Detection of Multiple Intracellular Bacterial Pathogens in Haemaphysalis flava Ticks Collected from Hedgehogs in Central China

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Abstract: Tickborne intracellular bacterial pathogens including Anaplasma, Coxiella burnetti, Ehrlichia, and Rickettsia cause emerging infectious diseases worldwide. PCR was used to amplify the genes of these pathogens in Haemaphysalis flava ticks collected from hedgehogs in Central China. Among 125 samples including 20 egg batches, 24 engorged females, and 81 molted male and female adult ticks, the DNA sequences and phylogenetic analysis showed that the minimum infection rate of the ticks was 4% (5/125) for A. bovis, 3.2% (4/125) for C. burnetti, 9.6% (12/125) for E. ewingii, and 5.6% for Rickettsia including R. japonica (3.2%, 4/125) and R. raoultii (2.4%, 3/125), respectively. The prevalence of these pathogens was significantly higher in dead engorged females (83.3%, 20/24) than in eggs (5%, 1/20) and molted ticks (8.6%, 7/81). Our study indicated that H. flava ticks could be infected with multiple species of tickborne pathogens including Anaplasma, C. burnetti, Ehrlichia, and Rickettsia in Central China, and the prevalence of these pathogens was reduced during transovarial and transstadial transmission in ticks, suggesting that ticks may not be real reservoirs but only vectors for these tickborne pathogens.

Keywords: Haemaphysalis flava; hedgehogs; Anaplasma bovis; Coxiella burnetti; Ehrlichia ewingii; Rickettsia raoultii; Rickettsia japonica and China

1. Introduction

Tickborne intracellular bacteria including Anaplasma spp., Ehrlichia spp., Coxiella burnetti, and Rickettsia spp. cause severe human diseases [1–4]. Ixodidae, the hard body ticks, play an important role in the maintenance and transmission of these tickborne pathogens [5]. Approximately 100 Ixodidae species have been identified in China. Because of the vast territory, complex geography, and different climates of China, tickborne diseases are prevalent in most parts of China and pose a serious public health threat [6–8].

Haemaphysalis flava ticks are widely distributed throughout Asia including in China, Japan, South Korea, and Vietnam [9–12]. Hosts of H. flava include domesticated animals such as horses (Equus caballus), pigs (Sus scrofa domestica), dogs (Canis lupus familiaris), sheep (Ovis aries), cattle (Bos taurus), and wild animals such as hedgehogs (Erinaceinae), pandas (Ailuropoda melanoleuca), Siberian chipmunks (Eutamias sibiricus), Raccoon dogs (Nyctereutes procyonoides), water deer (Hydropotes inermis), and eastern roe deer (Capreolus capreolus) [13–16]. Previous studies had demonstrated that H. flava were positive to bacterial pathogens such as Anaplasma bovis, Borrelia spp., Bartonella, Francisella tularensis, Rickettsia japonica, parasites such as Babesia spp. and Toxoplasma gondii, and viruses such as severe
fever with thrombocytopenia virus and tickborne encephalitis virus [17–25]. These studies about *H. flava* tickborne pathogens were mainly carried out in Japan and South Korea. In China, only two studies in the Jiangxi and Hubei Provinces demonstrated that *H. flava* were positive to *R. slovaca*, *K. japonica*, and unclassified *Ehrlichia* species [26,27].

The *Haemaphysalis flava* tick is widely distributed, and it is important to know the pathogens carried by *H. flava* in China. In this study, we investigated the prevalence of intracellular bacterial pathogens in *H. flava* ticks collected from hedgehogs in Hubei Province in Central China.

2. Materials and Methods

2.1. Tick Samples

Ticks were pulled parallel to the skin surface by using fine-tipped tweezers from hedgehogs (*Erinaceus amurensis*) collected in October 2018 [28]. Hedgehogs were captured from forests near the cesspools in Xinning City, Hubei Province, China [29]. Xinning is located at 29°87′ north latitude and 114°28′ east longitude in Central China. The temperature ranges from −7 °C to 40 °C, with an annual average of 16.8 °C. All ticks were morphologically identified as *H. flava* [15], and nine ticks were randomly selected to amplify the 16S rRNA gene (*rrs*) with PCR for species confirmation as previously described [30]. Engorged ticks were kept in an incubator with 85% relative humidity at 25 °C for oviposition or molting [31].

