Epidemiology and Infection

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Zoonotic pathogens identified in rodents and shrews from four provinces, China, 2015–2022

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Original Paper

Cite this article: Tian S, Jiang B-G, Liu W-S, Chen H-R, Gao Z-H, Pu E-N, Li Y-Q, Chen J-J, Fang L-Q, Wang G-L, Du C-H and Wei Y-H (2023). Zoonotic pathogens identified in rodents and shrews from four provinces, China, 2015–2022. *Epidemiology and Infection*, **151**, e174, 1–12
https://doi.org/10.1017/S0950268823001450

Received: 09 January 2023
Revised: 20 May 2023
Accepted: 04 July 2023

Keywords:
co-infection; phylogenetic analysis; rodent; shrew; zoonotic pathogens

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Abstract

Rodents and shrews are major reservoirs of various pathogens that are related to zoonotic infectious diseases. The purpose of this study was to investigate co-infections of zoonotic pathogens in rodents and shrews trapped in four provinces of China. We sampled different rodent and shrew communities within and around human settlements in four provinces of China and characterised several important zoonotic viral, bacterial, and parasitic pathogens by PCR methods and phylogenetic analysis. A total of 864 rodents and shrews belonging to 24 and 13 species from *RODENTIA and EULIPOTYPHLA orders* were captured, respectively. For viral pathogens, two species of hantavirus (Hantaan orthohantavirus and Caobang orthohantavirus) were identified in 3.47% of rodents and shrews. The overall prevalence of *Bartonella* spp., Anaplasmataceae, *Babesia* spp., *Leptospira* spp., Spotted fever group Rickettsiae, *Borrelia* spp., and *Coxiella burnetii* were 31.25%, 8.91%, 4.17%, 3.94%, 3.59%, 3.47%, and 0.58%, respectively. Furthermore, the highest co-infection status of three pathogens was observed among *Bartonella* spp., *Leptospira* spp., and Anaplasmataceae with a co-infection rate of 0.46%. Our results suggested that species distribution and co-infections of zoonotic pathogens were prevalent in rodents and shrews, highlighting the necessity of active surveillance for zoonotic pathogens in wild mammals in wider regions.

Introduction

Emerging infectious diseases (EIDs) are a serious challenge to public health and economic development in the world. Over 70% of zoonotic EID events originate in wild animals [1]. Rodents and shrews live in close contact with humans and act as a bond among humans, domestic animals, and arthropod vectors [2]. As two common types of wild mammals, they are recognised as reservoir hosts for many zoonotic pathogens, for example, *Anaplasma*, *Babesia*, spotted fever group Rickettsiae (SFGR), *Borrelia,* and hantavirus [3, 4]. What is more, about 10% of the 2,277 living rodent species are hosts for 66 zoonotic pathogens, including viruses, bacteria, fungi, worms, and protozoans [5]. However, the links between these pathogens are still rarely investigated. Thus, a comprehensive understanding of zoonotic pathogens prevalence in rodents and shrews, as well as the geographical distribution and co-infection status, will be valuable for preventing and controlling wildlife-origin EIDs.

China is a megadiversity country and harbours various rodents and shrews. The rodents and shrews belong to the RODENTIA and EULIPOTYPHLA orders, and there are 235 species of 12 families and 92 species of 3 families in the RODENTIA and EULIPOTYPHLA orders in China, respectively [6]. Rodents and shrews can transmit zoonotic pathogens to human directly when humans consume food, water, or air that is contaminated with faeces and bodies [7] or indirectly when humans are bitten by arthropod vectors [8–13]. Especially, China is the most severe endemic country of haemorrhagic fever with renal syndrome caused by hantaviruses, with 20,000–50,000 human cases reported annually in China [14]. Most of the previous studies only focus on one or a few pathogens in rodents and shrews, and the source areas of samples are limited [15–18]. In this study, we screened representative zoonotic pathogens in a great variety of rodents and shrews from four provinces in China to identify the prevalence and co-infection of zoonotic pathogens.

Materials and methods

Sample collection

All animals were trapped in mist nets or harp traps using fried foods as bait at night between June 2015 and January 2022, and they were kept alive after being captured. A total of 864 wild rodents and shrews were captured in Guangdong, Henan, Inner Mongolia, and Yunnan provinces in China. The species of rodents and shrews were initially identified by experienced field biologists and then confirmed by sequencing of mitochondrial cytochrome b gene [19]. All animals were anesthetised with ether before they were executed, and all efforts were made to minimise the pain. Within 12 hours of placement of the capture tool, the captured animals were killed at the site of cervical dislocation. The heart, liver, spleen, lung, and kidney samples of each animal were collected and stored at liquid nitrogen until further use.

Nucleic acid extraction

The samples for further nucleic acid extraction were prepared by mixing equal amounts of tissues from five different organs, including heart, liver, spleen, lung, and kidney. And then the nucleic acid of each animal was extracted using AllPrep DNA/RNA Mini Kit (Qiagen, Cat. No. 80204, Hilden, Germany) according to the manufacturer's instructions. All DNA/RNA samples were stored at −80°C until use.

Hantavirus screening

The oligonucleotide primers used for nested reverse transcription-polymerase chain reaction (RT-PCR) and polymerase chain reaction (PCR) product sizes are shown in Supplementary Table S1 [20]. The second (nested) PCR was performed using the same reaction solution as the first PCR, and the first PCR product used as the template for the second PCR. Positive and negative controls were included in each PCR run. The first PCR was performed in a total volume of 25 μL, including 1 μL of PrimeScript One-Step Enzyme Mix, 12.5 μL of 2X One-Step Buffer (Dye Plus) (Takara, Cat. No. 057A, Maebashi, Japan), 1 μL of LF-1 forward primer and 1 μL of LR-1 reverse primer, 2 μL RNA, and 7.5 μL dH_2O. The first amplification of PCR products consisted of 38 cycles (30 s at 94°C, 30 s at 54°C, and 45 s at 72sC) was performed using PCR System 9700 (Applied Biosystems, San Francisco, CA). Then, the 2 μL DNA product amplified by the first PCR was used as the template, and the forward (LF-2) and reverse (LR-2) primers were used for PCR detection under similar amplification conditions [20].

Bacterial and parasitic pathogens screening

The amplification conditions and primers of PCR used in this study can be found in Supplementary Table S1 [21–27]. For the nested PCR assays, both the first and second reactions were run to a final volume of 25 μL, including 12.5 μL of DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA), 0.5 μL of 2 μM forward and reverse primers (Sangon Biotech, Shanghai, China), 9.5 μL RNase-free ddH_2O, and 2 μL of genomic DNA sample for the first PCR or 2 μL of the first PCR product for the second PCR. Positive and negative controls were run alongside the samples in each assay to eliminate the interference of false negative and false positive.

