Co-infection of Anaplasma and Ehrlichia in Hedgehogs from China.

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Research article

Keywords: Hedgehogs, tick, Anaplasma, Ehrlichia, Candidatus. Neoehrlichia, Rickettsia.

Posted Date: September 16th, 2019

DOI: <https://doi.org/10.21203/rs.2.14515/v1>

Abstract

Hedgehogs (*Erinaceus amurensis*) is an insectivorous mammal frequently observed in the wild and around the residential areas. However, information about tick-borne diseases in this species is not well known. We investigated tick-borne rickettsial pathogens including *Anaplasma*, *Ehrlichia*, *Candidatus* *Neoehrlichia* and *Rickettsia* in hedgehogs collected in central China. Hedgehogs were captured from Hubei and Jiangxi Province with living traps. PCR amplification and DNA sequencing showed that 26% (19/73) hedgehogs were positive to *Anaplasma bovis*, 20.5% (15/73) were positive to a tentative new *Ehrlichia* species; in addition, 13.7% (10/73) hedgehogs were positive to *A. bovis* and *Ehrlichia* simultaneously. *Candidatus* *Neoehrlichia* and *Rickettsia* species was not detected among the 73 hedgehogs. We concluded that hedgehogs from central China were widely infected with *Anaplasma* and *Ehrlichia*, suggesting hedgehogs may play a role in the ecology of *Anaplasma* and *Ehrlichia*.

Background

Bacteria of the order Rickettsiales (*Alphaproteobacteria*) are obligate intracellular parasites of eukaryotes. At present, the order contains three established families (*Rickettsiaceae*, *Holosporaceae*, and *Anaplasmataceae*) as well as one proposed family (*Candidatus* *Midichloriaceae*) [1, 2]. Most described species of Rickettsiales are well known as emerging or reemerging zoonotic pathogens that may cause life-threatening diseases, including rickettsioses, ehrlichiosis, anaplasmosis and scrub typhus in humans and also be linked with devastating agricultural losses by infecting meat and milk producing animals [3, 4]. The number of newly discovered organisms in the Rickettsiales has markedly increased over the last 20 years and even bacteria that had previously been considered nonpathogenic are now associated with human disease [5, 6]. Rickettsiales are associated with a diverse host range, including diverse protists, annelids, arthropods, mammals and birds [5, 7, 8]. Although numerous studies conducted worldwide have focused on the ecology of Rickettsiales, investigation related to vector-pathogen interaction and the role of vertebrate hosts in the maintenance and dissemination of Rickettsiales in nature remain scarce [9].

Previous investigations showed that ectoparasites in hedgehogs carried several rickettsial agents [10, 11]. *Rickettsia helvetica*, *Candidatus* *Neoehrlichia mikurensis* and *Anaplasma phagocytophilum* were also detected in European hedgehogs (*Erinaceus europaeus*) in Europe [10, 12-14]. *Erinaceus europaeus* has also been suggested as reservoir hosts for *A. phagocytophilum* and different genospecies of the *Borrelia burgdorferi* sensu lato complex [15, 16]. Thus, hedgehog may play an important role in the transmission cycle of Rickettsiale species as well as acting as a reservoir. The aim of this study was to investigate the prevalence of *Rickettsia*, *Anaplasma* and *Ehrlichia* in hedgehogs from central China.

Results

Anaplasma in hedgehogs

PCR amplification with *Anaplasma rrs* primers showed that 19 (26%) hedgehogs were positive. DNA sequences analysis revealed that the sequences were 98.6-100% homologous to each other and they were 98.6-100% identical to *A.bovis* sequences derived from the blood of cattle and goat in Shaanxi Province, China (GenBank: MH255934 and MH255938). Phylogenetic analysis of two representative *rrs* sequences showed sequences from hedgehogs were in the same cluster with *A. bovis* detected in China, Japan, Korea and Malaysia (Fig.2). Additionally, all *Anaplasma rrs* positive samples were further genotyped using semi-nested PCR by targeting the *groEL* gene. A total of 18 highly identical (97.3%-100%) *groEL* sequences were finally obtained. BLAST analysis indicated all sequences were 97.86%-99.64% identical to the partial *groEL* sequence of *A.bovis* derived from a goat in Shaanxi Province (GenBank: MH255898). Phylogenetic analysis showed two representative *groEL* sequences obtained in the present study clustered with *A.bovis* detected in goats/cattle/ticks/mosquitos from Shaanxi and Hubei Province. They clearly formed a distinct clade and differ from *A.bovis* in other parts of China and the world (Fig.2).

