Identification of tick-borne pathogen diversity by metagenomic analysis in Haemaphysalis longicornis from Xinyang, China

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Abstract

Background: A wide variety of pathogens could be maintained and transmitted by Haemaphysalis longicornis. The aim of this study is to systematically examine the variety of pathogens carried by Haemaphysalis longicornis, an important vector, in tick-borne diseases epidemic area, and to estimate the risk of human infection imposed by tick bites.

Methods: Adult questing ticks were collected in Xinyang, central China. Genomic DNA and RNA were extracted from 144 H. longicornis ticks individually, and sequenced respectively as the templates for high-throughput sequencing. Clean reads were compared against the database of NCBI nucleotide collection and specific PCR was performed to confirm the presence of pathogen. Phylogenetic analysis was performed to explore the evolutionary status of pathogens.

Results: The assignment of reads to taxa based on BLASTN results revealed the existence of several potential pathogens, including Anaplasma spp., Rickettsia spp., Babesia sp., as well as severe fever with thrombocytopenia syndrome bunyavirus (SFTSV). Confirmatory PCR assays revealed the existence of Anaplasma bovis (13/144, 9.03%), Anaplasma centrale (2/144, 1.39%), Rickettsia helongiangensis (3/144, 2.08%), Rickettsia sp. LON-13 (1/144, 0.69%), Rickettsia raoultii (5/144, 3.47%), Babesia sp. (1/144, 0.69%). SFTSV accounted for the highest detected pathogen with a positive rate of 18.75% (27/144). Three of the ticks (2.08%) were co-infected with SFTSV and A. bovis.

Conclusion: Our study provided a broadened list of microorganism that harbored by H. longicornis. In previously unrecognized endemic regions, prokaryotic and eukaryotic infection including Anaplasma spp., Rickettsiae spp., and Babesia spp. should be considered, along with the well-known SFTSV for patients with tick bites history. A novel Babesia species was identified in local natural foci, which needs further investigation in the future.

Keywords: Haemaphysalis longicornis, Pathogens, High-throughput sequencing

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Background
*Haemaphysalis longicornis* is one of the most important tick species that imposes the risks of tick-borne disease infection, which is widely distributed in the Asia-Pacific region, including Korea, Japan, Australia, the Pacific Islands, and New Zealand [1–5]. Common hosts of *H. longicornis* include goats, cattle, sheep, *Bos mutus*, donkeys, pigs, *Cervus elaphus*, cats, *Rattus norvegicus*, *Musculus*, *Erinaceus europaeus*, *Mustela sibirica*, *Trichosurus vulpecula*, and some birds, along with human beings, which is commonly considered as the definitive host [5–7]. A wide variety of pathogens can be maintained and transmitted by *H. longicornis*. A remarkable example is the novel phlebovirus in the Bunyaviridae family (severe fever with thrombocytopenia syndrome virus, SFTSV), which was newly identified to be a causal agent of severe fever with thrombocytopenia syndrome (SFTS) in China and other neighbouring countries [8–10]. SFTS is largely considered to be a tick-associated disease, as high proportion of patients had tick exposure before disease onset [8]. *H. longicornis*, the most prevalent tick species that infests human in SFTS-endemic areas, was determined to be a competent vector of SFTSV by an experimental maintenance and transmission study [11]. In this context, we performed a metagenomic analysis to provide an inventory of predicted and unexpected pathogenic agents carried by *H. longicornis* ticks, captured in Xinyang Administrative Area, Henan Province in central China.

Xinyang is the region mostly heavily afflicted by SFTSV, reporting 48% of SFTS cases in China [12]. The region has a humid subtropical climate with annual precipitation of around 1100 mm. The southern part of Xinyang, stretching across the Dabie Mountain range, is an important habitat for *H. longicornis* ticks, captured in Xinyang Administrative Area, Henan Province in central China.

Xinyang is also reported to be epidemic area of many tick-borne diseases such as human granulocytic anaplasmosis, spotted fever and typhus fever [14, 15]. These findings indicated that *H. longicornis* might be a potential vector of causative agents. Therefore we expect the metagenomic analysis in this region might provide a broad list of the pathogens carried by this important vector, thereby offering the potential humans infection risk imposed by tick bites.

