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Molecular detection and identification of "*Candidatus* Ehrlichia hainanensis", a novel *Ehrlichia* species in rodents from Hainan province, China

Peipei Liu
Chinese Center for Disease Control and Prevention
Hongqing Zhao
National Institute for Communicable Disease Control and Prevention
Feng Xue
Zhejiang Mariculture Research Institute
Yunfeng Gao
Yuhe National Nature Reserve
Miao Lu
National Institute for Communicable Disease Control and Prevention
Kun Li (ecoli1984@sina.cn)
National Institute for Communicable Disease Control and Prevention

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Abstract

Background: *Ehrlichia* is a genus of Rickettsiales bacteria closely related to human/animal disease and it plays an important role in public health. Naturally they are hosted by mammals such as canines, bovines, wild rodents and vectored by ticks.

Results: In this study, we collected 121 rodent samples including 67 *Niviventer fulvescens*, 27 *Rattus tanezumi*, 24 *Chiromyscus* sp., 2 *Rattus nitidus* and 1 *Leopoldamys edwardsi* from Hainan province, which consists of the second largest island in China. The presence and genetic diversity of *Ehrlichia* was evaluated and characterized by amplifying and sequencing the 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 show highest 99.20%, 89.87% and 83.86% identity to known *Ehrlichia* sequences. In the phylogenetic trees they form a distinct cluster from any other species.

Conclusions: We propose that this species present a putative novel *Ehrlichia* species nominated as '*Candidatus* Ehrlichia hainanensis'. Its pathogenicity to human is still to be further researched and molecular surveillance in local populations is needed.

Background

Ehrlichia is a genus of obligatory intracellular bacteria belonging to family Anaplasmataceae, order Rickettsiales. Until today, six formally recognized *Ehrlichia* species [1] and some Candidatus species have been reported [2-6]. They are mainly vectored by ticks and many of them are pathogenic to human or animals. *E. canis*, *E. ruminantium* and *E. muris* are pathogens for canines, ruminants and mice, respectively [7-9]. *E. chaffeensis*, *E. ewingii*, *E. muris-like* agent and *E. canis* have been reported to infect human and cause syndromes ranging from febrile to severe multiple organ failure [10-14]. The common symptoms of human ehrlichiosis are fever, headache, myalgia, malaise, weakness, nausea, leukopenia, vomiting, diarrhea, and abdominal pain [15]. These uncharacteristic symptoms cause difficulties in diagnosis and may lead to misdiagnosis clinically. In China, multiple *Ehrlichia* species have been identified including *E. chaffeensis*, *E. canis*, *E. ruminantium*, etc, from ticks, mammals, mosquitoes and leeches [16-19] Until today, *E. chaffeensis* is the only known human *Ehrlichial* pathogen in China. Human granulocytic ehrlichiosis (HGE) disease caused by *E. chaffeensis* has been proved to be widespread in several provinces of China [16, 20-21]. Rural residents, especially farmers, are at substantially increased risk of *Ehrlichia* exposure [22].

Small mammals such as rodents have been proved to be the reservoirs of many *Ehrlichia* species. *Ehrlichia* sp. HF group has been identified in *Apodemus argenteus*, *A. speciosus*, *Eothenomys smithi* and *Myodes rufocanus bedfordiae* [23-25]. *Candidatus* E. khabarensis was identified and characterized from *Myodes rutilus*, *M. rufocanus* and *Sorex araneus* in Russian Far East [26]. *E. muris* was first isolated from the tissue of a wild mouse (*Eothenomys kageus*) in Japan [27], while a human pathogenic *E. muris*-like agent was identified from *Peromyscus leucopus* in US [28]. In the host-vector ecosystem, these *Ehrlichia*

bacteria can be horizontally transmitted to ticks that infesting on the hosts, meanwhile, spillover into human populations may also occasionally occur. However, it is still unclear whether they pose a threat to public health. The solid evidence of their involvement in the human ehrlichiosis is still to be further studied.

