Ticks and tick-borne Anaplasma spp. and Rickettsia spp. from hilly area in central China

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Research

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Abstract

Background

Ticks are widely distributed in diverse habitats across the globe. Different tick habitats breed different tick varieties and tick-borne diseases. Pingdingshan City, located in central China, is a hilly area where livestock grazing is an everyday activity. Till now, the prevalence of tick-borne Rickettsiales around Pingdingshan City has not yet been explored.

Methods

We used PCR and nest-PCR detected the 16S rRNA gene of ticks, 16S rRNA gene of Anaplasma spp. and 17 KDa gene and gltA gene of Rickettsia spp. in the ticks collected from livestock and vegetation in hilly area of central China, Pingdingshan City.

Results

In this study, we observed a high infection rate of a dominant tick, H. longicorni (98.22%, 166/169), with Anaplasma spp. (48.8%), Rickettsia spp. (30.72%), and co-infection (9.04%, 15/166). It suggests a high public health risk. 16S rRNA sequencing revealed that the A. phagocytophilum, which is present in H. longicorni was more than 99.74% homologous to (MN044900.1) from Zhejiang and (KT276565.1) from Shandong in China, and more than 98.23% homologous to (KR611598.1, KC422267.1) from South Korea. Besides, A. central showed 100% homology to (GU064903.1) from South Korea. Ca. R. jingxinese was detected from 17 kDa gene amplification, and it showed 99.08%-99.77% homology to (MH932039.1) from Xian, China, and 100% homology to Candidatus R. jingxinensis from north-eastern China (KT899089.1).

Conclusions

In the hilly area of central China, a high prevalence of H. longicorni, and high infection rate of Anaplasma spp. and Rickettsia spp., even co-infection, necessitated the current study. Furthermore, an in-depth investigation is required to unravel its pathogenicity. Besides, other tick-borne disease-causing pathogens in H. longicorni, which pose a significant threat to humans and livestock, demand further assessment.

Introduction

The recent decade has witnessed a steep rise in infectious disease cases across the globe. A majority of human pathogens are transmitted through animals. As livestock are present in close vicinity to humans, they spread pathogens more quickly as compared to wild animals. Usually, in developing countries, people lack awareness about livestock or vector transmitted diseases. Vectors, such as ticks, play a crucial role in wildlife and livestock mediated transmission of pathogens to humans [1].

Ticks (Acari: Ixodidae) are notorious sanguisuge ectoparasites of humans, domestic, and a wide range of wild animals. Till now, more than two hundred tick-borne pathogens have been identified, which includes viruses, bacteria, protozoa, and helminths [2]. The distribution of ticks within a specific habitat depends on several
environmental and climatic factors, such as annual rainfall, atmospheric temperature and relative humidity (RH), vegetation cover, altitude, and host availability [3]. In the hilly area of central China, climatic conditions are hot and rainy in summer, dry and cold in winter, clear in spring and autumn. Due to undulating terrains, this area is inapt for crop farming, and so, in this area, livestock grazing is commonly exercised. Hence, in the hilly area of central China, ticks and tick-borne diseases have a specific pattern, which affects human health.

However, the epidemiological investigations on ticks and tick-borne infectious agents such as Anaplasma spp. and Rickettsia spp.[4] have not been investigated so far in Pingdingshan City, the hilly area in central China. In this study, we collected ticks from livestock and vegetation from six counties of the Pingdingshan City, identified the tick species, and investigated the prevalence of Anaplasma spp. and Rickettsia spp. through 16S rRNA gene sequencing for ticks and Anaplasma spp., and 17 kDa and gltA gene sequencing for Rickettsia spp.

**Material And Methods**

**Ticks collection**

Ticks were collected from the surface of livestock, including goat, sheep, cattle, and vegetation from 11 sampling sites of six counties of Pingdingshan City (see Fig. 1) during the period of June-July, 2018. These ticks were starved for 2–3 days before the morphological examination, as described in a previous study [5, 6]. The findings were validated by amplification, sequencing, and analysis of the 16S rRNA partial sequences [7]. The details of this experiment are demonstrated in Table 1. All ticks were stored at -80 °C until DNA extraction.