2.2. PCR Amplification of Tickborne Pathogens in Ticks

For the detection of tickborne bacteria, the egg batches from each female were processed together, the dead engorged females were processed individually, and molted ticks (females and males) were processed in groups of six or seven ticks. Ticks were washed with distilled water and dried before DNA was extracted with the AllPrep DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Tick DNA was dissolved in 100 µL of DNase free water and stored at −80 °C. Tick DNA was used for the PCR amplification of tickborne pathogens. The primers listed in Table 1 were used to amplify the *Anaplasma* 16S rRNA gene (*rrs*) and heat shock protein GroEL (*groEL*) genes, the *C. burnetti* outer membrane protein (*omp*) gene and isocitrate dehydrogenase (*icd*) gene, the *Ehrlichia* *rrs* and GltA (*gltA*) genes, and the *Rickettsia* 17-kDa protein gene, outer membrane protein A (*OmpA*) gene, *gltA* and *rrs*. The PCR cycles of outer and inner primers for each gene were one cycle of 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C, and a final extension step of 10 min at 72 °C. Nuclease-free water was used as negative controls for each experiment.

PCR products were analyzed using 1.2% agarose gel electrophoresis and were detected with ethidium bromide staining under UV light. PCR products with the expected size were excised from the gels and extracted with a Gel Extraction Kit (Omega, Norcross, Georgia). The purified PCR products were cloned into PMD 19-T vectors (TaKaRa, Shiga, Japan), and the recombinant plasmids were sequenced bidirectionally.

**Table 1.** Primers for the amplification of sequences of *Coxiella burnetti*, *Anaplasma* spp., *Ehrlichia* spp., and *Rickettsia* spp. from ticks.

| Organisms     | Primary/Nested | Primers | Primer Sequences | Target Gene | Amplicon Size | Reference |
|---------------|----------------|---------|------------------|-------------|---------------|-----------|
| *Coxiella burnetti* | Primary | Omp1     | AGTGAAGCATCCAAAGGATTTG | *omp*       | 438 bp        | [32]      |
|                |             | Omp2     | TGGCTCTAGCTGAACGATTAG | | | |
|                |             | Omp3     | GAAGCGCAAACAGGAAGAACA | | | |
|                | Primary     | BicdF1   | CGGAGCTAAGCCGAGCCCTCCA | *icd*       | 651 bp        | [33]      |
|                |             | BicdR1   | CCGTGAATTCTGACATGTTACCCTTTT | | | This study |
|                |             | BicdF2   | AGTTAACCCGGAGATCCCATC | | | |
|                |             | BicdR2   | CTAACGGCTCTGCCCCCTT | | | |
### Table 1. Cont.