Sequencing and phylogenetic analysis

The amplified products were first detected by 1.5% agarose gel electrophoresis and then sequenced by the Sanger method. The sequences were assembled by CLC Genomics Workbench 3, and the low-signal sequences at both ends were discarded and entered into NCBI for homology search by BLAST. The multiple alignments were done by the MAFFT version 7 programme with default parameters. Relationships between individuals were assessed using a maximum-likelihood method with nucleotide distance with 1,000 replications for a bootstrap test. The phylogenetic trees of nucleotide sequences (547-bp L segment of hantavirus (GTR + G + I), 210-bp ITS gene of *Bartonella* spp. (HKY + G), 280-bp 16S rRNA gene of Anaplasmataceae (K2 + G), 153-bp 18S rRNA gene of *Babesia* spp. (HKY + G), 1,230-bp 16S rRNA gene of *Leptospira* spp.(K2 + G + I), 347-bp OmpA gene of SFGR (T92 + G), 347-bp 16S rRNA gene of *Borrelia* spp.(K2 + G), and 710-bp 16S rRNA gene of *Coxiella burnetii* (HKY + G + I)) were constructed using the MEGA X software.

Statistical analysis

The chi-square or Fisher's exact test was used for the prevalence comparison of the zoonotic pathogens among the different geographic region. Statistical analyses were performed by R software (version 3.5.3). All statistical tests were two-tailed, and *P*-value of lower than 0.05 was considered significant.

Result

Sample collection and species identification

From June 2015 to January 2022, a total of 864 wild small mammals, including rodents and shrews, were captured in Guangdong, Henan, Inner Mongolia, and Yunnan provinces in China (Figure 1). Species identification showed that these captured animals included 24 species of Cricetidae, Muidae, Sciuridae, and Spalacidae families from the RODENTIA order, and 13 species of Erinaceidae, Soricidae, and Talpidae families of the EULIPOTYPHLA order. Regarding the sampling provinces, we found that the most abundant species of rodents and shrews were collected from Yunnan province (27 species), followed by Henan (8), Guangdong (5), and Inner Mongolia (4) (Figure 1 and Table 1).

Prevalence and distribution of zoonotic pathogens in rodents and shrews

All 864 small mammals from 37 species were screened for eight different zoonotic pathogens. All eight zoonotic pathogens can be detected in Henan province, whereas *Leptospira* spp. and *C. burnetii* were not found in Guangdong, Yunnan, and Inner Mongolia provinces (Figure 2 and Table 1). In detail, we found that 30 (3.47%) samples from rodents and shrews were positive for hantavirus. The overall number and prevalence of *Bartonella* spp., Anaplasmataceae, *Babesia* spp., *Leptospira* spp., SFGR., *Borrelia* spp. and *C. burnetii* were 270 (31.25%), 77 (8.91%), 36 (4.17%), 34 (3.94%), 31 (3.59%), 30 (3.47%), and 5 (0.58%), respectively. The zoonotic pathogens were detected from 33 species (89.2%), including 22 species (91.7%) of the RODENTIA order and 10 species (76.9%) of the EULIPOTYPHLA order.

https://doi.org/10.1017/S0950268823001450 Published online by Cambridge University Press

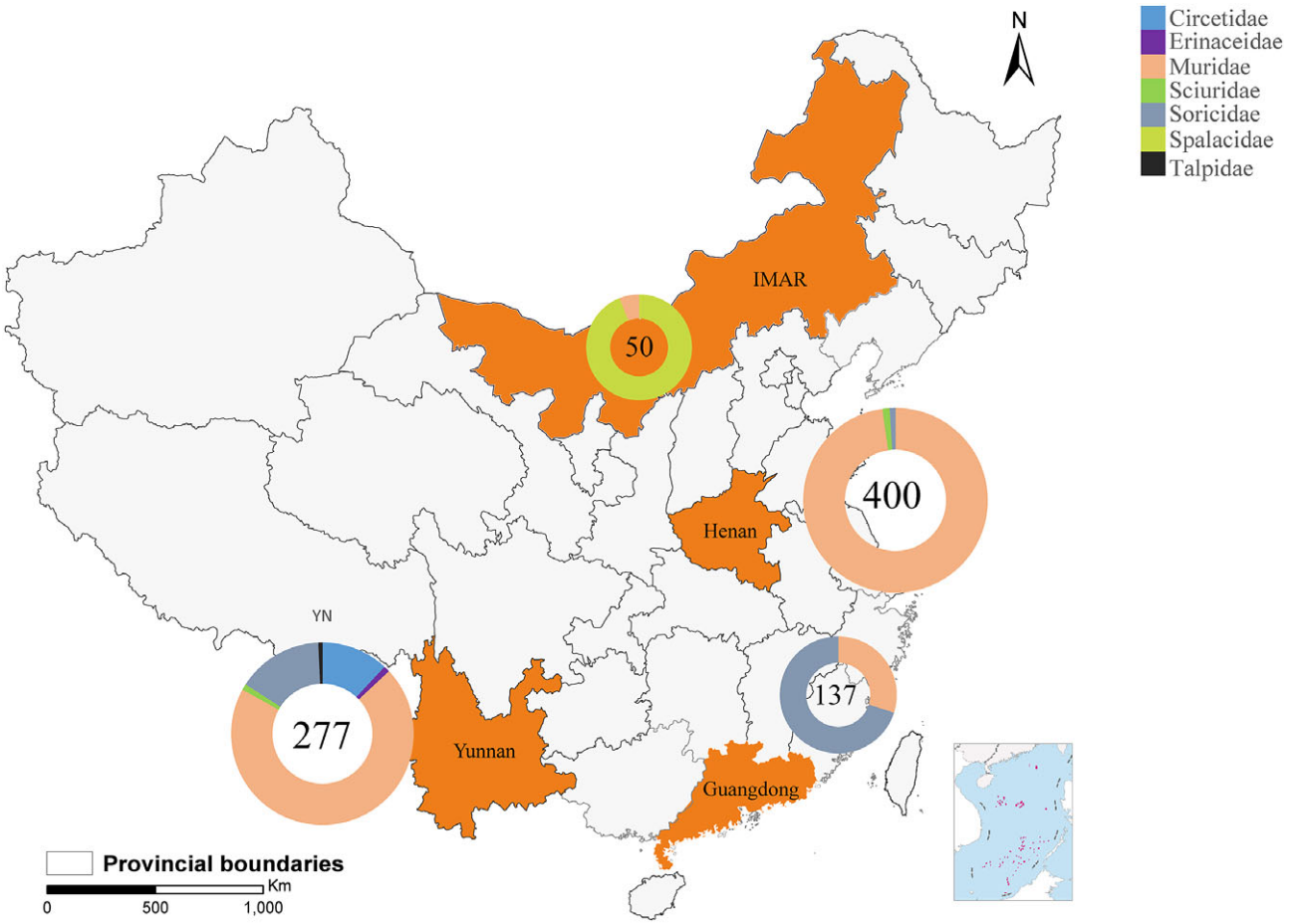

Figure 1. Geographical distribution of rodents and shrews in four provinces of China. From June 2015 to January 2022, small wild animals were collected in different parts of China. The total number of small wild animals and the proportion at the level of family collected from each region were shown.

The distribution of the zoonotic pathogens in different species and provinces can be found in Table 1. Hantavirus were detected in eight (8/37, 21.62%) species, including six species of the RODENTIA order and two species of the EULIPOTYPHLA order. The statistical analysis demonstrated that no significant difference of hantavirus prevalence was found between samples from EULIPOTYPHLA and RODENTIA (Supplementary Table S3). The number of positive results against hantavirus was mostly detected in *Rattus tanezumi* (10 animals), *Anourosorex squamipes* (6), and *Apodemus chevrieri* (5), respectively. Yunnan province had the highest prevalence of 90% (27/30) for hantavirus.