**Table 1**

| Ticks and tick-borne agent | Target gene | Primer | Sequences (5'-3') | Tm (°C) | Length (bp) | Ref |
|----------------------------|-------------|--------|-------------------|---------|-------------|-----|
| Ticks                      | 16S rRNA    | 16 s F | CTGCTCAATGATTTTTAATTGCTGTGG | 54      | 460         | [7] |
|                            |             | 16 s R | CCGGTCTGAACCTCAGATCAAGT |         |             |     |
| Anaplasma spp.             | 16S rRNA    | out1   | TTGAGAGTTTGATCCTGGCTCAGAACG | 55      | 650         | [9] |
|                            |             | out2   | CACCTCTACACTAGGAATTCCGCTATC |         |             |     |
|                            |             | HGA1   | GTCGAACCGGATTATTCTTTATAGCTTG | 55      | 390         |     |
|                            |             | HGA2   | TATAGGTACCGTCATTATCTTCCCTCAC |         |             |     |
| Rickettsia spp.            | 17 kDa      | 17KDF-out | GCTTTACAAAAATTCTAAAAACCATATA | 58      |             | [10]|
|                            |             | 17KDR-out | TGTCTATCAATTCAAAACTTGCCGTT |         |             |     |
|                            |             | 17KDF-inner | GCTCTTTGAAACTTTATAGTT | 61      | 430         |     |
|                            |             | 17KDR-inner | CATTGTTCGTCAGGTTGCG |         |             |     |
|                            | gltA        | CS2dF   | ATGACCATGAAAAATAATAAT | 50      | 1170        | [11]|
|                            |             | RpCS.1258n | ATTGCAAAAGTACAGTGAACA |         |             |     |

**DNA extraction**
DNA was extracted from ticks, as described previously [8]. Ticks were washed and disinfected thrice with 75% ethanol and sterile deionized water, 5 min each time. DNA was extracted by using the TIANamp Genomic DNA Kit (TIANGEN Biotech Co., Ltd., Beijing, catalog no: DP304), as per the manufacturer's instruction.

**Detection of Anaplasma spp. and Rickettsia spp. by nested PCR**

The molecular identification of ticks and *Anaplasma* spp. was made through the 16S rRNA gene (390 bp) amplification by using nested PCR [9] and sequencing. *Rickettsia* spp. was identified by the amplification of *Rickettsia* spp. specific 17 kDa gene by using nested PCR [10], and *gltA* gene amplification was used to validate the positive samples [11]. The details of these experiments are demonstrated in Table 1.

The PCR products of the 16S rRNA gene of tick and *Anaplasma* spp., and 17 kDa gene, *gltA* gene of *Rickettsia* spp. were visualized by 1.5% agarose gel electrophoresis. Positive bands were cut and sent with the appropriate primers to BGI (The Beijing Genomics Institute) for sequencing by ABI 3730 with the Sanger sequencing method.

**Sequence analysis**

NCBI BLAST was used to analyze the gene sequences. To understand the relationship between different sequences, Mega 5.0 [12] was used to construct the phylogenetic tree with bootstrap 1000 [13] by the Neighbor-Joining (NJ) method [14].

**Results**

**Collection of ticks**

The altitude of small hills ranges from 200–800 meters in Pingdingshan City. The livestock grazing around the hill is widespread from April to October or even later when the bush and grass grow copiously. Around 686 ticks were collected from livestock and vegetation during the period of June-July in 2018 from six counties in Pingdingshan City, Henan Province, central China. These ticks were classified into 169 groups by morphological examination, and DNA was extracted from all the 169 groups.

Morphological examination and the 16S rRNA gene amplification results revealed that the majority of these ticks were 98.22% (166/169) homologous to *Haemaphysalis longicornis*. A 99.51%-100% homology to *H. longicornis* from Yunnan (JX051064.1), Sichuan (JF979373.1), Shandong (KC203360.1), Gansu (FJ712721.1), Hebei (JF979374.1), Beijing (KC203355.1), Shanghai (KP324925.1), Guizhou (KU986723.1) in China, and Aomori, Japan (AB819205.1) was also observed. The rest of the three samples (1.78%, 3/169) were identified as *Rhipisephalus microplus* (*R. microplus*). They showed 98.67%-99.76% homology to *R. microplus* from Yunnan (JX051062.1) and Sichuan (JF97938.1) in China, and (MK621328.1) from India.