| Organisms | Primary/Nested | Primers | Primer Sequences | Target Gene | Amplicon Size | Reference |
|-----------|----------------|---------|------------------|-------------|---------------|-----------|
| **Anaplasma** | **Primary** | EC9 | TACCTTGTTACGACTT | rrs | 477 bp | [34] |
| | | EC12A | TAAAGGTCCGTTCAGACAGGC | rrs | 477 bp | [34] |
| | | EM87F | CGATTTGAATGCTCAAGAC | | | |
| | | EM84R | TGCCTGTTCAGCAG | | | |
| | | fD1 | GCAGTGTCGGTTCACGAC | | | |
| | | Rp2 | CGATTTGAATGCTCAAGAC | | | |
| | **Nested** | EHR16SD | ACGACCACCTTGAGTTTACGCTT | groEL | 473 bp | This study |
| | | EHR16SR | TGGGACCGGATCCCATCACC | | | |
| | | agroELnf | TGAGGGGTGGAAGCATTGAGCA | | | |
| | | agroElwr | AGAGTGATACAGACAGCAC | | | |
| | **Primary** | agroELwf | TTGTGTCGAAGCATTGAGCA | | | |
| | | agroELwr | AGAGTGATACAGACAGCAC | | | |
| **Ehrlichia** | **Primary** | EC9 | TACCTTGTTACGACTT | rrs | 477 bp | [34] |
| | | EC12A | TAAAGGTCCGTTCAGACAGGC | rrs | 477 bp | [34] |
| | | EM87F | CGATTTGAATGCTCAAGAC | | | |
| | | EM84R | TGCCTGTTCAGCAG | | | |
| | | HF51F | AAGTCGAACGGACAATTACC | | | |
| | | HF954R | GCTCTTGCAACTTCTATGTT | | | |
| | **Nested** | e-gltawf | TCTCCACGGTGTTGAGTTTGCAGAC | gltA | 411 bp | This study |
| | | e-gltawr | AATTGCAAGGATCAGAGACAG | | | |
| | | e-gltanf | TCTCCACGGTGTTGAGTTTGCAGAC | | | |
| | | e-gltanr | AATTGCAAGGATCAGAGACAG | | | |
| **Rickettsia** | **Primary** | R17F1 | TTACAAAGATCTCAAACCATCAT | 17-kDa protein gene | 410 bp | [37] |
| | | RR | TCAATTCACCGCTGCTG | | | |
| | | Rf2 | TCAATTCACCGCTGCTG | | | |
| | | RfR | TCAATTCACCGCTGCTG | | | |
| | **Nested** | S1 | TTCTTGCAACCTTTATATA | | | |
| | | S2 | TTCTTGCAACCTTTATATA | | | |
| | | S3 | TTCTTGCAACCTTTATATA | | | |
| | | S4 | TTCTTGCAACCTTTATATA | | | |
| | **Primary** | gltA1 | TGAAGGTTAATCAGCAG | | | |
| | | gltA2 | TGAAGGTTAATCAGCAG | | | |
| | | gltA3 | TGAAGGTTAATCAGCAG | | | |
| | | gltA4 | TGAAGGTTAATCAGCAG | | | |
| | **Nested** | Rr190.70p | ATGGCGAATATTTCCAAAA | ompA | 631 bp | [39] |
| | | Rr190.70n | ATGGCGAATATTTCCAAAA | | | |
| | **Primary** | Rglta1 | ATGACCAGAAGGAATATAATT | gltA | 341 bp | [40] |
| | | Rglta2 | ATGACCAGAAGGAATATAATT | | | |
| | | Rglta3 | ATGACCAGAAGGAATATAATT | | | |
| | | Rglta4 | ATGACCAGAAGGAATATAATT | | | |

*p Degenerate primer: Y = C or T.

### 2.3. Phylogenetic Analysis

The sequence chromatograms and analysis were examined with Chromas and BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi), respectively. Sequences were aligned and trimmed with MEGA7 (Philadelphia, PA, USA), and phylogenetic trees were constructed with MEGA7 using the maximum-likelihood method, with nucleotide sequences and bootstrap values that were calculated with 1000 replicates [38,41]. Only bootstrap values >50% were shown.

### 2.4. Statistical Analysis

All the statistical analyses was performed by Fisher’s exact test with SPSS (version 17.0) (Armonk, NY, USA), and $p < 0.05$ was considered to be a statistically significant difference.

### 3. Results

#### 3.1. Tick Species

A total of 125 ticks was collected from 15 hedgehogs, out of which 44 ticks were engorged adult females and 81 ticks were engorged nymphs. Of the engorged females,
20 females had oviposited and 24 females died before oviposition. All 81 engorged nymphs had molted into adult ticks regardless of sex. Ticks were morphologically identified as *H. flava* and confirmed with PCR amplification and DNA sequencing of the *rrs* gene.

