Original Article

First Molecular Evidence of *Anaplasma phagocytophilum* in Rodent Populations of Nanchang, China

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SUMMARY: In this study, systematic surveillance of rodent populations in Nanchang of China and determination of *Anaplasma phagocytophilum* infection in rodents were performed. Between 2011 and 2015, 110,084 rodent snap traps were set in 4 counties and in the city center of Nanchang, China. Finally, 942 rodents were captured, with a relative density of 0.86%. The densities varied considerably by geographical area with Anyi being the most rodent-infested County. Frequently captured rodents were sewer rats (*Rattus norvegicus*), house mice (*Mus musculus*), and *Rattus flavipectus*. The *Anaplasma* genera were investigated by PCR in 19 live rodents trapped by welded cages in Anyi, 6 rodents were assessed as positive based on amplification of 16S rRNA. Sequence analysis revealed 3 variants of *A. phagocytophilum* in Nanchang. PCR analysis of the *gltA* (citrate synthase) gene found 1 sample that was positive for *A. phagocytophilum* infection. The sequence of *A. phagocytophilum gltA* gene formed a clade with and showed 99% identity to *A. phagocytophilum* that has been previously described in rodents from South-Eastern China. Taken together, our research indicated that commensal rodents are potential hosts for *A. phagocytophilum* and controlling the rodent population may facilitate subsequent prevention of human granulocytic anaplasmosis in Nanchang, China, in the future.

INTRODUCTION

Rodents are important destructive animals globally that compete with humans for food, particularly through pre-harvest damage to rice crops. Some rodents occur commensally in human residential areas, easily contaminating the human environment by the production of metabolic excrement, faeces, and urine. Important pathogens like hemorrhagic fever with renal syndrome virus can be transmitted directly to humans by contact with objects and inhalation of air contaminated by rodents. Other pathogens, like *Borrelia* spirochetes, can indirectly infect humans through the bites caused by ectoparasites, such as lice, fleas, and ticks on the rodents. The research mentioned above was reviewed and documented in previous works (1,2).

Human granulocytic anaplasmosis (HGA) is considered a rodent-borne disease caused by *Anaplasma phagocytophilum*, which is an emerging obligate intercellular bacterial pathogen (3). HGA is increasingly recognized as an important and frequent cause of fever worldwide (4). Anaplasmosis was first recognized as a disease of humans in the United States in the mid-1990s. Cases of anaplasmosis have generally increased from

350 cases in 2000 to 1,163 cases in 2009. The number of reported cases increased by 52% between 2009 and 2010 (5). By the end of 2015, more than 100 HGA cases had been reported in Mainland China, covering the north and south of the Yangtze River. The cases were mainly distributed in Shandong and Beijing (6). In 2010, an HGA case was reported in a healthy young man from Nanchang of Jiangxi Province, China. The patient noticed an unknown arthropod bite one day before the initiation of fever, and died 10 days later (unpublished data). Clinical manifestations in humans range from mild self-limiting febrile illness to fatal infections. The most frequent manifestations in patients are non-specific influenza-like symptoms with fever, headache, myalgias, and malaise. Few patients have arthralgia or involvement of the gastrointestinal tract, respiratory tract, liver, or the central nervous system (4).

A. phagocytophilum appears to be a generalist, infecting a wide range of animals, such as humans, domestic animals, and wildlife, including rodents, throughout the world (7–10). Previous studies showed that various field and domestic animals are considered as important reservoirs of *A. phagocytophilum*. Roe deer play a key role in the endemic cycles of the pathogen in Germany (11). Dogs from both urban and rural habitats in Brazil were found to be positive for *A. phagocytophilum* (12). In addition, goats and sheep were highly infected with *A. phagocytophilum* in China (9). In Nanchang, however, a previous examination could not detect the presence of *A. phagocytophilum*. In the survey, 328 dogs and goats were found to be negative for *A. phagocytophilum* infection (unpublished data), suggesting that domes-

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tic animals might have a limited role as hosts for *A. phagocytophilum* in Nanchang.