Bartonella spp. was detected from 28 (28/37, 75.68%) species, including 20 species of the RODENTIA order and 8 species of the EULIPOTYPHLA order. The number of positive results against *Bartonella* spp. was mostly detected in *R. tanezumi* (51 animals), *Suncus murinus* (46), and *Apodemus ilex* (35), respectively. Yunnan province had the highest prevalence of 46.30% (125/270) for *Bartonella* spp. (Table 1). The prevalence of *Bartonella* spp. was significantly higher in samples from RODENTIA than those from EULIPOTYPHLA (Supplementary Table S3).

Anaplasmataceae was found from 14 (14/37, 37.8%) species, including 12 species of the RODENTIA order and 2 species of the EULIPOTYPHLA order. Anaplasmataceae was mostly detected in *A. ilex* (26 animals) and *R. tanezumi* (17), respectively. Yunnan province had the highest prevalence of 53.25% (41/77) for Anaplasmataceae (Table 1). No significant prevalence of Anaplasmataceae was observed in samples from EULIPOTYPHLA and RODENTIA (Supplementary Table S3).

Babesia spp. was found from eight (8/37, 21.62%) species, including six species of the RODENTIA order, two species of the EULIPOTYPHLA order. *Babesia* spp. was mostly found in *A. ilex* (19 animals) and *R. tanezumi* (11), respectively. All the positive samples against *Babesia* spp. were collected from Yunnan province, with the prevalence of 86.11% (31/36) (Table 1). There was no significant difference of *Babesia* spp. prevalence in samples between EULIPOTYPHLA and RODENTIA (Supplementary Table S3). *Leptospira* spp. was only found from four (4/37, 10.8%) species of the RODENTIA order. The number of positive results against *Leptospira* spp. was mostly detected in *Apodemus agrarius* (14 animals) and *R. tanezumi* (13), respectively. All the positive samples against *Leptospira* spp. were collected from Henan province (Table 1 and Supplementary Table S3).

Borrelia spp. was found from 10 (10/37, 27%) species, including 8 species of the RODENTIA order and 2 species of the EULIPOTYPHLA order. *Borrelia* spp. was mostly detected in *R. tanezumi* (eight animals) and *S. murinus* (seven animals). Henan province had the highest prevalence of 45.16% (14/31) for *Borrelia* spp. (Table 1).

SFGR was only found from six (16.2%) species of the RODENTIA order and was mostly detected in *R. tanezumi* (19 animals). Henan province had the highest positive rate of 7.25% (29/400) for SFGR (Table 1).

https://doi.org/10.1017/S0950268823001450 Published online by Cambridge University Press

Table 1. Detection rate of rodent species with different pathogens in Henan, Guangdong, Inner Mongolia, and Yunnan from 2015 to 2022