### 3.2. Phylogenetic Analysis of Different Tickborne Intracellular Bacteria

**Rickettsia:** Rickettsia sequences were obtained from seven ticks and tick pools by PCR with primers of the 17-kDa protein gene. The DNA sequence analysis indicated that the 17-kDa protein gene positive samples were divided into two groups. Group 1 consisted of four sequences, which had the highest homology with *R. japonica* (GenBank: CP032049) (99.3–99.5% homologous), and group 2 consisted of three sequences, which had the highest homology with *R. raoultii* (GenBank: MH932036) (98.8–99.5%). Further amplification of the 17-kDa gene positive ticks and tick pools with primers of *ompA*, *gltA*, and *rrs* showed that ticks in group 1 had one, two, and three samples that were positive, respectively; and one tick pool in group 2 was positive with *gltA* primers. The rickettsial sequences in group 1 obtained with primers of *ompA*, *gltA*, and *rrs* had the highest homology with *R. japonica* with a 99.7%, 99.7–100%, 96.1%, and 99.4% homology to *R. japonica*, respectively, and the sequence in group 2 obtained with *gltA* primers had the highest homology with *R. raoultii* (99.1%). The phylogenetic analysis was performed with only one tick sample for each group, with the R23 tick representing group 1 and R50 tick representing group 2. A phylogenetic analysis with concatenated sequences of the 17-kDa protein gene and *gltA* indicated that the R23 formed a cluster with *R. japonica* and *R. heilongjiangensis*; and R50 was in the same cluster as *R. raoultii* (Figure 1A). Due to the difficulty in differentiating between *R. japonica* and *R. heilongjiangensis*, they were further analyzed by using more sequences including all four genes we obtained in tick R23. A phylogenetic analysis with concatenated sequences of *rrs*, 17-kDa protein gene, *gltA*, and *ompA* showed that R23 was in the same cluster with *R. japonica* and *R. heilongjiangensis*, but closer to *R. japonica* (Figure 1B).

**Ehrlichia:** Ehrlichia sequences were obtained from nine individual ticks and three tick pools by PCR with *rrs* primers. The *rrs* sequences had the highest homology with *E. ewingii* (GenBank: U96436) (98.8–99.8%). The *rrs* positive ticks and tick pools were further amplified with *gltA* primers. The sequences obtained with *gltA* primers also had the highest homology with *E. ewingii* (GenBank: DQ365879) (90.3–90.9%). A phylogenetic analysis with concatenated sequences of *rrs* and *gltA* indicated that all 11 *Ehrlichia* species were in the same cluster as *E. ewingii*, but in a distinct group, suggesting that this *Ehrlichia* species was a novel *Ehrlichia* species (Figure 2).

**Coxiella:** Coxiella sequences were obtained from three individual ticks and one tick pool by PCR with *omp* primers. The *omp* sequences from ticks had the highest homology with *C. burnetii* (GenBank: CP014563) (99.5–99.8%). The *omp* positive tick samples were further amplified with *icd* primers, which showed that all four tick samples were positive. The *icd* sequences from ticks were 99.8–100% homologous with *C. burnetii* (GenBank: CP040059). A phylogenetic analysis with concatenated sequences of *omp* and *icd* indicated that the four sequences from ticks were in the same cluster with *C. burnetii* (Figure 3).

**Anaplasma:** Anaplasma sequences were obtained from five ticks by PCR with *rrs* primers. Because the *rrs* sequences were short and had a poor specificity at first, the semi-nested primers were designed to prolong the *Anaplasma rrs* sequences of the positive ticks (Table 1). The *rrs* sequences were prolonged in four of five *rrs* positive ticks. The *rrs* sequences from ticks had the highest homology with *E. bovis* (GenBank: U03775) (96.3–100%). The *rrs* positive ticks were further amplified with *groEL* primers, which showed that only one tick sample was positive. The *groEL* sequence obtained from a tick was 84.6% homologous with *A. bovis* (GenBank: MH255898). A phylogenetic analysis based on the concatenated sequence of *rrs* and *groEL* indicated that the concatenated sequence was in the same cluster as *A. bovis* (Figure 4).
Figure 1. Phylogenetic tree of the *Rickettsia* species. The phylogenetic trees were constructed using (A) the concatenated sequences of the 17-kDa protein gene and *gltA* and (B) the concatenated sequences of *rrs*, 17-kDa protein gene, *gltA*, and *ompA*. The tree was generated using the Maximum Likelihood method, the Kimura 2-parameter model, and 1000 replicates for bootstrap testing in MEGA 7.0 software. Only bootstrap values > 50% were shown. *Rickettsia* sequences obtained in this study are shown with dots. The scale bar indicates nucleotide substitutions per site. The *Rickettsia* species’ name and complete genome GenBank accession numbers of reference sequences are shown in each line.
Ehrlichia: Ehrlichia sequences were obtained from nine individual ticks and three tick pools by PCR with rrs primers. The rrs sequences had the highest homology with E. ewingii (GenBank: U96436) (98.8–99.8%). The rrs positive ticks and tick pools were further amplified with gltA primers. The sequences obtained with gltA primers also had the highest homology with E. ewingii (GenBank: DQ365879) (90.3–90.9%). A phylogenic analysis with concatenated sequences of rrs and gltA indicated that all 11 Ehrlichia species were in the same cluster as E. ewingii, but in a distinct group, suggesting that this Ehrlichia species was a novel Ehrlichia species (Figure 2).