Detection of Rickettsia spp. and Anaplasma spp.

*Rickettsia* spp. and *Anaplasma* spp. are tick-borne pathogens, and nested-PCR analysis showed that the tick samples collected in this study were positive for *Rickettsia* spp. and *Anaplasma* spp. We determined the proportion of tick samples that were positive for *Rickettsia* spp., *Anaplasma* spp., and the co-infection, which were collected from counties in Pingdingshan City (Table 2). The co-infection rate was 9.04% (15/166) in *H. longicornis* and 0/3 in *R. microplus*. *Anaplasma* spp. infection rate was 48.8% (81/166) in *H. longicornis* and 66.67% (2/3) in *R. microplus*. *Rickettsia* spp. infection rate was 30.72% (51/166) in *H. longicornis* and 0/3 in *R. microplus*. 

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Prevalence of *Anaplasma* spp. and *Rickettsia* spp. in *H. longicornis* and *R. microplus* in Pingdingshan City, hilly area of central China.

| Sampling Time   | County   | Sampling sites | Altitude (m) | Tick species | No. of samples tested | Pathogen | Anaplasma spp. | Rickettsia spp. | Co-infection |
|-----------------|----------|----------------|--------------|--------------|-----------------------|----------|---------------|----------------|-------------|
| June 5, 2018    | Wugang   | 1, 2, 3, 4     | 234–269      | *H. longicorni* | 38 (63.16)            | 24 (63.16) | 5 (13.16)     | 1 (2.63)       |             |
|                 |          |                |              | *R. microplus* | 2 (100)              | 2 (100)  | 0 (0)         | 0 (0)          |             |
| July 3, 2018    | Baofeng  | 5              | 578          | *H. longicorni* | 19 (42.11)            | 8 (42.11) | 10 (52.63)    | 0 (0)          |             |
| July 13, 2018   | Lushan   | 6              | 476          | *H. longicorni* | 24 (41.67)            | 10 (41.67)| 5 (20.83)     | 0 (0)          |             |
| July 18, 2018   | Jiaxian  | 7, 8           | 377          | *H. longicorni* | 59 (47.46)            | 28 (47.46)| 7 (11.86)     | 4 (6.78)       |             |
|                 |          |                |              | *R. microplus* | 1 (0)                | 0 (0)    | 0 (0)         | 0 (0)          |             |
| July 30, 2018   | Xinhua   | 9              | 114          | *H. longicorni* | 4 (75)               | 3 (75)   | 4 (100)       | 3 (75)         |             |
| July 30, 2018   | Ruzhou   | 10, 11         | 209          | *H. longicorni* | 22 (36.36)            | 8 (36.36)| 20 (90.90)    | 7 (31.82)      |             |
| **Total**       |          |                |              | *H. longicorni* | 166 (48.80)           | 81 (48.80)| 51 (30.72)    | 15 (9.04)      |             |
|                 |          |                |              | *R. microplus* | 3 (66.67)            | 2 (66.67)| 0 (0)         | 0 (0)          |             |

**Genetic and phylogenetic analysis**

To decipher the correlation of the sample sequences with the reference sequences, we constructed a phylogenetic tree based on the tick 16S rRNA sequences (Fig. 2) from *Anaplasma* spp. (Fig. 3) and 17 kDa and gltA from *Rickettsia* spp. (Fig. 4).

We obtained seven sequences from the *Anaplasma* spp. 16S rRNA gene. Out of the seven sequences, six were identified as *A. phagocytophilum*, one as *A. central*. Out of the six *A. phagocytophilum* sequences, three sequences showed 99.74% homology to *A. phagocytophilum* (MN044900.1, Zhejiang; KT276565.1, Shandong), one showed 100% homology to *A. phagocytophilum* (MN044900.1, Zhejiang; KT276565.1, Shandong), one showed 98.48% homology to KC422267.1 while the other one showed 98.23% homology to KR611598.1 from South Korea. The 16S rRNA gene sequence of *A. central* showed 100% homology to GU064903.1 from South Korea.