Order	Family	Species	Hantavirus		Anaplasmataceae		*Bartonella* spp.		*Borrelia* spp.		*Coxiella burnetii*		*Leptospira* spp.		SFGR		*Babesia* spp.	
			Positive number (GD/HN/IMAR/YN)	Total (Positive %)	Positive number (GD/HN/IMAR/YN)	Total (Positive %)	Positive number (GD/HN/IMAR/YN)	Total (Positive %)	Positive number (GD/HN/IMAR/YN)	Total (Positive %)	Positive number (GD/HN/IMAR/YN)	Total (Positive %)	Positive number (GD/HN/IMAR/YN)	Total (Positive %)	Positive number (GD/HN/IMAR/YN)	Total (Positive %)	Positive number (GD/HN/IMAR/YN)	Total (Positive %)
EULIPOTYPHLA	Erinaceidae	*Hylomys suillus*	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2
		Neotetracus sinensis	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1
			0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/0	0/3
	Soricidae	*Anourosorex squamipes*	0/0/0/6	6/15 (40.0)	0/0/0/0	0/15	0/0/0/7	7/15(46.7)	0/0/0/1	1/15 (6.67)	0/0/0/0	0/15	0/0/0/0	0/15	0/0/0/0	0/15	0/0/0/1	1/15 (6.67)
		Blarinella wardi	0/0/0/0	0/4	0/0/0/0	0/4	0/0/0/1	1/4 (25.0)	0/0/0/0	0/4	0/0/0/0	0/4	0/0/0/0	0/4	0/0/0/0	0/4	0/0/0/0	0/4
		Crocidura attenuata	0/0/0/0	0/5	0/0/0/0	0/5	0/0/0/1	1/5 (20.0)	0/0/0/0	0/5	0/0/0/0	0/5	0/0/0/0	0/5	0/0/0/0	0/5	0/0/0/0	0/5
		Crocidura dracula	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2
		Crocidura tanakae	0/0/0/0	0/5	1/0/0/0	1/5 (20.0)	0/0/0/0	0/5	0/0/0/0	0/5	0/0/0/0	0/5	0/0/0/0	0/5	0/0/0/0	0/5	0/0/0/0	0/5
		Cryptotis niausa	0/0/0/1	1/1 (100)	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1
		Episoriculus macrurus	0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/1	1/3 (33.3)	0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/0	0/3
		Sorex bedfordiae	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/1	1/8 (12.5)	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/0	0/8
		Suncus murinus	0/0/0/0	0/99	5/0/0/1	6/99 (6.06)	44/0/0/2	46/99 (46.5)	7/0/0/0	7/99 (7.07)	0/0/0/0	0/99	0/0/0/0	0/99	0/0/0/0	0/99	1/0/0/0	1/99 (1.01)
			0/0/0/7	7/142 (4.93)	6/0/0/1	7/142 (4.93)	44/0/0/13	57/142 (40.1)	7/0/0/1	8/142 (5.6)	0/0/0/0	0/142	0/0/0/0	0/142	0/0/0/0	0/142	1/0/0/1	2/142 (1.41)
	Talpidae	*Uropsilus atronates*	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/1	1/1 (100.0)	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1
		Uropsilus nivatus	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/1	1/1 (100.0)	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1
			0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/2	2/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2
	Total		0/0/0/7	7/147 (4.76)	6/0/0/1	7/147 (4.76)	44/0/0/15	59/147 (40.1)	7/0/0/1	8/147 (5.44)	0/0/0/0	0/147	0/0/0/0	0/147	0/0/0/0	0/147	1/0/0/1	2/147 (1.39)
RODENTIA	Cricetidae	*Eothenomys cachinus*	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1
		Eothenomys eleusis	0/0/0/1	1/8 (12.5)	0/0/0/1	1/8 (12.5)	0/0/0/2	2/8 (25.0)	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/1	1/8((12.5)
		Eothenomys miletus	0/0/0/1	1/16 (6.25)	0/0/0/0	0/16	0/0/0/5	5/16 (31.3)	0/0/0/2	2/16 (12.5)	0/0/0/0	0/16	0/0/0/0	0/16	0/0/0/0	0/16	0/0/0/0	0/16
		Eothenomys proditor	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/2	2/8 (25.0)	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/0	0/8
			0/0/0/2	2/33 (6.06)	0/0/0/1	1/33 (3.03)	0/0/0/9	9/33 (27.3)	0/0/0/2	2/33 (6.06)	0/0/0/0	0/33	0/0/0/0	0/33	0/0/0/0	0/33	0/0/0/1	1/33 (3.03)
	Muridae	*Apodemus agrarius*	0/0/0/0	0/39	0/4/0/0	4/39 (10.3)	0/20/0/0	20/39 (51.3)	0/0/0/0	0/39	0/1/0/0	1/39 (2.56)	0/14/0/0	14/39 (35.9)	0/2/0/0	2/39 (5.13)	0/1/0/0	1/39 (2.56)
		Apodemus chevrieri	0/0/0/5	5/56 (8.93)	0/0/0/1	1/56 (1.79)	0/0/0/34	34/56 (60.7)	0/0/0/0	0/56	0/0/0/0	0/56	0/0/0/0	0/56	0/0/0/0	0/56	0/0/0/0	0/56
		Apodemus ilex	0/0/0/5	5/80 (6.25)	0/0/0/26	26/80 (32.5)	0/0/0/35	35/80 (43.8)	0/0/0/1	1/80 (1.25)	0/0/0/0	0/80	0/0/0/0	0/80	0/0/0/0	0/80	0/0/0/19	19/80 (23.8)
		Berylmys bowersii	0/0/0/0	0/1	0/0/0/1	1/1 (100)	0/0/0/1	1/1 (100)	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1
		Chiropodomys gliroides	0/0/0/1	1/1 (100)	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1
		Melomys burtoni	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2
		Micromys minutus	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/1	1/1 (100)	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1
		Mus musculus	0/0/0/0	0/60	0/0/0/0	0/60	0/0/0/0	0/60	0/4/0/0	4/60 (6.67)	0/0/0/0	0/60	0/0/0/0	0/60	0/4/0/0	4/60 (6.67)	0/0/0/0	0/60
		Mus pahari	0/0/0/0	0/6	0/0/0/1	1/6 (16.7)	0/0/0/5	5/6 (83.3)	0/0/0/0	0/6	0/0/0/0	0/6	0/0/0/0	0/6	0/0/0/0	0/6	0/0/0/0	0/6
		Niviventer andersoni	0/0/0/0	0/4	0/0/0/1	1/4 (25.0)	0/0/0/3	3/4 (75.0)	0/0/0/0	0/4	0/0/0/0	0/4	0/0/0/0	0/4	0/0/0/0	0/4	0/0/0/0	0/4
		Niviventer confucianus	0/0/0/0	0/20	0/12/0/0	12/20 (60.0)	0/8/0/0	8/20 (40.0)	0/3/0/0	3/20 (15.0)	0/1/0/0	1/20 (5.00)	0/6/0/0	6/20 (30.0)	0/3/0/0	3/20 (15.0)	0/0/0/0	0/20
		Niviventer niviventer	0/0/0/0	0/9	0/2/0/0	2/9 (22.2)	0/1/0/6	7/9 (77.8)	0/0/0/0	0/9	0/1/0/0	1/9 (11.1)	0/0/0/0	0/9	0/0/0/0	0/9	0/0/0/0	0/9
		Rattus andamanensis	0/0/0/0	0/11	0/0/0/0	0/11	3/0/0/0	3/11 (27.3)	1/0/0/0	1/11 (9.09)	0/0/0/0	0/11	0/0/0/0	0/11	0/0/0/0	0/11	0/0/0/0	0/11
		Rattus norvegicus	0/0/0/0	0/46	1/2/0/0	3/46 (6.52)	1/4/0/0	5/46 (10.9)	1/1/0/0	2/46 (4.35)	0/0/0/0	0/46	0/1/0/0	1/46 (2.17)	0/1/0/0	1/46 (2.17)	0/1/0/0	1/46 (2.17)
		Rattus pyctoris	0/0/0/0	0/1	0/0/0/0	0/1	0/0/1/0	1/1 (100)	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1	0/0/0/0	0/1
		Rattus tanezumi	0/3/0/7	10/292 (2.40)	1/7/0/9	17/292 (5.82)	2/37/0/12	51/292 (17.5)	1/6/0/1	8/292 (2.74)	0/2/0/0	2/292 (0.68)	0/13/0/0	13/292 (4.45)	0/19/0/0	19/292 (6.51)	0/1/0/10	11/292 (3.77)
			0/3/0/18	21/629 (3.34)	2/27/0/39	68/629 (10.8)	6/70/1/97	174/629 (27.7)	3/14/0/2	19/629 (3.02)	0/5/0/0	5/629 (0.79)	0/34/0/0	34/629 (5.41)	0/29/0/0	29/629 (4.61)	0/3/0/29	32/629 (5.09)
	Sciuridae	*Callosciurus erythraeus*	0/0/0/0	0/5	0/0/0/0	0/5	0/1/0/0	1/5 (20.0)	0/0/0/0	0/5	0/0/0/0	0/5	0/0/0/0	0/5	0/0/0/0	0/5	0/0/0/0	0/5
		Tamiops swinhoei	0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/3	3/3 (100)	0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/0	0/3	0/0/0/0	0/3
			0/0/0/0	0/8	0/0/0/0	0/8	0/1/0/3	4/8 (50)	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/0	0/8	0/0/0/0	0/8
	Spalacidae	*Myospalax aspalax*	0/0/0/0	0/2	0/0/0/0	0/2	0/0/1/0	1/2 (50.0)	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2	0/0/0/0	0/2
		Myospalax psilurus	0/0/0/0	0/45	0/0/1/0	1/45 (2.22)	0/0/23/0	23/45 (51.1)	0/0/2/0	2/45 (4.44)	0/0/0/0	0/45	0/0/0/0	0/45	0/0/1/0	1/45 (2.22)	0/0/1/0	1/45 (2.22)
			0/0/0/0	0/47	0/0/1/0	1/47 (2.13)	0/0/24/0	24/47 (51.1)	0/0/2/0	2/47 (4.26)	0/0/0/0	0/47	0/0/0/0	0/47	0/0/1/0	1/47 (2.13)	0/0/1/0	1/47 (2.13)
	Total		0/3/0/20	23/717 (3.21)	2/27/1/40	70/717 (9.76)	6/71/25/109	211/717 (29.4)	3/14/2/4	23/717 (3.21)	0/5/0/0	5/717 (0.70)	0/34/0/0	34/717 (4.74)	0/29/1/0	30/717 (4.18)	0/3/1/30	34/717 (4.74)
			0/3/0/27	30/864 (3.47)	8/27/1/41	77/864 (8.91)	50/71/25/124	270/864 (31.3)	10/14/2/5	31/864 (3.59)	0/5/0/0	5/864 (0.58)	0/34/0/0	34/864 (3.94)	0/29/1/0	30/864 (3.47)	1/3/1/31	36/864 (4.17)

*HN: Henan; GD: Guangdong; IMAR; Inner Mongolia; YN; Yunnan.