**Figure 2.** Phylogenetic tree of Ehrlichia species. The phylogenetic tree was constructed using the concatenated sequences of rrs and gltA. The tree was generated using the Maximum Likelihood method, the Kimura 2-parameter model, and 1000 replicates for bootstrap testing in MEGA 7.0 software. Only bootstrap values >50% were shown. *Ehrlichia* sequences obtained in this study are shown with dots. The scale bar indicates nucleotide substitutions per site. The *Ehrlichia* species' name and GenBank accession numbers of reference sequences are shown in each line. For the *Ehrlichia* species without complete genome sequences, the GenBank accession numbers in the order of rrs and gltA were DQ365879.1 and M73227.1 for *E. ewingii*; DQ365879.1 and U96436.1 for *E. ewingii*; MN685612.1 and MN658719.1 for *E. muris*; MN685612.1 and MN658719.1 for *E. muris*; and NR 148800.1 and JX629807.1 for *E. minasensis*.

Coxiella: Coxiella sequences were obtained from three individual ticks and one tick pool by PCR with omp primers. The omp sequences from ticks had the highest homology with C. burnetti (GenBank: CP014563) (99.5–99.8%). The omp positive tick samples were further amplified with icd primers, which showed that all four tick samples were positive. The icd sequences from ticks were 99.8–100% homologous with C. burnetii (GenBank: CP000107.1 for *E. canis*; CP0025749.1 for *E. canis*; NR 148800.1 and JX629807.1 for *E. minasensis*).
Figure 3. Phylogenetic tree of the *Coxiella* species. The phylogenetic tree was constructed based on the concatenated sequences of *omp* and *icd*. The tree was generated using the Maximum Likelihood method, the Kimura 2-parameter model, and 1000 replicates for bootstrap testing in MEGA 7.0 software. Only bootstrap values >50% were shown. *Coxiella* sequences obtained in this study are shown with dots. The scale bar indicates nucleotide substitutions per site. The *Coxiella* species' name and complete genome GenBank accession numbers of reference sequences are shown in each line.

Figure 4. Phylogenetic tree of the *Anaplasma* species. The phylogenetic tree was constructed based on the concatenated sequences of *rrs* and *groEL*. The tree was generated using the Maximum Likelihood method, the Kimura 2-parameter model, and 1000 replicates for bootstrap testing in MEGA 7.0 software. Only bootstrap values >50% were shown. *Anaplasma* sequences obtained in this study are shown with dots. The scale bar indicates nucleotide substitutions per site. The *Anaplasma* species' name and complete genome GenBank accession numbers of reference sequences are shown in each line.
3.3. Infection Rate of Tickborne Intracellular Bacteria in Ticks