We obtained eighteen sequences of 17 kDa gene, which were the 99.08%-99.77% homologous to *Candidatus R. jingxinensis* (MH932039.1) (Fig. 4A). To validate this identification, three sequences of gltA from the 17 kDa positive samples were obtained, which showed 100% homology to *Candidatus R. jingxinensis* (KT899089.1) (Fig. 4B).
Discussion

Ticks are widely distributed across the globe, and this distribution depends on several environmental and climatic factors [3]. *H. longicornis* mostly occurs in temperate secondary forests, mountainous and hilly fringe areas, [15] and transmits the virus, bacteria, spirochetes, rickettsia, blood protozoa, chlamydia, and other disease-causing and emerging infectious pathogens [1, 16]. In our research, *H. longicornis* is the dominant tick species in hilly area in Pingdingshan. Not only in the hilly area we studied, in other hilly areas, such as Shandong Province [17], the hilly area in eastern Liaoning Province [18], the hilly area in Hebei Province [19, 20], southwest forest in Beijing [21], Huichun and Tumen in Jilin Province [22], Huaian in Jiangsu Province [23], Anhui Province [24], Jiangxi Province [25] in China, even in Japan (ref) and South Korea (ref), *H. longicornis* is the dominant tick species. One common feature of these areas is that they are all hilly areas. Therefore, the distribution characteristics of *H. longicornis* remind us that the hill area is a unique ecological environment (Habitat), in which *H. longicornis* was bred. In other words, the hilly area and *H. longicornis* are a natural pair. Meanwhile, *H. longicornis* transmits different pathogens, and inhabits the body of livestock close to humans, suggesting it may be a substantial threat to healthy human beings in the hilly area.

*R. microplus* is also widely distributed in China and abroad (ref ?). We found a small amount of *R. microplus* in the collected ticks, which is consistent with the report of XX (Ref). We believe that the proportion of *R. microplus* in this area is relatively small, far lower than that of *H. longicornis*, so that some research teams have not found the existence of *R. microplus* in the whole Henan Province, including Pingdingshan (Ref.). In addition, the main hosts we collected were goats and sheep, which were not the main hosts of *R. microplus* (Ref.), which may be the reason for the low proportion of tick. Therefore, we believe that although *R. microplus* is not the main tick species in the region, its proportion may be slightly larger than that detected by us. In other words, the tick in the area and the diseases it spreads still cannot be underestimated.

The genus *Anaplasma* includes six species: *A. phagocytophilum*, *A. marginale*, *A. centrale*, *A. bovis*, *A. ovis*, and *A. platys*. All the *Anaplasma* members are tick-borne, obligatory, intracellular, Gram-negative bacteria, which infects humans and/or domestic animals [26, 27], culminating in severe infections. *Anaplasma phagocytophilum* causes granulocytic anaplasmosis (HGA) in humans and anaplasmosis in ruminants, horses, dogs, and cats [26], which is vectored mainly by *Ixodes* [28], sheep and goat [29, 30]. In the ticks found in China, 16S rRNA gene sequences of *A. phagocytophilum* were detected in *Ixodes persulcatus*, *Dermacentor silvarum*, *H. concinna*, *Dermacentor nuttalli*, and *H. longicornis* [31–33]. In the current study, the *A. phagocytophilum* infection rate (48.8%) was higher than *H. longicornis* in the tick samples found at the border between China and Russia 2.5% (13/515) [34], followed by samples from Hubei Province in China (4.22%) [35], Baoding, Qinhuangdao, Tangshan in Hebei Province, China (14.6%, 39/267) [31], and Laizhou in Shandong Province in China (43.5%) [36]. The infection rate is similar to Yiyuan County in Shandong Province (48.1%) [17]. *H. longicornis* emerged as dominant tick species, and more than 40% *A. phagocytophilum* infection rate was seen in Henan and Shandong Province, which may be caused by similar latitude, altitude, and climate, in other words, hilly area. Interestingly, the infection rate in the areas surrounding the Henan and Shandong provinces in China was far less; however, the underlying mechanism remains unclear.