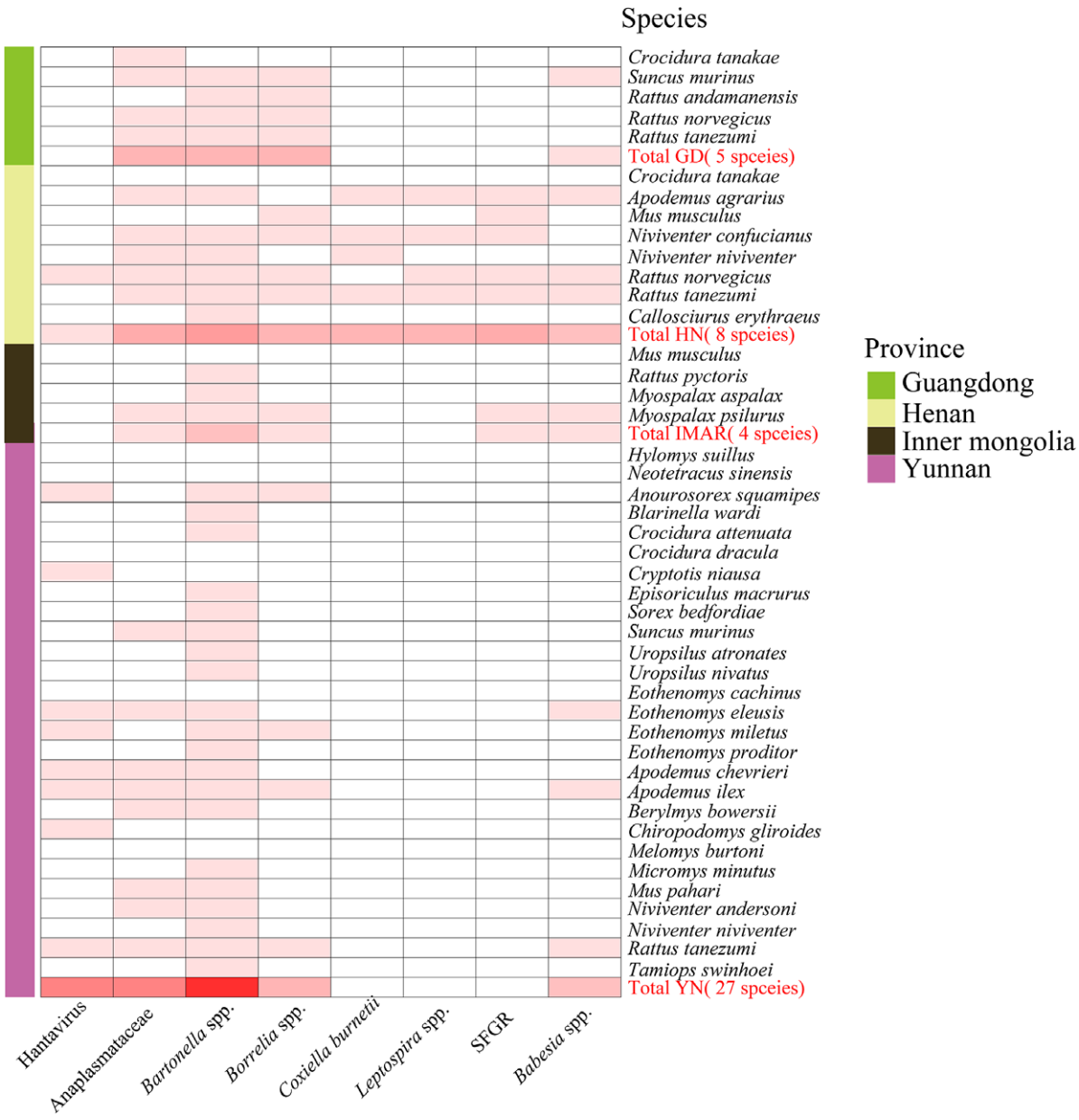

Figure 2. The carrying status of eight pathogens in rodents and shrews from four provinces.

C. burnetii was only found from four (10.8%) species of the RODENTIA order and was mostly detected in *R. tanezumi* (two animals). All the positive samples against *C. burnetii* were collected from Henan province (Table 1).

Co-infection status

A total of 127 samples (14.70%) possessed more than one zoonotic pathogen. And, two, three, four, and five different pathogens were found in 127, 24, 5, and 1 samples. Statistical analysis of all samples revealed that the highest co-infection status among three pathogens was *Bartonella* spp., *Leptospira* spp., and Anaplasmataceae with co-infection rate of 0.46%, and the top three co-infections with two zoonotic pathogens were *Bartonella* spp.–Anaplasmataceae (3.36%), Anaplasmataceae–*Babesia* spp. (2.08%), and *Bartonella* spp.–*Leptospira* spp. (1.50%). SC-21-14 (*Niviventer confucianus*, Henan) was co-infected with five pathogens at the same time. Relatively low co-infection rate was observed among other pathogens (Figure 3).

Phylogenetic analysis

Hantavirus

Phylogenetic analysis of the partial L gene showed that the hantavirus identified in the study formed into two distinct lineages with nucleotide identities of 71.4%–85.7% (Figure 4A). Seven strains in our study shared high sequence similarity and were closely related to the Caobang orthohantavirus found in Vietnam, in 2006, with 85.7% nucleotide identities and other six strains shared high sequence similarity and were closely related to the Hantaan orthohantavirus identified in China, in 2006, with 99.2%–99.7% nucleotide identities.

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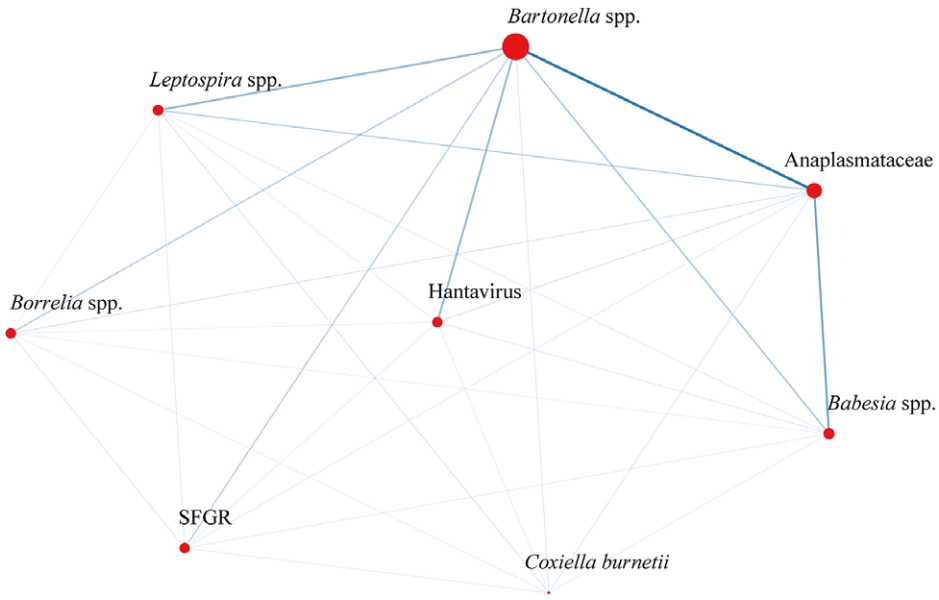

Figure 3. Co-infection between pathogens. The size of the circle represents the number of infections, and the thickness of the line represents the number of samples in which co-infections were present.

Bartonella spp.