***Ehrlichia* in hedgehogs**

PCR amplification with *Ehrlichia rrs* primers showed that 15 hedgehogs were positive. The 15 *rrs* positive hedgehogs were further amplified for the *groEL* and *gltA* genes. All 15 hedgehogs were positive to the *groEL*, but only 4 were *gltA* positive. BLAST analysis showed that the *rrs* sequences from hedgehogs were 99.13-99.35% identical to an *Ehrlichia* sequence (GenBank: KJ410252) derived from ticks in Xinjiang Province in western China. The four *gltA* sequences were 99.5-100% homologous and the *groEL* sequences detected from 15 hedgehogs were 99.1-100% identical to each other. In consistence with the *rrs* sequences, the *gltA* and *groEL* were highly homologous to *Ehrlichia* detected in ticks from various places in China including Xinjiang, Zhejiang, and Hubei in China, and *E. ewingii* (93.79%-95.04% for *groEL* and 91%-91.47% for *gltA*). Phylogenetic analysis based on the *rrs* showed that *Ehrlichia* sequences detected from hedgehogs formed a clade together with uncultured *Ehrlichia* species from ticks in Daishan County, Xinjiang provinces and *E. ewingii* (Fig.3). Phylogenetic analysis of *groEL* and *gltA* genes also showed that *Ehrlichia* detected in hedgehogs clustered together with uncultured *Ehrlichia* that was previously reported in ticks in Wuhan City, Xinjiang and Zhejiang provinces of China, and *E. ewingii* (Fig.4). The results indicated that the *Ehrlichia* from hedgehogs appeared to be a tentative new species which is taxonomically closely related to *E. ewingii*.

The infection rates of hedgehogs

The overall infection rate of *A.bovis* and *Ehrlichia* in hedgehogs was 26% and 20.5%, respectively. Co-infections were found in 10 hedgehogs (13.7%) (Table 2). No genomic DNA of *Ca. Neoehrlichia* and *Rickettsia* was detected in our survey.

Histopathological examination of a road-killed hedgehog

A seemingly intact but seriously road-injured adult hedgehog was found near a collection site in Wuhan city. The animal was later dead and dissected, tissue samples from liver, lung, kidney and spleen were

fixed in 10% formalin for further analysis and subsequent use (Fig.5). After 72 h, the samples were dehydrated, embedded in paraffin and cut into 5-mm thickness and stained with hematoxylin & eosin (H&E) (Fig.6).The animal was later molecularly screened positive both for *Anaplasma* and *Ehrlichia*.

Discussion

To the best of our knowledge, this is the first identification of *A. bovis* and a tentative new *Ehrlichia* sp. in peripheral blood of hedgehogs. These results suggest that hedgehogs could play a role in the circulation of *A. bovis* and this tentative new *Ehrlichia* species. In several studies, *Candidatus* Neoehrlichia mikurensis were identified in mosquitoes, rodents and humans in China [9, 27]. *Rickettsia japonica* is widely distributed in China [28, 29]. We tested the primers for *Rickettsia* by PCR with positive *R. japonica* sample and demonstrated that all primers worked well, suggesting that the hedgehogs tested in this study were truly negative to *Rickettsia* species. We do not have *Ca. Neoehrlichia* DNA and did not test the *Ca. Neoehrlichia* primers with positive control in our laboratory although the primers has been repeatedly demonstrated to work well by other investigators [24] . Therefore, we do not know the hedgehogs were truly negative or not to *Ca. Neoehrlichia*. This need to be further investigated by calibrating the primers and increasing sample size. *Anaplasma bovis* cause disease in livestock affecting animal health and economy. The major clinical symptoms of *A. bovis* infection include fever, anemia, drowsiness, convulsions, weight loss, and enlargement of lymph nodes [30-32]. There are several reports of ruminants, raccoons, cats and deer infected with *A. bovis* worldwide [33-36], but information about the epidemiology of this agent is scarce in small mammals. Previous phylogenetic analysis based on *groEL* gene revealed that all currently available *A. bovis* sequences in GenBank database were divided into four lineages, and all sequences reported in China were classified into three lineages, suggesting a great diversity of *A. bovis* in and outside China [20] .

