Detection of a Novel *Ehrlichia* Species in *Haemaphysalis longicornis* Tick from China

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Abstract

We collected 2460 *Haemaphysalis longicornis* ticks from vegetation in Jiaonan County, Shandong Province, in June of 2013 and Daishan County, Zhejiang Province, China, in May of 2015. The tick DNA was subsequently amplified with nested polymerase chain reaction using *Ehrlichia* common 16S rRNA gene primers and *Ehrlichia ewingii* species-specific *groEL* and *gltA* primers. We found 0.4% (3/780) of the ticks from Zhejiang Province contained *Ehrlichia* DNA that was different from all known *Ehrlichia* species, but most closely related to *E. ewingii*. We concluded that a novel *Ehrlichia* species exists in *H. longicornis* ticks in China.

Key Words: *Ehrlichia*—*Haemaphysalis longicornis*—Tick-borne diseases—China.

Introduction

ERLICHIA ARE TICK-BORNE obligatory intracellular bacteria, which infect humans and animals. The currently known *Ehrlichia* species include *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia muris*, and *Ehrlichia ruminantium*. All species of *Ehrlichia* have been reported to cause human infection. *E. chaffeensis* and *E. canis* cause monocytic infection (Buller et al. 1999, Anderson et al. 1992, Dawson et al. 1991), *E. ewingii* causes neutrophil infection (Anderson et al. 1992, Dawson et al. 1991, Perez et al. 2006, Buller et al. 1999), and the target cell of *E. muris* in humans has not been identified. Symptoms of *Ehrlichia* infection might include fever, headache, myalgia, progressive leukopenia, thrombocytopenia, and anemia. The illness occurs during the spring and summer months when the ticks are active. *Ehrlichia* species were now recognized as significant agents of emerging human zoonoses worldwide. *Ehrlichia* infection has been reported in Europe, Asia, Africa, and the United States (Magnarelli and Anderson 1993, Brouqui et al. 1994, Heppner et al. 1997, Petrovec et al. 1997, Ndip et al. 2009). *Ehrlichia* species are transmitted through the bite of an infected nymphal or adult tick vector that had been previ-

ously infected in the larval or nymphal stage while feeding on *Ehrlichia*-infected animals (usually wildlife) known as a reservoir host (Nicholson et al. 2010). Disease distribution in humans and animals correlates with the distribution of those vector ticks. In China, the distribution and species of *Ehrlichia* are not clear across a massive land and different climates. A few studies reported that *E. chaffeensis* and *E. canis* exist in animals and ticks in China (Cao et al. 2000, Pan et al. 2000, Dong et al. 2013). In this study, we used ticks as sentinel species to detect the risk of *Ehrlichia* infection to humans in China.

Material and Method

Study sites

Ticks were collected from Jiaonan County of Shandong Province (119°30'–120°11' E, 35°35'–36°8'N) and Daishan County of Zhejiang Province (121°31'–123°17' E, 30°07'–30°38'N) (Fig. 1). Both sites are in East China with Jiaonan County in the north and Daishan County in the south, and the distance between the two areas is ~1000 KM. Jiaonan County is located on the coast of the Yellow Sea, and Daishan County is located on islands in the East China Sea. The

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FIG. 1. Map of China. Stars indicate tick collection sites: Jiaonan County in Shandong Province and Daishan County in Zhejiang Province.

climate of the two sites is the maritime monsoon type with four distinct seasons. The annual average temperature is below 12.1°C and 16.2°C, and the average rainfall is 798 mm and 1400 mm, respectively, in the two sites.

Tick collection

We collected 2460 *Haemaphysalis longicornis* ticks by flagging from vegetation in Jiaonan County, Shandong Province, in June of 2013 and in Daishan County, Zhejiang Province, in May of 2015. The ticks were classified morphologically and molecularly as described previously (Luo et al. 2015). The ticks were frozen at -80°C until DNA extraction.

DNA preparation and polymerase chain reaction amplification of *Ehrlichia* DNA

Total tick nucleic acids were extracted simultaneously by using the AllPrep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Ticks were pooled with each pool consisting of 50 larvae, 20 nymphs, or 5 adult ticks and were homogenized using metal beads (Tissue Lyser; Qiagen)

in the RLT buffer (Qiagen). Tick DNA was used as template for polymerase chain reaction (PCR) amplification of *ehrlichia* DNA. PCR primers for the 16S rRNA gene and amplification conditions were described previously (Rar et al. 2005, Kim et al. 2013). PCR primers for *groEL* and *gltA* gene were designed using *E. ewingii* *groEL* and *gltA* genes as templates in this study. The sequences of all primers are in Table 1. No *Ehrlichia* DNA was used as positive control, and distilled water was used as negative control. The amplification cycles of outer primers for each gene were DNA denaturation step at 95°C for 5 min, followed by 35 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C, and a final extension step of 7 min at 72°C. The PCR protocol for the inner primers was a DNA denaturation step at 95°C for 5 min, followed by 35 cycles for 1 min at 95°C, 1 min at 56°C, and 45 s at 72°C, and a final extension step of 7 min at 72°C.

