human monocytic ehrlichiosis cases caused by *E. chaffeensis* have been frequently reported in China. Furthermore, *E. ruminantium* as well as several closely related *Ehrlichia* species, *E. ewingii*, *E. canis* and *E. muris-like* agent have also been demonstrated to infect humans[2-5,10]. Although other *Ehrlichia* species have been characterized from ticks or animals in recent years, few studies have been performed on their pathogenicity to humans. In this study, owing to the distant relationship between *Candidatus* E. hainanensis and other *Ehrlichia* species, it is difficult to speculate whether this species might infect humans, and its pathogenicity to humans remains to be further researched in this area.

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Supplementary Table S1. Genbank numbers of bacterial sequences used in phylogenetic analysis

No.	Gene	Genbank number	Bacterial strain
1	16S	AY135531.1	Ehrlichia sp. 'Rattus strain'
2	16S	AY098730.1	Ehrlichia sp. Belluno
3	16S	KR063138.1	Candidatus Ehrlichia khabarensis strain m3
4	16S	AB074459.1	Candidatus Ehrlichia shimanensis
5	16S	KY425523.1	Candidatus Ehrlichia sp. AG-2017 isolate Y272
6	16S	NR074513.2	Ehrlichia ruminantium strain Welgevonden
7	16S	DQ324367.1	Ehrlichia sp. P-Mtn
8	16S	CP000236.1	Ehrlichia chaffeensis str. Arkansas
9	16S	KX505292.1	Ehrlichia chaffeensis isolate X1
10	16S	AB454074.2	Ehrlichia sp. NS101
11	16S	NR121714.1	Ehrlichia muris AS145
12	16S	CP007474.1	Ehrlichia sp. HF
13	16S	GU227701.1	Ehrlichia sp. Yunnan
14	16S	NR044747.1	Ehrlichia ewingii strain Stillwater
15	16S	KF728345.1	Uncultured Ehrlichia sp. clone SY36
16	16S	AF311968.1	Ehrlichia sp. EHt224
17	16S	AF414399.1	Ehrlichia sp. Tibet
18	16S	AF497581.1	Ehrlichia sp. EBm52
19	16S	KM995821.1	Uncultured Ehrlichia sp. clone Tajikistan-4
20	16S	DQ324547.1	Ehrlichia sp. Fujian
21	16S	JX402605.1	Uncultured Ehrlichia sp. clone Xinjiang158-10
22	16S	KF843826.1	Candidatus Ehrlichia regneryi clone Camel_17
23	16S	AF318946.1	Ehrlichia ovina
24	16S	U26740.1	Ehrlichia canis
25	16S	AB723708.1	Ehrlichia canis isolate W-134
26	gltA	DQ513396.1	Ehrlichia ruminantium strain Senega
27	gltA	KR063140.1	Candidatus Ehrlichia khabarensis strain m3
28	gltA	JX629807.1	Ehrlichia minasensis strain UFMG-EV
29	gltA	AY647155.1	Ehrlichia canis
30	gltA	KP719095.1	Ehrlichia ovina isolate T2002
31	gltA	AF304142.1	Ehrlichia chaffeensis Arkansas
32	gltA	DQ647319.1	Ehrlichia sp. HF
33	gltA	MN685601.1	Ehrlichia muris
34	gltA	AF311966.1	Ehrlichia sp. EHt224

Continued

No.	Gene	Genbank number	Bacterial strain
35	gltA	KX987356.1	Ehrlichia sp. strain WHBMXZ-40
36	gltA	JX402606.1	Uncultured Ehrlichia sp. clone Xinjiang116-7
37	gltA	KJ410275.1	Ehrlichia sp. TC248-16
38	groEL	KJ930193.1	Uncultured Ehrlichia sp. clone Tajikistan-3
39	groEL	JX402613.1	Uncultured Ehrlichia sp. clone Xinjiang157-6
40	groEL	KY705065.1	Ehrlichia sp. isolate YNT
41	groEL	AF195273.1	Ehrlichia ewingii
42	groEL	KJ410296.1	Ehrlichia sp. TC251-2
43	groEL	DQ647010.1	Ehrlichia ruminantium strain Mara87/7
44	groEL	AB074462.1	Candidatus Ehrlichia shimanensis
45	groEL	FJ966353.1	Candidatus Ehrlichia khabarensis isolate Khabarovsk 1931
46	groEL	U96731.1	Ehrlichia canis
47	groEL	JX629806.1	Ehrlichia minasensis strain UFMG-EV
48	groEL	DQ672553.1	Candidatus Ehrlichia ovata
49	groEL	KU214846.1	Ehrlichia sp. EMLA
50	groEL	MN685610.1	Ehrlichia muris