Our study revealed that *A. bovis* infection is common in hedgehogs and the infected individuals may be subclinical since all the molecularly detected agents were found in apparently healthy animals. The histopathological examination of the infected road-killed hedgehog also supported this inference. The maintenance of *Ehrlichia* involve complex zoonotic systems including ticks and reservoir hosts. Previous studies indicated wide distribution and genetic diversity of *Ehrlichia* in Hubei [9, 21, 37, 38]. However, the natural system of *Ehrlichia* in this region is still little known. In traditional Chinese medicine, the skin of hedgehogs has long been used in treatments for hemorrhoids, thus this species is frequently taken from the wild and eaten across central China. Also, hedgehogs frequently forage long distances and hibernate near areas where humans and domestic animals live. Thus, hedgehogs may spread ticks and tick-borne diseases to humans and animals around and in long distance.

Conclusions

We first time detected *A. bovis* and a tentative new *Ehrlichia* species in hedgehogs collected from central China, suggesting that hedgehogs may be important in the ecology of these tick borne intracellular bacteria.

Methods

Hedgehog samples

From April, 2018 to June, 2019, hedgehogs were captured using traps baited with meat, and road killed or injured individuals were also permitted to be collected from Wuhan and Xianning cities, Hubei Province and Jiujiang city, Jiangxi Province of China (Fig.1). The hedgehogs were morphologically identified as *Erinaceus amurensis* as described previously [17]. Animals were classified into two age groups: young and adults. Age was estimated from the appearance of the animal, following the criteria set out by Robinson [18], gender was identified and recorded. Captured hedgehogs were anesthetized by an intramuscular injection (20 mg/kg) of Ketamine. One of the front feet was sterilized immediately after sedation, and one nail was clipped 3 mm short. Blood samples were immediately collected with 5ml centrifuge tubes and disposable plastic transfer pipettes. The sample volume ranged from 0.2-1ml. All blood samples were preserved with dry ice, and later stored in -80°C for further use. All sampled individuals were later released back to the wild.

PCR amplification of *Rickettsia*, *Anaplasma*, *Ca.Neoehrlichia* and *Ehrlichia*

Hedgehog blood DNA was extracted with the Qiagen DNA Kit (Qiagen, Hilden, Germany). DNA concentration and purity were measured with an absorbance ratio of 260 to 280 nm by using DeNovix DS-11 spectrophotometer (DeNovix, Wilmington, DE, USA) and were stored at -20°C until used. Mean quantity of DNA obtained was 38 ± 9.7 ng/ul and the 260/280nm ratio of all samples were 1.66 ± 0.27 . Blood DNA samples were used as templates for PCR amplification of *Ehrlichia*, *Ca.Neoehrlichia*, *Rickettsia* and *Anaplasma* DNA with primers described in Table 1. *Anaplasma* 16S rRNA (*rrs*) gene was amplified with a nested PCR by using the primers EHR1/EHR2 in the first-round reaction, and EHR3/EHR4 in the second-round reaction [19]. For *rrs* positive samples, *groEL* gene was targeted for further genotyping as described previously [20]. For *Ehrlichia*, nested PCR amplifications of *rrs*, heat shock protein gene (*groEL*) and citrate synthase gene (*gltA*) were performed [21, 22]. For detection of *Rickettsia*, nested PCR amplifications of *Rickettsia* *rrs*, *gltA*, and outer membrane protein B gene (*ompB*) were performed [23]. For *Ca.Neoehrlichia* detection, 16S rRNA were targeted [24]. DNA isolated from *A.bovis*, *E. chaffeensis* and *R. japonica* was served as positive controls. To avoid contamination, all steps were performed in separate rooms. Negative control with distilled water was run for each reaction.