Phylogenetic analysis of the partial ITS gene showed that the *Bartonella* spp. identified in the study formed into four distinct lineages with nucleotide identities of 47%–92.5% (Figure 4B). Nine strains shared high sequence similarity and were closely related to the *Candidatus Bartonella thailandensis* identified in Thailand, with 58.8%–91.5% nucleotide identities. YunL-39B (*Niviventer andersoni*, Yunnan) was closely related to the *Bartonella callosciuri* identified in Thailand, with 95.5% nucleotide identity. TC-19 (*R. tanezumi*, Yunnan) and YL-60A (*A. chevrieri*, Yunnan) were closely related to the *Bartonella silvatica* found in Japan, with 84.4%–92.5% nucleotide identities. 118A (*A. chevrieri*, Yunnan) was closely related to the *Bartonella japonica* identified in Japan, with 97.8% nucleotide identity. SC-21-123 (*R. tanezumi*, Henan) was closely related to the *Bartonella coopersplainsensis* found in Australian, with 100% nucleotide identity. TC-37 (*R. tanezumi*, Yunnan) was closely related to the *Bartonella elizabethae*, with 99.5% nucleotide identity.

Anaplasmataceae

Phylogenetic analysis of the partial 16S rRNA gene showed that the Anaplasmataceae identified in the study formed into three distinct lineages with nucleotide identities of 92.2%–95.4% (Figure 4C). Seven strains shared high sequence similarity and were closely related to the *Anaplasma* sp. identified in China, with 98.5%–100% nucleotide identities. Another seven strains were closely related to the *Ehrlichia minasensis* identified in Egypt, with 100% nucleotide identity, and four strains were closely related to the *Ehrlichia* sp. identified in China, with 100% nucleotide identity. In addition, four strains shared high sequence similarity and were closely related to the *Candidatus* Neoehrlichia mikurensis identified in China, with 98.5%–100% nucleotide identities.

Babesia spp.

Phylogenetic analysis of the partial 18S rRNA gene showed that the *Babesia* spp. identified in the study formed into two distinct lineages with nucleotide identities of 90.1%–91.6% (Figure 4D). Three strains were closely related to the *Babesia felis* identified in Romania, with 100% nucleotide identity, and another three strains shared high sequence similarity and were closely related to the *Babesia microti* found in Japan, with 97.4%–98.1% nucleotide identities.

Leptospira spp.

Phylogenetic analysis of the partial 16S rRNA gene showed that all *Leptospira spp.* identified in the study clustered into the same lineage and were closely related to the *Leptospira interrogans* identified in the Federation of Saint Kitts and Nevis, with 98.5%–100% nucleotide identities (Figure 4E).

Spotted fever group Rickettsiae

Phylogenetic analysis of the OmpA gene showed that the SFGR identified in the study formed into two distinct lineages with nucleotide identities of 91.7%–93.8% (Figure 4F). Five strains shared high sequence similarity and were closely related to the *Rickettsia raoultii* found in Russia, with 98.4%–100% nucleotide identities, and four strains shared high sequence similarity and were closely related to the *Rickettsia heilongjiangensis* identified in Russia, with 99.5%–100% nucleotide identities.

Borrelia spp.

Phylogenetic analysis of the partial 16S rRNA gene showed that the *Borrelia* spp. identified in the study formed into two distinct lineages with nucleotide identities of 92.2%–97% (Figure 4G). Four strains shared high sequence similarity and were closely related to the *Borrelia* spp. identified in Australia, with 99.2%–100% nucleotide identities. The nucleotide homology of the remaining two JP-14 (*A. squamipes*, Yunnan) and Yunl-26 (*A. ilex*, Yunnan) with *Borrelia miyamotoi* found in Japan was 97.9% and 98.3%, respectively. SG21-1 (*Rattus andamanensis*, Guangdong) was closely related to the *Borrelia yangtzensis* found in Japan, with 98.3% nucleotide identity.

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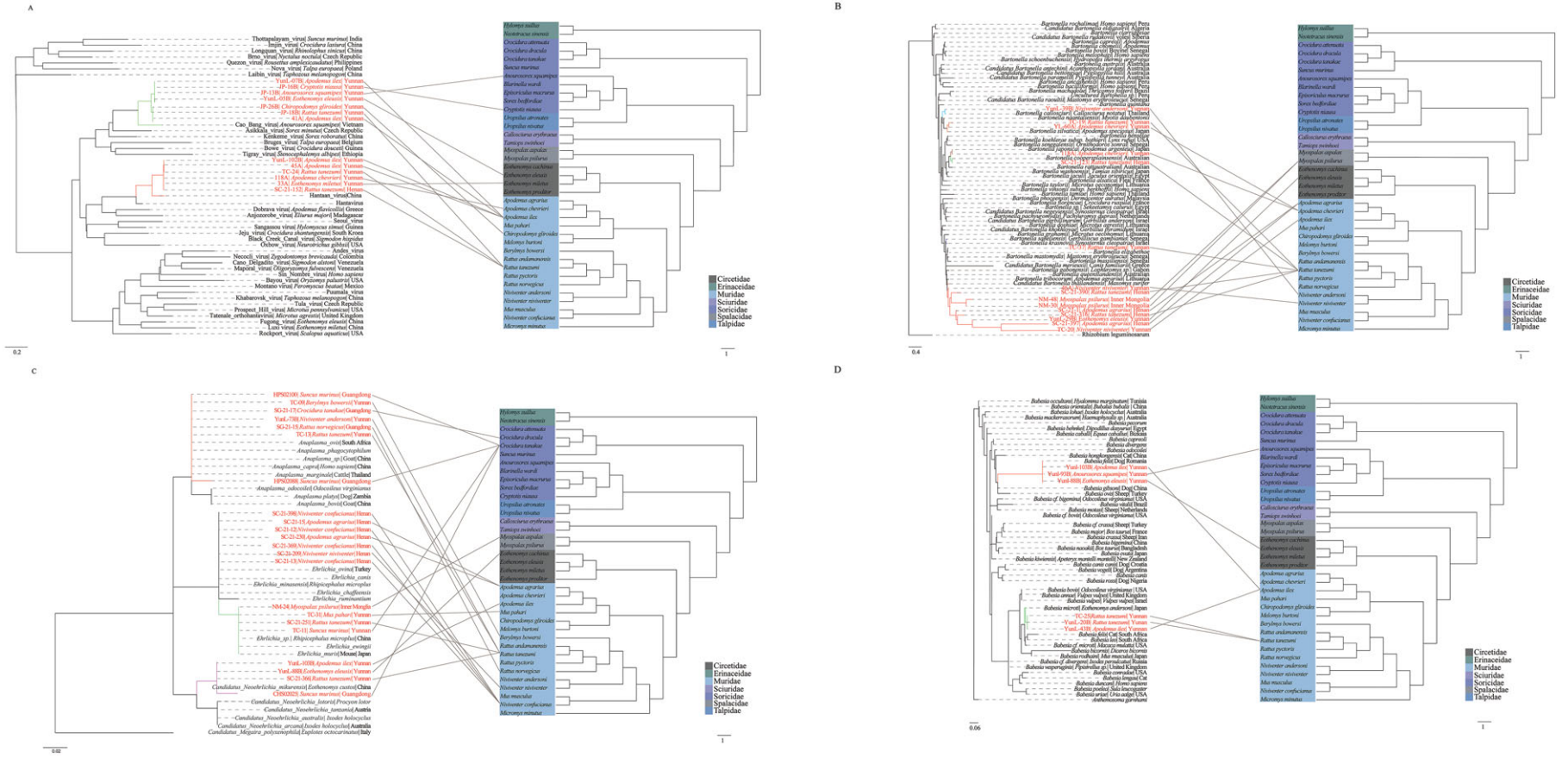

Figure 4. Rodents detect phylogenetic trees of pathogens. Phylogenetic trees based on the sequence of this study and the known species of this pathogen. The sequences obtained in this study are marked in red. (A) Hantavirus L segment. (B) *Bartonella* spp. ITS gene. (C) Anaplasmataceae 16S rRNA. (D) *Babesia* spp. 18S rRNA. (E) *Leptospira* spp. 16S rRNA. (F) SFGR OmpA gene. (G) *Borrelia* spp. 16S rRNA. (H) *Coxiella burnetii* 16S rRNA. The maximum likelihood tree was constructed under the best model, which was selected based on the BIC scores. Phylogenetic tree construction based on the MAGE X software. The link between the host and the detection of the pathogens.