Methods
Ticks collection and DNA/RNA extraction
The questing ticks were captured from 10 sampling sites across Xinyang. Sample sites were selected from representative geographical areas where patients got tick bite in Dabie Mountain area and plain area. (Dabie Mountain area: 31.69 N, 115.45E; 31.59 N, 115.30E; 31.76 N, 115.27E; 31.70 N, 114.82E; 31.66 N, 115.01E; plain: 32.48 N, 115,31E; 32.40 N, 115.24E; 32.38 N, 115.45E; 32.25 N, 114.90E; 32.33 N, 115.11E), Henan Province, in May 2013. The ticks were collected by dragging over the vegetation layer during daytime. Morphologic features were used to identify the species and developmental stage of ticks by an entomologist (Sun Y) [16]. 10–15 *H. longicornis* from each site were randomly selected and totally 144 ticks were included in our study. The predominant tick species was confirmed to be *H. longicornis*. Live *H. longicornis* ticks were subsequently sterilised in 75% ethanol and then washed up with deionised water for 5 min each to remove environmental contaminants. DNA and RNA were extracted from single tick using TIANamp DNA/RNA extraction kit (Tiangen, Beijing, China) according to the manufacturer’s instructions.

Library preparation for high-throughput sequencing
The DNA/RNA extracted from 144 ticks were pooled respectively as the templates for library preparation. For prokaryotic pathogen screening, the pooled DNA was amplified in 50 μl reactions: 26.5 μl pure water, 10 μl 5 × phusion HF buffer (Thermo scientific, Hudson, NH, USA), 1 μl 10 mmol/L dNTPs (Thermo scientific), 2 μl of the 16S F and 16S R primers [17] (Invitrogen Corp., Carlsbad, CA), 0.5 μl Phusion High-Fidelity DNA polymerase (Thermo scientific), and 10 μl DNA(≈ 50 ng). The amplification was conducted according to a protocol involving initial denaturation for 30s at 98 °C and 35 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. Agarose gel with target fragments was purified using TIANgel Midi Purification Kit (Tiangen, Beijing, China) according to the manufacturer's instruction. For eukaryotic pathogen screening, the pooled DNA was amplified as previously described using primers BTH-1F: cctgmracrccccggttacctacact and BTH-R: ttggcaccatctccccca [18]. For virus screening, the pooled RNA was reverse transcribed with random hexmers to cDNA using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (Thermo, Waltham, USA). The purified 16S and 18S PCR product, cDNA from RNA and DNA were sequenced with Ion Personal Genome Machine (PGM) System as described by Vogel and others [19]. Quality of the library was analysed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Bioinformatics analysis
After sequencing, the individual sequence reads were filtered within the PGM software to remove low quality sequences. Sequences matching the PGM 3’ adaptor were
also automatically trimmed. Sequences that were shorter than 100 bp were deleted with an in-house python script. Each clean read was compared against the NCBI nucleotide collection (non-redundant nt database) using Blastn with default parameters (−v 5, −b 5, −w 35). The hit with the highest “Max Score” for every query was picked up, and the resulting hits were grouped by species according to its GI number. The number of reads and the total matched length of each species were calculated.

**Specific PCR for detection of three pathogens in ticks**

Based on the results from the alignments, specific PCR was performed to confirm the presence of pathogens in the DNA and RNA of individual ticks. The genes used for phylogenetic analysis were as below: 16S rDNA for *Anaplasma* spp., *gltA* gene for *Rickettsia* spp., 18S rDNA for *Babesia* spp. and the S segment for SFTSV. Total RNA from each sample (0.1–1 μg) was used for reverse transcription using the SuperScript III First-Strand Synthesis System (Invitrogen). DNA extracted from each sample and cDNA reverse-transcribed from each sample were used as PCR template.

The targeted genes were amplified from template in 30 μL PCR mixtures containing 120 mmol/L of each primer (Table 1) [20–24], 60 mmol/L of each dNTP, 3 μL of 10 × *Taq* PCR buffer (Takara, Dalian, China), and 1.5 U of *Taq* DNA polymerase (Takara, Dalian, China). Amplification cycling conditions were as follows: denaturation for 3 min at 94 °C and 35 cycles of 94 °C for 45 s, 56 °C for 35 s, 72 °C for 1 min, followed by a final extension at 72 °C for 15 min. Amplified products were visualized with SYBR® Safe (Thermo, Waltham, USA) after electrophoresis in 2% agarose gel. For nested PCR, the second round were performed using the same reaction and cycling conditions as described above, and 1 μL of the first-round PCR production were used as template.

The primers for amplification in this study are presented in Table 1. The PCR amplicons were directly sequenced using an ABI 3730 machine (Applied Biosystems, Foster City, CA, USA). To reveal the evolutionary status of identified pathogens of interest, the phylogenetic analysis was performed using the Mega 5.0 software (http://www.megasoftware.net). The alignment was made under default parameters. Phylogenetic analysis was performed by the Maximum Likelihood method. All positions containing alignment gaps and missing data were deleted (complete-deletion).