PCR was performed with the Taq DNA polymerase reagent kit (Promega). Negative control with sterilized distilled water was run simultaneously. The amplified DNA was separated by electrophoresis in a 1.5% agarose gel and visualized under UV light. The desired band was purified from the gel using a Gel Extraction Kit (Qiagen). The purified PCR product was ligated into the pMD 19-T vector (Takara Bio, Inc.) according to the manufacturer's instructions. Positive clones were sequenced on both strands.

Phylogenetic analysis

The sequences of the PCR products were aligned with sequences in GenBank using BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic trees were constructed with the neighbor-joining method in MEGA5 (Tamura et al. 2007, Tamura et al. 2011). The robustness of the trees was tested with 1000 bootstrap replications.

Result

Tick collection

A total of 2460 ticks collected in Jiaonan and Daishan counties were used in this study, and the number of ticks of each developmental stage at each site is listed in Table 2. All ticks were identified as *H. longicornis*.

TABLE 1. NESTED PCR PRIMERS FOR *EHRLICHIA* 16S rRNA GENE, *GLTA*, AND *GROEL*

Name	Target gene	Sequence 5' → 3'	Product size (bp)
EHR1-out	16S rRNA	GAACGAACGCTGGCGGCAAGC	691
EHR2-out		AGTA[T/C]CG[A/G]ACCAGATAGCCGC	
EHR3-in		TGCATAGGAATCTACCTAGTAG	
EHR4-in	<i>gltA</i>	CTAGGAATTCCGCTATCCTCT	524
5GltA-out		GGCATTTCCTGATGTGCATGAT	
3GltA-out		ATACCATTGAGCCGACCAGCC	
5GltA-in	<i>groEL</i>	AGCAGTGTCTCAAATTGCAGG	426
3GltA-in		ATCCTATGGCCAAAACCCATTA	
5GroEL-out		GTACGGCTGGACCTAAAGGA	
3GroEL-out		AGTGCTGAGAGCTTCACCTTC	701
5GroEL in		ATGGGGCACCAGAAGTTACA	
3GroEL -in		CCACGATCAAATTGCATACCATCA	
			422

in, inside primer; out, outside primer; PCR, polymerase chain reaction.

TABLE 2. *HAEMAPHYSALIS LONGICORNIS* TICKS FROM JIAONAN AND DAISHAN COUNTIES OF CHINA

Study site	Larvae	Nymph	Adult	Total
Daishan	0	630	150	780
Jiaonan	120	1200	360	1680
Total	120	1830	510	2460

Detection of Ehrlichia DNA in ticks

With *Ehrlichia* species common 16S rRNA gene primers, *Ehrlichia* DNA was detected in three pools of nymphal ticks from Daishan County of Zhejiang Province, but none of the tick pools from Shandong Province was positive by PCR amplification. Assuming that a positive pool of ticks contained one infected tick, the minimal *Ehrlichia* infection rate

of ticks from Zhejiang Province was 0.4% (3/780). DNA sequence analysis revealed that the sequence from the ticks was 98.3–99.8% homologous to the 16S rRNA genes of *Ehrlichia* species, with the highest homology to *E. ewingii* (99.8%).

We designed primers from the *groEL* and *gltA* of *E. ewingii* to amplify these genes from the tick pools that contained the ehrlichial 16S rRNA gene by nested PCR. Fragments of the *groEL* and *gltA* genes were amplified from all three pools of ticks that contained ehrlichial 16S rRNA gene DNA sequences revealing that the homology of the *Ehrlichia* species from the Chinese ticks to the known *Ehrlichia* species was 89.6–93.8% for the *groEL* gene and 68.5–90.4% for the *gltA* gene. Again, *E. ewingii* is the closest species related to the *Ehrlichia* species from the ticks with both *groEL* and *gltA* genes.

The phylogenetic analysis based on 16S rRNA gene sequences showed that the *Ehrlichia* species from ticks were

FIG. 2. Phylogenetic analysis of *Ehrlichia* species. The trees were constructed using the 16S rRNA gene (A), *groEL* gene (B), *gltA* gene (C), and the concatenated sequences of the 16S rRNA gene, *gltA*, and *groEL* of each *Ehrlichia* species (D). The number in each line was GenBank accession number for each sequence in (A–C). The GenBank accession numbers (in the order of 16S rRNA gene, *groEL*, and *gltA*) for each *Ehrlichia* species in (D) are the following: Daishan *Ehrlichia* (KT886409, KT886408, KT886407), *E. chaffeensis* (AF416764, KJ907753, AF304142), *Ehrlichia muris* (GU358691, CP006917, CP006917), *Ehrlichia canis* (KJ513194, JN391408, AY647155), *Candidatus E. khabarensis* (KR063138, KR063139, KR063140), *Ehrlichia ewingii* (NR 044747, KJ907744, DQ365879), *Ehrlichia ruminantium* (NR 044831, CR925677, DQ513396), and *Anaplasma phagocytophilum* (HM366586, KC800986, AY464138). Numbers at nodes represented bootstrap values. Scale bar represented nucleotide substitutions per site. Dot represented the new *Ehrlichia* species from ticks collected in vegetation of Daishan County of Zhejiang Province. The trees were rooted with the sequences of the corresponding genes of *A. phagocytophilum*.