Although the HGE has been common in China, the geographical distribution and genetic diversity of various *Ehrlichia* species have not yet been well studied. In Hainan Province, the investigations and studies on *Ehrlichia* have been very few [29]. Hainan province consists of the second largest island of China locating in Southern China Sea. It has an area 33, 900 square kilometers and a population of about 9.34 million. Owing to the tropical climate and outstanding landscape, it is becoming a great tourist attraction and is receiving tens of millions of tourists each year. For better understanding on the *Ehrlichia* distribution and its potential risk to public health in Hainan Province, we examined the molecular evidence of *Ehrlichia* in wild rodents and a novel *Ehrlichia* species was characterized in this study.

Methods

Sample collection and treatment

From November to December 2019, rodents were trapped by cages using bait. In total, 121 rodents were captured in Qiongzhong autonomous county, Hainan province. All animals were captured alive, and then anesthetized and euthanized to minimize suffering. The tissue samples were collected and stored in RNAlater. After washing twice with PBS, the liver samples were subjected to total DNA extraction using DNA/RNA isolation kit (Omega Bio-Tek, USA) according to the manufacturer's instructions. The extracted DNA was stored at -20◦C prior to the species identification and *Ehrlichia* detection. The rodent species were identified by sophisticated field biologists and then confirmed by sequencing the cytochrome oxidase subunit 1 (COI) gene.

Ehrlichia detection and key genes amplification

Ehrlichia was detected using hemi-nested PCR primers targeting a conserved region of 16S rRNA gene as described [18]. The length of PCR fragments was 546 bp. Amplification parameters for both the first and second PCR included 94◦C×3min, followed by 40 cycles of: 94◦C×30 s, 49◦C×40 s, and 72◦C×45 s.

For better phylogenetic analysis, a 16S rRNA fragment of 1256 bp was obtained from the positive samples using primers as previously reported (18). The heat shock protein gene (*groEL*) fragments of 1116 bp in length were amplified using hemi-nested PCR primers [30]. The 970 bp citrate synthase gene (*gltA*) fragment was amplified using primers as described [30]. The PCR reactions were performed using Sensoquest PCR System LabCycler Standard P (Germany). PCR amplicons were analyzed by electrophoresis in 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 pMD19-T cloning vector (TaKaRa), transformed into *E. coli* and plated onto culture dish. The obtained clones were picked and sent for Sanger sequencing.

Phylogenetic analysis

Ehrlichia sequences (16S rRNA, *groEL* and *gltA* sequences) obtained in this study, as well as those retrieved from GenBank (Table S1), were aligned using the Clustal W method implemented in the MEGA program, version 6.0 [31]. Nucleotide sequence identities were calculated by MegAlign program available within the DNASTAR Lasergene package, version 7.0 (DNASTAR, Inc., Madison, WI).

Phylogenetic trees of basing on these three genes were constructed using the maximum likelihood (ML) method in the PhyML v3.0 package [32] based on the best-fit GTR+I+Γ4 model of nucleotide substitution as determined by jModeltest. Bootstrap values higher than 70% were considered significant. The best-fit evolutional model was determined using jModel Test version.

Results

Detection of *Ehrlichia* in rodents and amplification of key genes

During 2019, a total of 121 rodents were captured in Qiongzhong autonomous county, Hainan province, Southern China (Fig. 1). These rodents represent 5 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 *Ehrlichial* DNA in 121 liver tissue samples. Consequently, PCR products of the expected size (546 nt) were recovered from 5 *N. fulvescens* (5/67, 7.46%). The result was confirmed by DNA sequencing. No positive result was detected from other rodent samples.

Genetic and phylogenetic analysis

Partial 16S rRNA (1256 bp), *groEL* (1116 bp) and *gltA* (970 bp) sequences were successfully obtained from 3 randomly selected samples. Basing on BLAST and the MegAlign analysis, the 16S gene sequences of the 3 strains have a nucleotide identity of 99.92-100% to each other, and they show highest 99.20% nucleotide identity with the *Ehrlichia* sp. EHt224 characterized from *Hyalomma truncatum* in Niger (AF311968.1) [33] and *Ehrlichia* sp. EBm52 from *Rhipicephalus microplus* in Thailand (AF497581.1) [34], respectively. The *gltA* gene shows highest 83.86% nucleotide identity to those from *Ehrlichia* sp. TC248-16 reported in Xinjiang [35], while highest 89.87% nucleotide identity with *Ehrlichia* sp. TC248-16 was identified for the *groEL* gene. In all the 3 phylogenetic trees, the sequences obtained in this study form a distinct cluster far from any other *Ehrlichia* species. We propose that it represents a novel species and we nominate it as *Candidatus* E. hainanensis. All the sequences have been submitted to Genbank (Accession numbers: MT875365-MT875373).