### Results

**Taxonomic classification**

The DNA and RNA extraction from a total of 144 adult *H. longicornis* ticks were respectively pooled into one sample, and subject to the high throughput sequencing. The fragments length of the constructed library ranged between 300 to 400 bp (Fig. 1). Totally 826,561 kb data were obtained, while 706,862 kb were of high quality (>Q20). A total of 6,926,470 reads were obtained with mean length of 233 bp (Fig. 2). The assignment of unassembled sequence reads to taxa based on BLASTN results revealed the existence of several pathogens, including *Anaplasma* spp., *Rickettsia* spp., *Babesia* spp., as well as SFTSV (Table 2).

**Confirmation of the pathogens and phylogenetic analysis**

The remaining DNA/RNA from the 144 *H. longicornis* ticks were individually detected for the presence of these pathogens by PCR or RT-PCR (Additional file 2: Figure S1). The sequence analysis revealed the existence of *Anaplasma bovis* (Ehrlichia bovis, 13/144, 9.03%), *Anaplasma centrale* (2/144, 1.39%), *Rickettsia helongiangensis* (3/144, 2.08%), *Rickettsia* sp. LON-13 (1/144, 0.69%), *Rickettsia raoultii* (5/144, 3.47%), *Babesia* sp. (0.69%, 1/144) (Table 3). Three ticks were co-infected with SFTSV and *A. bovis*.

By phylogenetic analysis, the *Anaplasma* spp. (KY007144-KY007156, KX817983, KX817984) identified in the *H. longicornis* ticks were shown to be clustered with *A. centrale* and *A. bovis* (Fig. 3a). *Rickettsia*, which is recognized as medically important arthropod-vectored pathogens, was found involved in symbiosis within 9 *H. longicornis* ticks, and clustered into three different branches (KX817986-KX817988, KX965745-KX965757).

### Table 1 Primers used in this study

| Target       | Gene       | Primer                        | Primer name              |
|--------------|------------|-------------------------------|--------------------------|
| *Anaplasma*  | 16S rRNA   | Out1                          | TTAGAGATTTGTCATATGCTTTGA |
|              |            | Out2(21)                      | CACCTCTACACTAGGAATCCGCTTC |
|              |            | Out2F                         | GAGTACGCGAATCTTAGTGTA    |
| *Rickettsia* | *gltA*     | 317Pan(20)                    | AAAGGAGTATCCAGC          |
| *Coxiella*   | transposase| Cox_trans-3                   | GTAAACGATCAGCCAGC        |
| *Babesia*    | 18S rDNA   | Piro0F                        | GCCGCTGATGTAGCTGTTGTA    |
|              |            | Piro1F                        | CCCATGCTGCTGTTGTA        |
|              |            | Piro5.5R                      | CCTYAAATGTAAGGGTCACAAACTT |
|              |            | Piro6R(22)                    | CTCCTTCCTAYAAGGGTACAAGGC |
| *SFTSV*      | S segment  | BNYS1-F                       | TCTTCCCATCAAAGAACAGC     |
|              |            | BNYS1-R(24)                   | ITTCGACAAATTAAGCATCC     |
R. heilongjiangensis, R. raoultii, and Rickettsia sp. LON-13 (Fig. 3b). One of the H. longicornis ticks was found carrying Babesia sp. (GenBank No. KX817985). The phylogenetic analysis showed the 18S rRNA gene was most close to Babesia sp. MA*361–1 (GenBank No. AB251610) and Babesia sp. SAP*091 (GenBank No. AB251609) (Fig. 3c). The represented SFTSV sequences (KX817989, KX965742–KX965744) obtained from four H. longicornis ticks were clustered with sequences obtained from local SFTS patients [25] (Fig. 3d).

**Discussion**

In recent years, due to intense research interest in SFTS, there has been an increasing number of pathogens that were detected from H. longicornis ticks in SFTS endemic region [26]. Our study, based on the Next Generation Sequencing (NGS) methods, provided a broadened list of the microorganism harboured by this tick species, including prokaryotic and eukaryotic pathogens and viruses. In addition to SFTSV, other tick-borne pathogens, A. bovis (13/144, 9.03%), A. centrale (2/144, 1.39%), R. heilongjiangensis (3/144, 2.08%), R. raoultii (5/144, 3.47%), were detected as well.