tightly clustered together with uncultured *Ehrlichia* species from Xinjiang Province, China, and *E. ewingii*, but were distantly related to other species of *Ehrlichia* (Fig. 2A). The sequences of *gltA* and *groEL* of *Ehrlichia* species from the ticks also clustered together with the uncultured *Ehrlichia* species from Xinjiang Province, China, and *E. ewingii*, and distantly clustered with other species of *Ehrlichia* (Fig. 2B, C). The phylogenetic analysis using concatenated sequences of 16S rRNA gene, *groEL* gene, and *gltA* gene was consistent with the results of phylogenetic analysis using each individual gene, which showed that *Ehrlichia* species from Daishan tick formed a monophyletic group with *E. ewingii*, but not other *Ehrlichia* species (Fig. 2D).

The sequence of each gene from all three pools of ticks from Daishan was identical, and only one sequence for each gene from the ticks was deposited in GenBank (Accession numbers: KT886407–9).

Discussion

In this study, we identified an *Ehrlichia* species in *H. longicornis* tick collected from southern China, most closely related to *E. ewingii*. However, despite the high homology of the 16S rRNA gene between the *Ehrlichia* organism in *H. longicornis* tick and *E. ewingii*, the *groEL* and *gltA* sequences of the *Ehrlichia* organism in *H. longicornis* tick were dramatically different from *E. ewingii*. The classification of the new species of *Ehrlichia* from this study needs to be further studied by isolation of the organism and comparison of complete genomes.

E. ewingii has been reported to infect humans, dogs, and deer and causes granulocytic ehrlichiosis in humans and dogs (Anderson et al. 1992, Breitschwerdt et al. 1998, Buller et al. 1999, Yabsley et al. 2002, Liddell et al. 2003). *E. ewingii* was reported only in the United States until recently when it was discovered to infect dogs in Cameroon (Ndip et al. 2005) and in Brazil (Oliveira et al. 2009). *E. ewingii* has been reported to be carried by *Amblyomma americanum* and *Dermacentor variabilis* ticks in the United States (Wolf et al. 2000, Steiert and Gilfooy 2002).

In China, *E. chaffeensis* has been detected from ticks, including *Amblyomma testudinarium*, *H. yengi*, *D. silvarum* ticks collected from cattle, dogs, wild rats, and wild mice (Cao et al. 2000, Dong et al. 2013); *E. canis* was detected in *Rhipicephalus sanguineus* and *Rhipicephalus microplus* (formerly *Boophilus microplus*), and canine ehrlichiosis was found in southern China (Pan et al. 2000). *E. ewingii* had not been found previously in China. A new species of *Ehrlichia* was detected in *H. longicornis* in this study, and *H. longicornis* is the major tick species in East China, which has attracted more attention in recent years because it carries a novel deadly bunyavirus—severe fever with thrombocytopenia virus (SFTSV) (Yu et al. 2011). *H. longicornis* has been reported to carry several human pathogens, including bunyavirus, SFTSV (Luo et al. 2015), *Rickettsia japonica* (Uchida et al. 1995), and *Anaplasma capra* (Sun, et al. 2015) and *Anaplasma phagocytophilum* (Kim et al. 2003). In China, farmers are encouraged by local governments to breed domestic animals, such as sheep, goats, and cattle, which have dramatically promoted tick population growth, especially *H. longicornis* because it primarily feeds on domestic animals. *Ehrlichia* infection in humans has not been investigated in

China. Infection with *Ehrlichia* generally results in mild-to-severe febrile disease in humans (Buller et al. 1999). Further investigation on human infection with *Ehrlichia* pathogens should be carried out in China.

We detected the novel species in ticks collected from Zhejiang Province in southern China, but not from ticks from Shandong Province in northern China. However, this does not suggest that this *Ehrlichia* does not exist in northern China. The minimal infection rate of the ticks was based on PCR targeting the 16S rRNA gene common primers for *Ehrlichia*, which is not optimized for sensitivity; therefore, we cannot guarantee to detect the novel species of *Ehrlichia* from all investigated ticks, which may underestimate the infection rate of ticks with the novel *Ehrlichia* species.

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Author Disclosure Statement

No competing financial interests exist.

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