Discussion

Rodents are the main reservoirs of a great many of human pathogens, and they play a key role in the transmission of zoonotic diseases such as hemorrhagic fever with renal syndrome (HFRS) [36], Lassa fever [37], rickettsiosis [38], etc. In this study, rodents including *R. tanezumi*, *N. fulvescens*, *Chiromyscus*

sp., *R. nitidus* and *L. edwardsi* were captured from Hainan province were detected for the presence of *Ehrlichia* using hemi-nested PCR targeting the the 16S rRNA genes. Subsequently, a novel *Ehrlichia* species which is most closely related to *Ehrlichia* sp. strain Tibet genetically was identified from *N. fulvescens* samples. *N. fulvescens* belonging to genus *Niviventer*, family *Muridae* is a widespread rodent in the mountain and forest area, Southern China. Although rodents have been well recognized as the reservoir of Rickettsiales bacteria, studies on Rickettsiales bacterial pathogens from *Niviventer* have been very few until now. This might be the first report of *Ehrlichia* species identified and characterized from *Niviventer* rodents. Although *N. fulvescens* are mainly distributed in mountain and forest areas, the ongoing urbanization and deforestation makes more opportunities for rodents to contact human. The high positive rate (7.46%) in *N. fulvescens* may suggest the potential risk of infecting human. Besides, as a reservoir of *Ehrlichia* bacteria, the *N. fulvescens* might be involved in transmission cycles of *Candidatus* E. hainanensis in the nature because free-living rodents are common hosts of ticks and they have the potential to transmit the endosymbiotic bacteria to ticks. It is still to be determined that whether ticks act as vectors in the cycle, which may further increase the exposure risk of human.

In this study, we obtained the partial sequence of the 16S rRNA gene (1256 bp), the *groEL* gene (1116 bp), and the sequence of a *gltA* gene fragment (970 bp) from three *Ehrlichia* strains. In all the three phylogenetic trees, the *Candidatus* E. hainanensis significantly differ from other *Ehrlichia* bacteria and form a distinct clade. Genetic analysis indicates that the highest similarity of nucleotide sequences between *Candidatus* E. hainanensis and other *Ehrlichia* species is 99.20% for the 16S rRNA, 89.87% for the *groEL* gene and 83.86% for the *gltA* gene. These results of molecular-genetic analysis sufficiently support our proposition that *Candidatus* E. hainanensis can represent a putative new *Ehrlichia* species.

This study provided a better understanding of the genetic diversity of *Ehrlichia* species in rodents from Southern China. *Ehrlichia* is a group of endosymbiotic bacteria closely related to human and animal diseases [39]. Until today, *E. chaffeensis* is the most prevalent *Ehrlichia* infecting human. HGE cases caused by *E. chaffeensis* have been frequently reported in China [16, 20-21]. Besides, *E. ruminantium* as well as several closely related *Ehrlichia* species, *E. ewingii*, *E. canis* and *E. muris-like* agent have also been proved to infect human [10-14, 40]. Although some *Ehrlichia* species were characterized from ticks or vertebrate animals in recent years [6, 41-45], few studies have been carried out on their pathogenicity to human beings. In this study, due to the far relationship between *Candidatus* E. hainanensis and other *Ehrlichia* species, it is difficult to speculate whether it may infect human or not. Its pathogenicity to human beings is still to be further researched in this area.

Conclusions

This study discovered a novel *Ehrlichia* species in *N. fulvescens* from Hainan Island and we named it as *Candidatus* E. hainanensis. Its pathogenicity is to be studied and the high positive rate (7.46%) may suggest a potential threat to public health.

Abbreviations

Human granulocytic ehrlichiosis: HGE, maximum likelihood: ML.

Declarations

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article or uploaded to GenBank.

Authors' contributions

KL conceived the study. PL and HZ performed the molecular identification of pathogens and wrote the first draft of the manuscript. FX and YG worked in fields and collected the samples. ML performed the phylogenetic analysis. KL reviewed the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interest.