Members of the genus Anaplasma include A. phagocytophilum, A. marginale, A. bovis, A. ovis, A. platys and A. centrale, all of which are obligate intracellular bacteria that infect a variety of cell types [27]. In China, wild and domestic ruminants play active roles as Anaplasma spp carriers and reservoirs. The presence of A. bovis in sheep and goats has been reported in Northwest, Central and Southern China [28, 29]. A. centrale has been reported in goats and sheep from South-eastern China [30]. Our results indicate the epidemic of A. bovis and A. centrale in central China. In the study, spotted fever group Rickettsia, including R. heilongjiangensis, R. raoultii and Rickettsia sp. LON-13 was detected in H. longicornis ticks in the same region. R. heilongjiangensis can cause spotted fever in humans, which was detected in Dermacentor. silvarum and H. longicornis ticks in Heilongjiang Province and Zhejiang Province [31–33]. R. raoultii, the predominant Rickettsia found in Dermacentor silvarum
Table 2 Potential pathogens presented by high-throughput sequencing

| Species                        | Superkingdom | Total lengtha   | Total numberb |
|--------------------------------|--------------|----------------|--------------|
| Anaplasma bovis                | Prokaryota   | 38 461         | 157          |
| Anaplasma ovis                 | Prokaryota   | 21 710         | 85           |
| Anaplasma phagocytophilum      | Prokaryota   | 2123           | 12           |
| Babesia bigemina               | Eukaryota    | 2225           | 19           |
| Babesia bovis                  | Eukaryota    | 1188           | 15           |
| Babesia canis                  | Eukaryota    | 22 734         | 199          |
| Babesia cf. divergens          | Eukaryota    | 2808           | 20           |
| Babesia divergens              | Eukaryota    | 24 651         | 95           |
| Babesia felis                  | Eukaryota    | 4036           | 16           |
| Babesia gibsoni                | Eukaryota    | 15 349         | 69           |
| Babesia microti                | Eukaryota    | 29 114         | 307          |
| Babesia occultans              | Eukaryota    | 31 359         | 233          |
| Babesia odocoilei              | Eukaryota    | 14 817         | 155          |
| Babesia ovata                  | Eukaryota    | 4636           | 22           |
| Babesia rossi                  | Eukaryota    | 2239           | 22           |
| Babesia sp. 28                 | Eukaryota    | 8 543          | 71           |
| Babesia sp. 4 NAN-2012         | Eukaryota    | 2 054 498      | 12 132       |
| Babesia sp. giraffe 1505       | Eukaryota    | 8 671          | 37           |
| Babesia sp. Kh-Hj143           | Eukaryota    | 68 397         | 241          |
| Babesia sp. MA#230             | Eukaryota    | 58 780         | 236          |
| Babesia sp. MA#361–2           | Eukaryota    | 56 703         | 252          |
| Babesia sp. NJ5                | Eukaryota    | 3230           | 21           |
| Babesia sp. NV-1               | Eukaryota    | 4606           | 34           |
| Babesia sp. RWF-2013           | Eukaryota    | 15 721         | 80           |
| Babesia sp. sable antelope/2005| Eukaryota    | 8 071          | 33           |
| Babesia sp. SAP#901            | Eukaryota    | 497 797        | 1919         |
| Babesia sp. SAP#131            | Eukaryota    | 177 543        | 813          |
| Babesia sp. UR1                | Eukaryota    | 5355           | 34           |
| Babesia sp. ‗venatorum‘        | Eukaryota    | 9688           | 59           |
| Babesia sp. YZ-2012            | Eukaryota    | 3 803 579      | 15 839       |
| Coxiella burnetii              | Prokaryota   | 3 480 573      | 13 807       |
| Rickettsia australis           | Prokaryota   | 25 868         | 93           |
| Rickettsia canorii             | Prokaryota   | 9 580          | 45           |
| Rickettsia helongiangensis     | Prokaryota   | 628 174        | 2387         |
| Rickettsia helongiangii        | Prokaryota   | 42 045         | 189          |
| Rickettsia japonica            | Prokaryota   | 5 718          | 23           |
| Rickettsia prowazekii          | Prokaryota   | 5 166          | 26           |
| Rickettsia rhizophilalii       | Prokaryota   | 3 309          | 14           |
| Rickettsia rickettsii          | Prokaryota   | 3 760 044      | 1519         |
| Rickettsia slovaca             | Prokaryota   | 2 357          | 16           |
| Rickettsia sp. BJ-90           | Prokaryota   | 9 265          | 38           |
| Rickettsia sp. MSeoKT1         | Prokaryota   | 2 799          | 18           |
| Rickettsia sp. T170-B          | Prokaryota   | 1 056 955      | 4112         |