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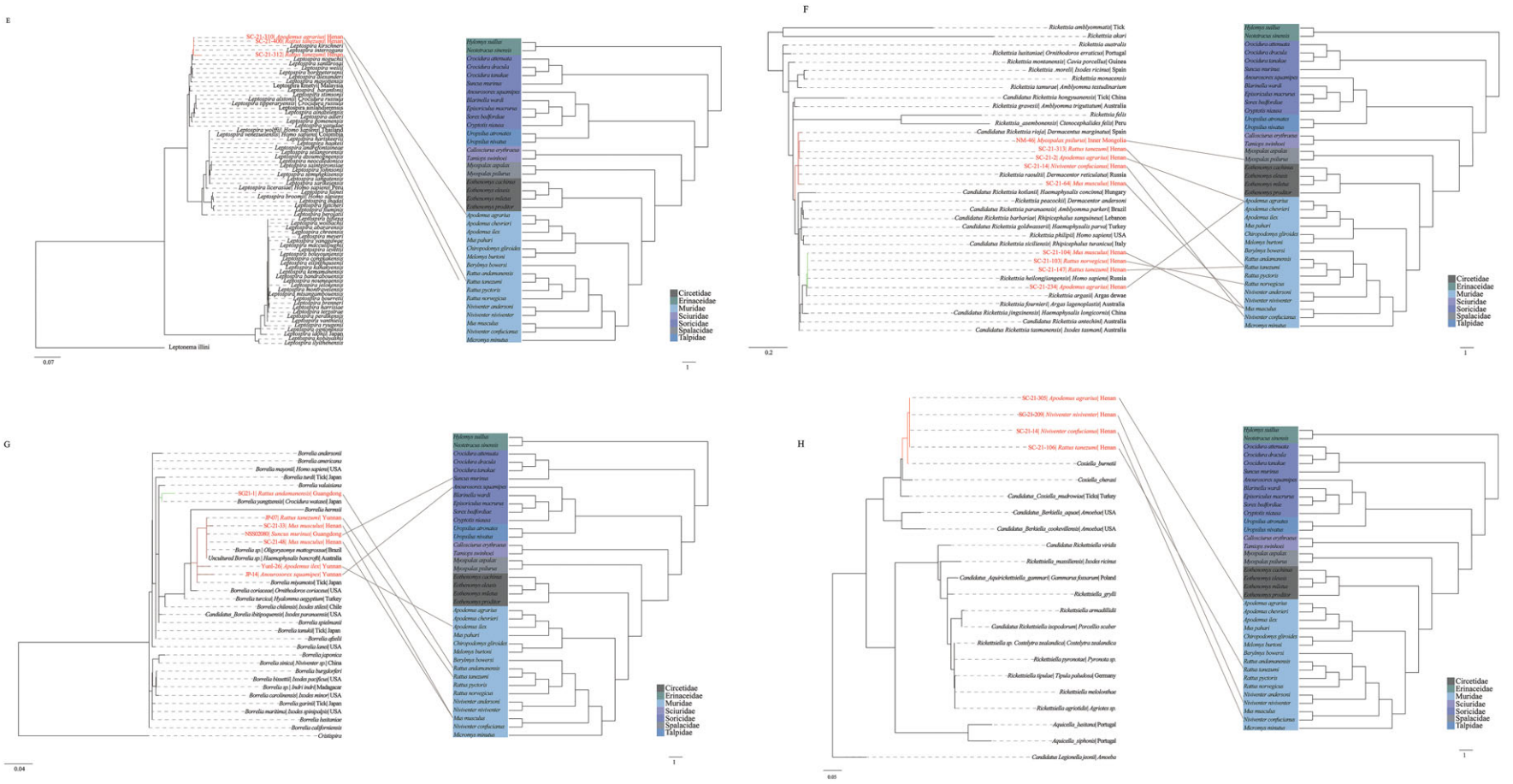

Figure 4. (Continued)

Coxiella burnetii

Phylogenetic analysis of the partial 16S rRNA gene showed that all *C. burnetii* identified in the study clustered into the same lineage and were closely related to the *C. burnetii* identified in Indonesia, in 2017, with 99.4% nucleotide identity (Figure 4H).

Discussion

Through active surveillance of eight zoonotic pathogens in rodents and shrews from four provinces in China, we found that zoonotic pathogens were ubiquitous in different species and the co-infection rates of zoonotic pathogens in the same samples were relatively high. The types of pathogens carried by samples from four provinces were slightly different. Eight, five, five, and four pathogen species carried by the sample were found in Henan, Inner Mongolia, Yunnan, and Guangdong, respectively. The highest positive results of hantavirus, Anaplasmataceae, *Bartonella* spp., and *Babesia* spp. were observed in Yunnan province, whereas Henan province had the most positive sample number against *Borrelia* spp., *C. burnetii*, *Leptospira* spp., and SFGR. Therefore, specific zoonotic pathogens surveillance should be conducted in different regions.

In recent decades, hantavirus is distributed globally and is mainly endemic in Asia, such as Seoul orthohantavirus and Hantaan orthohantavirus. More common in China are Seoul orthohantavirus and Hantaan orthohantavirus; Caobang orthohantavirus has not been found in mainland China but only in shrews from Taiwan, China [28]. Hantavirus has been found in 31 provinces in China, and the number of human cases in Zhejiang, Jiangsu, and other provinces has been on the rise since 2011 [29–33]. Our study found that the Caobang orthohantavirus were first identified in *A. squamipes* of Yunnan province. More importantly, the partial L gene of Caobang orthohantavirus identified in our study were clustered with but distinct from the previous Caobang orthohantavirus identified in Vietnam, with nucleotide identity lower than 90%. However, previous studies showed that the sero-positive rates of haemorrhagic fever with renal syndrome, Anaplasmosis, and Cat Scratch Disease in Yunnan were relatively low compared with those in other provinces [34–37]. Therefore, we should strengthen the surveillance of zoonotic pathogens in rodent and shrew species, and strengthen the protection of susceptible human groups by publicity, education, and vaccination. Moreover, the first report of Caobang orthohantavirus in *A. squamipes* in China expanded the currently known geographic scope, host types, and the genetic heterogeneity of Caobang orthohantavirus.