In addition, *Anaplasma central* (*A. central*) is a member of the genus *Anaplasma*. *A. central* causes a mild form of infectious disease. *A. marginale* is closely related to *A. central*, and widely used in immunization and to protect against virulent *A. marginale* challenge [37]. *A. centrale* primarily occurs in wild swine, deers [38, 39], sheep [40], and vectored by *Ixodes persulcatus* [41], *Rhipicephalus pumilio*, *Hyalomma asiaticum* [40]. In this study, *A. centrale*
was detected for the first time in *H. longicornis* in China. These outcomes draw attention to the increased risk of *A. centrale* infection in the areas where *H. longicornis* primarily occurs.

*Candidatus Rickettsia jingxinensis* (*Ca. R. jingxinensis*) is an uncultured *Rickettsia* spp. identified in *H. longicornis* from north-eastern China [42], and its *gltA* sequence (KU853023) was detected in a human subject. Besides, it was detected in *H. longicornis* from Japan [43], *H. longicornis* and rodents from South Korea [44], *H. longicornis* from Hebei Province [45], Liaoning Province [46] and *R. microplus* from Wuhan Province of Hubei [47] in China; also *H. longicornis* was found in Shaanxi Province [4], and *H. longicornis* in Yunnan Province [48] in China. In our research, 17 kDa and *gltA* gene sequences of *Ca. R. jingxinensis* were also detected in *H. longicornis* ticks. These results indicate that *Ca. R. jingxinensis* is widely distributed in *H. longicornis* in hilly area in east Asia and suggests potential health risk to human and animals.

**Conclusion**

In summary, in the current study, we investigated ticks and tick-borne *Anaplasma* spp. and *Rickettsia* spp. from livestock and vegetation from the hilly area in central China due to a high prevalence of *H. longicornis*, high infection rate of *Anaplasma* spp. and *Rickettsia* spp., even co-infection of ticks in this hilly area. Further epidemiological studies are required to assess the role of these disease-causing pathogens and other pathogens transmitted by *H. longicornis* in hilly area and its impact on human health with an emphasis on people related to animal husbandry and field workers. In addition, in hilly area, the pathogenicity of *A. phagocytophilum, A. central*, and *C. Ra. jingxinensis* demands further investigations.

**Declarations**

**Ethics approval and consent to participate**

All the experiments with animals were approved by the Animal Ethics Committee of Pingdingshan University, China (PDSAEC-2018-001).

**Availability of data and materials**

The nucleotide sequences were submitted to Genbank: *H. longicornis* 16S rRNA gene MT555302-MT555306, *R. microplus* 16S rRNA gene MT555307, *Anaplasma* 16S rRNA gene sequences, *Rickettsia* 17 kDa and *gltA* sequence.

**Competing interests**

The authors declare no conflict of interest.

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Author's contribution

Ke Zhang: study design, data analysis, writing, reviewing, and editing manuscript. Aijun Li, Jinjuan Zhang, Huijing Wang, Hongyu Wu: tick collection. Ruixuan Shi: morphological identification of ticks. Yujie Chen: molecular identification of ticks. Ying Ding: *Anaplasma* spp. detection. Tingting Tong: *Rickettsia* spp. detection. Suxiang Lu draw the map of sampling sites.

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Figures
Figure 1

Map of tick collection area, 11 sampling sites in six counties from Pingdingshan City, central China. The red circle represents sheep. The green circle represents goat. The black circle represents cattle. The purple circle represents vegetation.

Figure 2

Phylogenetic tree of ticks from Pingdingshan City, central China based on tick 16S rRNA (460 bp). Phylogenetic tree of (A) H. longicornis, (B) R. microplus. The bootstrap was 1000, and numbers at each node indicate bootstrap values. Argas persicus (GU355920) is the outgroup.
Figure 3
Phylogenetic tree of ticks from Pingdingshan City, central China based on tick 16S rRNA (460 bp). Phylogenetic tree of (A) H. longicornis, (B) R. microplus. The bootstrap was 1000, and numbers at each node indicate bootstrap values. Argas persicus (GU355920) is the outgroup.

Figure 4
Phylogenetic tree of Rickettsia spp. based on 17 kDa sequences (A) and gltA (1170 bp) sequences (B). The bootstrap was 1000, and numbers at each node indicate bootstrap values. Rickettsia bellii (KJ534308.1) is the outgroup in the Fig 3A, and Rickettsia bellii (AY375161.1) is the outgroup in the Fig 3B.