PCR products were separated with 1.2% agarose gel electrophoresis and visualized with UV light after ethidium bromide staining. PCR products with expected sizes were excised from gels and extracted using a Gel Extraction Kit (Promega, Madison, WI, USA), which were then cloned into the pMD19-T vector (TaKaRa, Shiga, Japan). M13F-47, M13R-48 Universal Primers were used for Sanger dideoxy sequencing in TingKe Biotech Company (Wuhan, China) on both strands.

Phylogenetic analysis

All sequences were searched using BLAST in the GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). After alignment by ClustalW with MEGA 7.0 [25], the datasets were analyzed by jModeltest2 and the best evolutionary models were chosen [26]. Phylogenetic trees were constructed using the Maximum Likelihood method with the best model in MEGA 7.0, and the robustness of the trees was tested with 1,000 bootstrap replications.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Wuhan University (2018010). Hedgehogs were handled in accordance with good animal practices required by the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Consent for publication

Not applicable

Availability of data and materials

The *Anaplasma* and *Ehrlichia* sequences obtained in this study were deposited in GenBank under the following accession numbers: MH900201, MH900209, MN199181, MN199182, MH893644, MH893645, MH893652, MH893657, MH893658, MH893659, MH985746, MH934951, MH879865, and MH879866.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was financially supported by grants from National Natural Science Funds of China (Nos. 31570167). The funder had no role in study design, data collection and analysis, interpretation of data or writing of the manuscript.

Authors' contributions

XX and XJY designed the study. HJH, RQ, JWL, XX, XRQ, SCL, MC, CMZ, LZP, XJM and XQG participated in hedgehog sampling and performed the experiments. HJH and RQ helped in data analysis. XX and XJY wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors would like to thank Li-Bin Duan for the assistance of hedgehog collection.

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Tables

Table 1. PCR primers used in this study.

	PCR method	Primer	Primer sequences (5'→3')	Target gene	Annealing temp (°C)	Amplicon size (bp)	References
a	Nested PCR	16S-EC9-F	TACCTTGTTACGACTT	<i>rrs</i>	41	1462	[24]
		16S-EC12A-R	TGATCCTGGCTCAGAACGAACG				
		16S-IS58-62f	GGAATAGCTGTTAGAAATGACA		54	488	
		16S-IS58-594r	CTATCCTCTCTCGATCTCTAGTTT				
	Nested PCR	EC9	TACCTTGTTACGACTT	<i>rrs</i>	52	1462	[21]
		EC12A	TGATCCTGGCTCAGAACGAACG				
		HF51f	AAGTCGAACGGACAATTACC		55	923	
		HF954r	GTTAGGGGGATACGACCTTC				
		5gltA-out	GGCATTITTCCTGATGTGCATGAT	<i>gltA</i>	60	897	[22]
		3gltA-out	ATACCATTGAGCCGACCAGCC				
		5gltA-in	AGCAGTGCTCTCAAATTGCAGG		56	426	
		3gltA-in	ATCCTATGGCCAAAACCCATTA				
		5GroELout	GTACGGCTGGACCTAAAGGA	<i>groEL</i>	60	701	[22]
		3GroELout	AGTGCTGAGAGCTTCACCTTC				
		5GroEL-in	ATGGGGCACCAGAAGTTACA		56	422	
		3GroEL-in	CCACGATCAAATTGCATACCATCA				
	Nested PCR	EHR1	GAACGAACGCTGGCGGCAAGC	<i>rrs</i>	57	691	[19]
		EHR2	AGTA[T/C]CG[A/G]ACCAGATAGCCGC				
		EHR3	TGCATAGGAATCTACCTAGTAG		60	524	
		EHR4	CTAGGAATTCCGCTATCCTCT				
	Semi-nested PCR	groEL-F1	GTTGCGCAGTATTTTGCCAGT	<i>groEL</i>	50		[20]
		groEL-R	CTGCRITCAGAGTCATAAATAC				
		groEL-F2	ATCTGGAAGRCCACTATTGAT		50	845	
		groEL-R	CTGCRITCAGAGTCATAAATAC				
	Nested PCR	S1	TGATCCTGGCTCAGAACGAAC	<i>rrs</i>	55	1486	[23]
		S2	TAAGGAGGTAATCCAGCCGC				
		S3	AACACATGCAAGTCGRACGG		52	1371	
		S4	GGCTGCCTCTTGCGTTAGCT				
		gltA1f	GATTGCTTTACTTACGACCC	<i>gltA</i>	52	1087	[23]
		gltA1r	TGCATTTCITTTCCATTGTGC				
		gltA2f	TATAGACGGTGATAAAGGAATC		53	667	
		gltA2r	CAGAACTACCGATTTCITTTAAGC				
		B1f	ATATGCAGGTATCGGTACT	<i>ompB</i>	56	1355	[23]
		B1r	CCATATACCGTAAGCTACAT				
		B2f	GCAGGTATCGGTACTATAAAC		56	843	
		B2r	AATTTACGAAACGATTACTTCCGG				