The minimum infection rate (MIR) of pooled ticks was calculated by assuming only one tick was infected in a positive group, and the maximum infection rate (MAR) was calculated by assuming that all ticks were positive in a positive group. The MIR of all tickborne intracellular bacteria in the tested ticks was 22.4% (28/125) (Table 2). The MIR for all tickborne bacteria was 5% (1/20) for tick egg batches, and 83.3% (20/24) for dead engorged females. For molted adult ticks (females and males), the MIR was 8.6% (7/81) and the MAR was 60.5% (49/81). For a comparison of differences according to intracellular bacterial species, the prevalence of the novel *Ehrlichia* (9.6%, 12/125) was significantly higher than *A. bovis* (4%, 5/125) (*p* < 0.01), *C. burnetti* (3.2%, 4/125) (*p* < 0.01), *R. japonica* (3.2%, 4/125) (*p* < 0.01), or *R. raoultii* (2.4%, 3/125) (*p* < 0.01), but there was no significant difference among the last four bacterial species. For a comparison of differences according to groups of developmental stage, the prevalence of tickborne intracellular bacteria in dead engorged ticks was significantly higher than in molted adult ticks (*p* < 0.01) or eggs (*p* < 0.01), but there was no significant difference between molted adult ticks and eggs (*p* = 1).

**Table 2.** Prevalence of intracellular tickborne pathogens in ticks collected from hedgehogs in Hubei Province, China.

| Tick Species       | Year of Tick Collection | Pathogens          | Egg Batches (%) n = 20 | Dead Engorged Females (%) n = 24 | Melted Adults (%) n = 81 | Total % n = 125 |
|-------------------|-------------------------|--------------------|------------------------|-------------------------------|-------------------------|-----------------|
| *H. flava*        | 2018                    | *Anaplasma bovis*  | 0                      | 20.8                          | 0                       | 0               |
| *H. flava*        | 2018                    | *Coxiella burnetti*| 0                      | 12.5                          | 1.2                     | 8.6             |
| *H. flava*        | 2018                    | *Ehrlichia ewingii*| 0                      | 37.5                          | 3.7                     | 25.9            |
| *H. flava*        | 2018                    | *Rickettsia raulii*| 5                      | 0                             | 2.5                     | 17.3            |
| *H. flava*        | 2018                    | *Rickettsia japonica*| 0                     | 12.5                          | 1.2                     | 8.6             |
| *Total*           | 5                       |                    |                        | 83.3                          | 8.6                     | 60.5            |

Note: MIR = the minimum infection rate of pooled ticks and MAR = the maximum infection rate of pooled ticks.

GenBank deposition: the sequences of tickborne pathogens obtained in this study were deposited in GenBank with accession numbers: *A. bovis* rrs: MW275984–MW275987 and MN148605, and groEL: MW226869; *E. ewingii* rrs MN148606–MN148617, and gltA: MW226861–MW226866; *C. burnetti* icd: MW226857–MW226860, and omp: MW226877–MW226880. *Rickettsia* 17-kDa protein gene: MW226870–MW226876, gltA: MW226867 and MW226868, rrs: MW275981–MW275983, and ompA MW265948.

4. Discussion

We collected 125 ticks from 15 hedgehogs in Hubei Province, Central China, and all ticks were *H. flava*. We found three genera of *Rickettsiales*, including: *Rickettsia*, *Ehrlichia* and *Anaplasma*, and *Coxiella burnetti* in different developmental stages of *H. flava*, including eggs, adult ticks, and dead engorged females. We hypothesized that ticks in different stages should have a similar infection rate of intracellular bacteria as they were all collected from 15 hedgehogs. However, our results showed that the prevalence of intracellular bacteria was significantly higher in dead engorged ticks than in eggs and adult ticks molted from nymphs, indicating that there were far more pathogens in every tick immediately after bloodmeal than after the transition to the next stage of development. The transovarial and transstadial transmission reduction in the prevalence of ticks can be observed in all detected nonrelated pathogens. The engorged females were not accidentally killed during harvesting from hedgehogs due to their large body size. Even if the ticks were accidentally killed, this could not explain why the infection rate of the intracellular bacteria was significantly higher in the dead ticks than in the live ticks. The significant difference in the infection rates between the dead engorged females, and the tick eggs or molted adult ticks may be explained by two possibilities. One possibility is the detrimental effect of intracellular bacteria on ticks, i.e., intracellular bacteria might be detrimental to the engorged adult females, causing the death of engorged female adult ticks during oviposition; another possibility is the transstadial blockage of intracellular bacteria in ticks, i.e., ticks obtained
intracellular bacteria during feeding on hedgehogs, and the intracellular bacteria were lost during oviposition or molting due to these bacteria failing to be effectively transmitted transovarially or due to a transstadial blockage occurring in the molting ticks. A previous study showed that only 6% of *Ixodes ricinus* larvae could be infected by the European strain of tickborne encephalitis virus through transovarian transmission and that the Kyasanur forest disease virus could successfully pass transovarially in 59% of *Haemaphysalis spinigera* larvae [42]. Our previous study also demonstrated that 70% of *Haemaphysalis longicornis* ticks transovarially transmitted SFTSV and that only 20% transstadially transmitted SFTSV [30]. A previous study showed that if infected ticks were maintained on infection-free hosts for several generations, their pathogens would permanently disappear after 2–3 generations [43]. Our study and previous study suggested that ticks are not real reservoirs but only vectors for these tickborne pathogens.