Table 2 Potential pathogens presented by high-throughput sequencing (Continued)

| Species                        | Superkingdom | Total lengtha   | Total numberb |
|--------------------------------|--------------|----------------|--------------|
| Rickettsia grylli              | Prokaryota   | 15 798         | 91           |
| Severe fever with thrombocytopenia syndrome virus | Viruses | 1420 | 5 |

*Species Superkingdom Total sequencing numberb
Total number: Number of reads that classified with the corresponding species
Total length: Sum of the reads that classified with the corresponding species

**Table 3** Comparison of DNA sequence similarities between pathogens detected in ticks and the number of infection

| Pathogen Genbank Match-Accession Number | Gene (Length) | Identity | Number of Infection (%) |
|----------------------------------------|--------------|----------|-------------------------|
| Ehrlichia bovis-JN558824                | 16S (1490 bp) | 99%      | 13 (0.03%)              |
| Anaplasma centrale-AB251610             | 16S (1490 bp) | 100%     | 2 (1.39%)               |
| Rickettsia helongiangensis-EU665234     | gltA (341 bp) | 100%     | 3 (2.08%)               |
| Rickettsia sp. LON-13-AB516964          | gltA (341 bp) | 100%     | 1 (0.69%)               |
| Babesia sp. M1#361–1-AB251610          | gltA (341 bp) | 100%     | 5 (3.47%)               |
| SFTSV-KC292288                          | 5 (491 bp)   | 100%     | 27 (18.75%)             |
Fig. 3 Phylogenetic analysis of confirmed pathogens of interests. Sequences were aligned using the MEGA5 (Version5.1) software package. Phylogenetic analysis was performed by the Maximum Likelihood method. All positions containing alignment gaps and missing data were deleted (complete-deletion). Bars indicate the percentage of sequence divergence. All positions containing alignment gaps and missing data were deleted (Complete-deletion). 

a) Phylogenetic tree of bacteria belonging to *Anaplasma*, inferred from comparison of the partial 16S rDNA gene sequences. b) Phylogenetic tree of bacteria belonging to *Rickettsia*, inferred from comparison of the partial gltA gene sequences. c) Phylogenetic tree of bacteria belonging to *Babesia*, inferred from comparison of the partial 18S rDNA gene sequences. d) Phylogenetic tree of bacteria belonging to SFTSV, inferred from comparison of the partial 5S rDNA gene sequences.
co-infections of various tick-borne pathogens should be considered for patients after tick bite. Since the discovery of SFTS in China, enormous efforts have been applied to identify SFTSV infection in both human being and the predominant tick species. However, other tick-borne pathogens were largely neglected. Due to nonspecific clinical presentation and less access to confirmatory laboratory findings, it is rather difficult to make diagnosis. In addition, novel *Rickettsia* and *Babesia* species of undetermined pathogenicity continue to be detected from ticks, highly possible to cause human illness. The current findings might have important application in determining the etiological determination in SFTS endemic region with *H. longicornis* as the predominant tick species, the most important tick-borne infectious disease not only in China, but also in countries where SFTSV infection has been reported.

Conclusions

In the study, a broadened list of the microorganism harboured by *H. longicornis* was provided. In SFTS region with abundant *H. longicornis*, prokaryotic infection including *Anaplasma* spp., *Rickettsiae* spp., and *Babesia* spp. should also be considered. The possibility of their co-infection with tick-borne viral pathogens in *H. longicornis* ticks, and dual infection in human, should be acknowledged by the clinicians. Specially, a novel *Babesia* species was identified in local natural foci, which needs further investigation in the future.

Additional files

**Additional file 1**: Multilingual abstracts in the five official working languages of the United Nations. (PDF 532 kb)

**Additional file 2**: Figure S1. Experimental confirmation of predicted pathogens of interest predicted by bioinformatics. A), PCR amplification of the *H. longicornis* ticks to confirm the predicted *Anaplasma* spp. S1 represented PCR amplification with primer Out1, Out2; S2 represented PCR amplification with primer Out2F, 317Pan. B), The second run of nested PCR (with primer Rpcf877F, Rpcf1258R) amplification of *H. longicornis* ticks to confirm the predicted *Rickettsia* spp., C), The second run of nested PCR (with primer Pir1F, Pir95SR) amplification of *H. longicornis* ticks to confirm the predicted *Babesia* spp.. D), The PCR (with primer BNy51F, BNy51-R) amplification of the *H. longicornis* ticks to confirm the predicted SFTSV. (JPEG 80 kb)

Abbreviations

SFTS: Severe fever with thrombocytopenia syndrome; SFTSV: Severe fever with thrombocytopenia syndrome virus

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