Consent for publication

Not applicable.

Ethics approval

This study was reviewed and approved by the ethics committee of the National Institute for Communicable Disease Control and Prevention of the Chinese CDC.

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Figures

Figure 1

A map of Qiongzhong autonomous county, Hainan province, showing the location where the rodents were collected. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

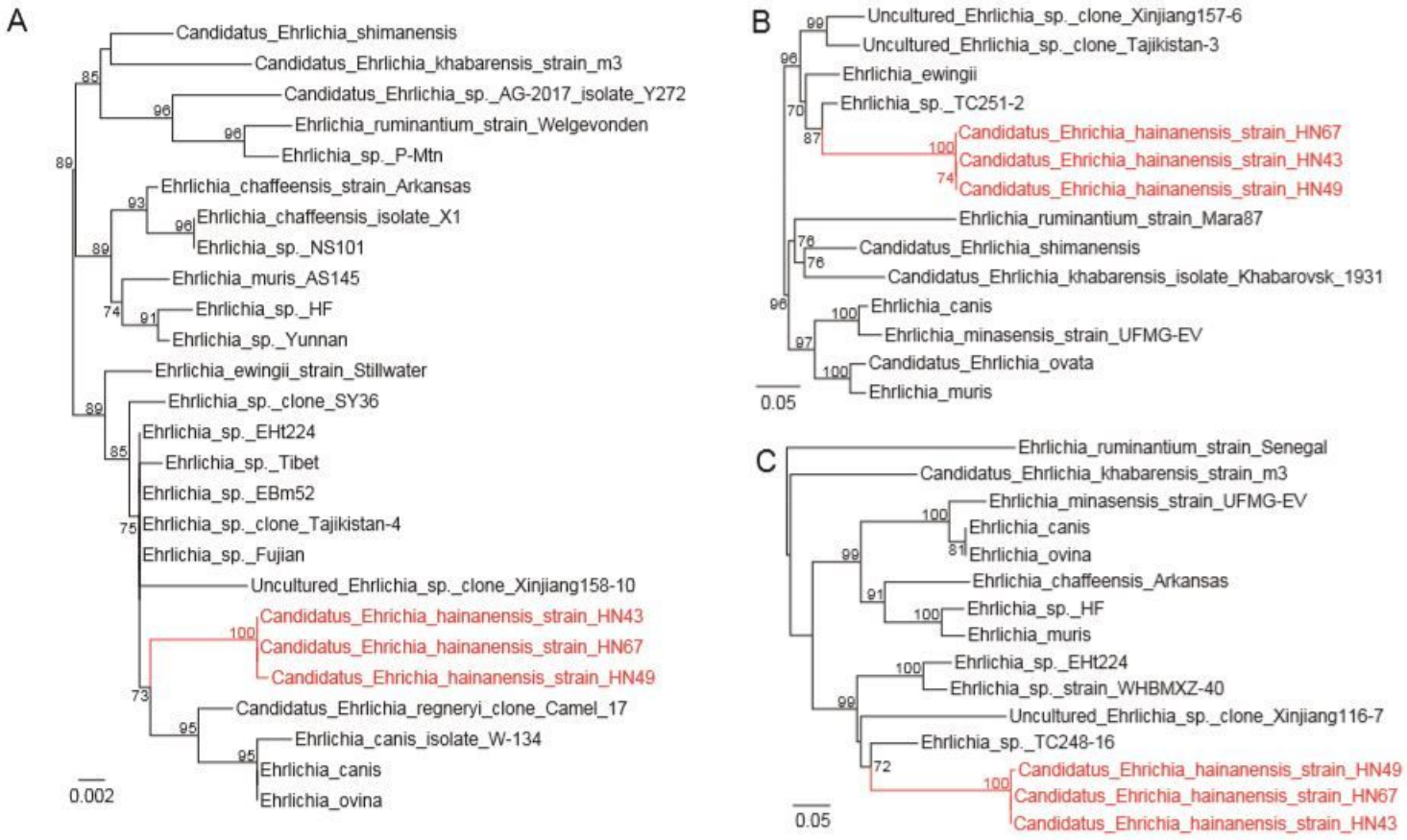

Figure 2

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

Supplementary Files

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- TableS1.doc