Anaplasmataceae mainly detected in ticks and domestic animals, which can cause an acute, nonspecific febrile illness of humans that named, Human granulocytotropic anaplasmosis [38]. In wild animals, the positive rates of Anaplasmataceae reported in Gansu and Xinjiang provinces were 0.7% and 19.2%, respectively [13, 39]. Our study showed that the positive rate of Anaplasmataceae from different provinces ranged from 2% to 14.8%.

Bartonella spp. positive samples were mostly identified in Yunnan province, but it was the dominant pathogen in samples from Inner Mongolia. Among all pathogens, *Bartonella* spp. had the highest positive rate (31.3%). Previous studies reported that the positive rates of *Bartonella* spp. in wild animals were 8.4%, 14.9%, 26.1%, and 57.7% in east, southeast, northwest, and northeast China, respectively [15, 40–43]. Consistent with those previous studies, the positive rate of *Bartonella* spp. from different provinces ranged from 17.8% to 50% in this study.

Between 1986 and 2020, a total of 2,584 confirmed human cases who infected by *Borrelia* spp. have been reported in 25 provinces of China [8]. Erythema migrans are often accompanied by discomfort, fatigue, headache, fever, and local lymph node enlargement when humans are infected by *Borrelia* spp. In wild animals, two previous studies showed that *Borrelia* spp. was found in 40.7% and 32% samples collected in Jiangxi province and Tianjin city [8]. However, only 3.6% samples in our study were detected positive for *Borrelia* spp.

Symptoms caused by *C. burnetii* usually manifest as pulmonary and influenza-like complications [44]. A previous study showed that the *C. burnetii* was only detected in *Rattus flavipectus* in Yunnan province in China with a positive rate of 15.9% [9]. Differently, our study found that the *C. burnetii* can also be found in four species in Henan province, with a positive rate of 0.58%, suggesting that the host range and geographical distribution of *C. burnetii* are much larger than ever reported.

Leptospirosis is a systemic disease of humans and domestic animals, characterised by fever, renal and hepatic insufficiency, pulmonary manifestations, and reproductive failure [45]. *L. interrogans* is the predominant *Leptospira* species in China and *A. agrarius* is the main animal host [46], and most of Leptospirosis cases were caused by *L. interrogans* [47]. Similarly, our study found that the *L. interrogans* was mostly detected in the species of *A. agrarius* in Henan province.

Clinical manifestations caused by SFGR include skin necrosis and acrogangrene, pneumonia, meningoencephalitis, and multiple organ failure [48]. Rodents play an important role in the interspecies transmission of SFGR from, and recent surveys have shown significant diversity, prevalence, and geographic distribution of Rickettsial bacteria in rodents [37, 49]. It was reported that the prevalence of SFGR were 13.95%, 16.05%, and 33.3% in southeast, southwest, and south of China, respectively [50–52]. However, SFGR was mostly found in the species of *R. tanezumi* in Henan province in our study, with a lower prevalence of 7.3%. Phylogenetic analysis demonstrated that *R. heilongjiangensis* was found in Henan, and *R. raoultii* was found in Inner Mongolia and Henan. *R. raoultii* and *R. heilongjiangensis* are both zoonotic pathogens. Rodents and shrews are extremely important natural hosts for the natural circulation of SFGR, posing increased threat to global public health [53].

Human babesiosis caused by *Babesia* spp. is an emerging tick-borne disease, and most of the cases are clinically asymptomatic [54]. *Babesia* spp. found in several provinces in China from 1940 to 2013, and a field investigation confirmed the presence of *Babesia* spp. in rodents near human cases infected in Henan province [55]. Previous surveillance showed that the positive rates of *Babesia* spp. in rodents were 3.94% and 12.1% in Beijing city and Fujian province, respectively [56, 57]. Similarly, our study showed that the positive rate of *Babesia* spp. may be vary in different provinces, ranged from 0.73% to 11.2%.

R. tanezumi was found to carry the largest number of zoonotic pathogen species (eight species), and it was distributed widespread in China [6]. However, due to the small number of animal samples collected in Inner Mongolia, *R. tanezumi* was captured in three provinces in our study, and the surveillance of *R. tanezumi* should be strengthened in the future. Our study also indicated that RODENTIA had significantly prevalence of *Bartonella* spp., *Leptospira* spp., and SFGR than EULIPOTYPHLA.

As rodents are important vectors for many pathogens, co-infection may have important consequences in terms of disease transmission and risk of zoonotic transmission [58]. A previous study of zoonotic pathogens in Austrian rodents reported double

https://doi.org/10.1017/S0950268823001450 Published online by Cambridge University Press

and triple infection rates of 6.4% and 1.8% [59]. Differently, our study found that the double and triple infection rates were 3.4% and 0.5%. The most common co-infection of the three pathogens, *Bartonella* spp.–*Leptospira* spp.–Anaplasmataceae, which was observed in four animals (three *N. confucianus* and one *A. agrarius*). The co-infection of four and five pathogens mainly occurred in *N. confucianus* in Henan province. The risk of host infection after interaction with the environment depends on the ability of zoonotic pathogens to persist, survive, and inter-species transmission [59, 60]. Surveillance should primarily focus on species of rodents or shrews that carried multiple pathogens.

There are three limitations in our study. First, we only screened several representative zoonotic pathogens in the rodents and shrews rather than for all pathogens through next-generation sequencing. Second, partial gene sequences were obtained rather than the whole genome for the zoonotic pathogens. Finally, the sampling number of rodents and shrews from different provinces and different species was varied.

In conclusion, our study expands the knowledge of the prevalence, distribution, and co-infection status against zoonotic pathogens in rodents and shrews from different provinces in China. The CaoBang orthohantavirus was first identified in the mainland of China, and prospective surveillance of zoonotic pathogens in wild animals is imperative.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0950268823001450.

Data availability statement. The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary Material.

Data availability statement. All data are incorporated into the article and its Supplementary Material.

Acknowledgements. We would like to thank all patients who contributed to the data and clinicians who collected the data. We gratefully acknowledge all authors from the submitting laboratories who contributed to sharing information to literature, books, and websites, as most of our analyses were made possible by the sharing of their work.

Author contribution. Y.-H.W. and C.-H.D. designed and supervised the research. Y.-H.W., B.-G.J., C.-H.D., W.-S.L., H.-R.C., Z.-H.G., E.-N.P., and Y.-Q.L. collected samples and data. S.T. and J.-J.C. performed laboratory testing. S.T., J.-J.C., and G.-L.W. analysed the data. S.T., G.-L.W., L.-Q.F., Y.-H.W., and C.-H.D. drafted the manuscript. All authors reviewed and approved the final manuscript.

Financial support. This work was supported by the Key Research and Development Program of China (Grant No. 2021YFC230200-12), Science and Technology Plan Grant of Guangzhou (Grant No. 202102080035), the Key Project of Medicine Discipline of Guangzhou (Grant No. 2021-2023-11) and the Basic Research Project of Key Laboratory of Guangzhou (Grant No. 202102100001). The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Competing interest. The authors declare no competing interest.

Ethical standard. The study protocol was approved by the Academy of Military Medical Sciences. All animals were treated by the guidelines in the regulations for the Administration of Laboratory Animals (Decree No. 2 of the State Science and Technology Commission of the People's Republic of China, 1988). All animals were treated strictly according to the guidelines for the rules for surveillance methods for vector density of Rodent (2009) from People's Republic of China Ministry of Health (GB/T23798-2009).

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