An *Ehrlichia* species identified in this study was most closely related to *E. ewingii*, but in a distinct phylogenetic group, suggesting that it was a novel *Ehrlichia* species that needed to be further investigated. *Ehrlichia ewingii* had been reported to infect dogs and cause canine fever, thrombocytopenia, anorexia, polyarthritis, and central nervous system abnormalities [44]. The susceptible animal for this new *Ehrlichia* species needed to be investigated. *Coxiella burnetii*, the causative agents of Q fever, are broadly distributed in the environment. Livestock were identified as main reservoirs, which may infect people through their contaminative urine, feces, milk, and birth products. Our previous study had demonstrated that 12.2% of hedgehogs in Hubei Province were PCR-positive to *C. burnetii* [29]. Our studies indicated that both hedgehogs and their surface parasite ticks could serve as the animal host and vector for *C. burnetii*. Many kinds of animals could be infected by *A. bovis*, which caused animal abortions and the reduction of milk production and body weight, and which frequently led to death [45]. A previous study indicated the *A. bovis* infection of monkeys, suggesting that *A. bovis* may infect humans [46]. *Rickettsia japonica*, which was widely distributed in China (including in Henan [40], Anhui [7], Zhejiang [47], Shandong [48], and Hubei [26]) and *R. raoultii*, which was mainly reported around the border of China (like the Northeastern [49], Northwestern [50,51], Southwestern [52], Inner Mongolia [53], and Central [40] areas) belonged to the spotted fever group rickettsiae and could cause human fever, vomiting, nausea, maculopapular rash, and occasionally eschars at the site of inoculation [54].

To our knowledge, this is the first report about *C. burnetti*, *E. ewingii*, and *R. raoultii* in *H. flava*. *R. japonica*, *R. heilongjiangensis*, *Candidatus R. principis*, *R. felis*, and *R. helvetica* have been reported in *H. flava* in China, South Korea, and Japan [55–59]. These studies indicate that *H. flava* could transmit multiple rickettsial pathogens in Asia.

In conclusion, *H. flava* ticks collected from hedgehogs in Central China were infected with multiple intracellular bacterial pathogens, including *R. raoultii*, *R. japonica*, *E. ewingii*, *C. burnetti*, and *A. bovis*. The diseases caused by these pathogens need to be monitored in China.

**Author Contributions:** L.-Z.F., S.-C.L. designed the study and performed the experiments. S.-C.L., X.X., and X.-Q.G. participated in ticks’ sampling. Z.-J.Y. had applied statistical techniques and J.-W.L. designed the methodology. L.-Z.F., S.-C.L., H.Y. revised manuscript, and X.-J.Y. involved in funding acquisition, conceptualization, and manuscript revision. All authors read and approved the final manuscript.

**Funding:** This research was funded by the National Natural Science Funds of China grant number 81971939.

**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Ethics Committee of Prevention Medicine of Wuhan University (protocol code 2018010).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.
**Conflicts of Interest:** The authors declare no conflict of interest.

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