Table 2. PCR detection of *A. bovis* and *Ehrlichia* from hedgehogs captured in central China from April, 2018-June, 2019.

Hedgehogs		<i>A. bovis</i> (%)	<i>Ehrlichia</i> sp. (%)	Co-infection (%)
Location				
Wuhan	31	6 (19.4)	6(19.4)	1 (3.2)
Xianning	21	7(33.3)	5(23.8)	5(23.8)
Jioujiang	21	6(28.6)	4(19)	4(19)
Total	73	19(26)	15(20.5)	10(13.7)
Age				
Adult	45	14(31.1)	10(22.2)	9(20)
Young	28	5(17.9)	5(17.9)	1(3.6)
Gender				
Male	42	12(28.6)	8(19)	7(16.7)
Female	31	7(22.6)	7(22.6)	3(9.7)

Figures

Figure 1

The location of collection sites (red) of hedgehogs in central China. The map was constructed using R 3.3.2 software (<https://www.r-project.org/>).

Figure 2

Maximum likelihood phylogenetic trees of *Anaplasma* based on the *rrs* and *groEL* genes. The trees were constructed with the *rrs* sequences (524bp) and *groEL* sequences (840bp) by using the Kimura 2-parameter model with MEGA 7.0 (<http://www.megasoftware.net>); we calculated bootstrap values with 1,000 replicates. Sequences number of *Anaplasma* detected in hedgehogs in this study are in bold print and marked by circles. Scale bar indicates nucleotide substitutions per site.

Figure 3

Maximum likelihood phylogenetic tree based on the *rrs* gene of *Ehrlichia*. The phylogenetic trees were constructed by using the Kimura 2-parameter model with MEGA 7.0 (<http://www.megasoftware.net>). The bootstrap values were calculated with 1,000 replicates. Sequences number of *Ehrlichia* detected in hedgehogs in this study are in bold print and marked by circles. Scale bar indicates nucleotide substitutions per site.

Figure 4

Maximum likelihood phylogenetic tree based on the *gltA* and *groEL* genes of *Ehrlichia*. The phylogenetic trees were constructed by using the Kimura 2-parameter model and general time reversible model respectively with MEGA 7.0 (<http://www.megasoftware.net>). The bootstrap values were calculated with 1,000 replicates. Sequences number of *Ehrlichia* detected in hedgehogs in this study are in bold print and marked by circles. Scale bar indicates nucleotide substitutions per site.

Figure 5

The physical examination of the road-killed hedgehog. a. Ticks bite on the skin; b. Normal body appearance; c. Lung; d. Unknown worms parasitized in liver.

Figure 6

The physical examination of the road-killed hedgehog. a. Ticks bite on the skin; b. Normal body appearance; c. Lung; d. Unknown worms parasitized in liver.