Rodents are common in both urban and rural human residential areas of Nanchang city (13). Furthermore, they can cause certain rodent-borne diseases, such as hemorrhagic fever with renal syndrome and leptospirosis (12,13). However, little information on the systematic statistics of the rodent population and the presence of *A. phagocytophilum* in commensal rodents is available. The objective of this study was to survey a wide range of commensal rodent populations monthly, covering all regions of Nanchang, between the period of 2011 and 2015. The highest rodent-infested county was then selected to monitor whether *A. phagocytophilum* infection could be found in rodents.

MATERIALS AND METHODS

Commensal rodent surveillance: Rodent population densities were determined using snap traps at 58 sites in Nanchang city from 2011 to 2015. The sites were distributed in 4 counties and the city center, and were randomly selected with regard to land use and geographic location (Fig. 1A). Rodent snap traps, with fried bread sticks as bait, were set in the evening and collected in the next morning. A total of 61,554, 12,088, 12,209,

12,352, and 11,881 traps were placed in the city center, Xinjian County, Nanchang County, Anyi County, and Jinxian County, respectively. Traps were spaced at approximately 5-meter intervals in the vertical direction and at 25-meter intervals in the lateral direction. All rodents captured were measured, weighed, examined for sex and developmental condition, and, finally, morphologically identified. The rodent population density was calculated according to the following equation:

$$R(\%) = \frac{N_r}{N_e} \times 100$$

Where: R = rodent population density:

N_r = number of traps with captured rodents:

N_e = number of effective traps collected; effective traps indicating non-exciting traps and the traps with rodents.

Occurrence of *A. phagocytophilum* in commensal rodents: Live rodents were trapped using welded cages in Anyi, the county with the highest rodent density in Nanchang city. Rodent capture sites were localized in 3 residential areas near 2 brick kiln industries, and a pig breeding base without optimal hygienic conditions (Fig. 1B). Rodents were captured from late March to early April of 2016. Traps were spaced 10 m apart along lines, with bait and were checked in the morning at 24 h and 48 h after setup. Captured rodents were characterized and morphologically identified before blood collection. Blood samples were collected through incision of the femoral artery and were then transferred to EDTA-treated tubes. All samples were stored at -80°C until analyses.

Genomic DNA was extracted from a volume of 200 μl of whole blood using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. One hundred micro-liter eluted DNA samples (30–90 $\text{ng}/\mu\text{l}$) were harvested and stored at -30°C for subsequent PCR analysis. DNA concentrations and purities were determined with a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The samples with A_{260}/A_{280} of 1.8–2.0 ratio and a concentration greater than 20 $\text{ng}/\mu\text{l}$ were used in the analysis. The presence of *Anaplasma* genus DNA was examined by a semi-nested PCR assay targeting 16S rRNA as previously described (14). DNA of *A. phagocytophilum* was detected by a nested PCR based on the *glfA* (citrate synthase) gene, according to a previous description (15).

Fig. 1. (A) Investigation sites. Filled triangles on the map show 58 rodent surveillance sites. (B) Rodent capture site. (i) Full view of a residential area; (ii) Outside view of a house; (iii) Indoor view of a room, household items were in chaotic arrangement. Rodent faeces and bites in food and household items, such as tables and shoes, were observed here and there. A black arrow with white margin shows a rodent gnaw mark on a table.

Fig. 2. The fluctuation of rodent population density in Nanchang.

Samples positive for *Anaplasma* genus (*phagocytophilum*) infection were randomly selected for cloning and sequencing of amplicons. For each specimen, at least 5 clones confirmed positive by colony PCR were multiplied and purified using a NucleoSpin Plasmid Quick Pure (Macherey-Nagel, Düren, Germany). Sequencing reactions were performed using ABI BigDye Terminator Kit v3.1 (Applied Biosystems, Foster City, CA, USA). DNA fragment sequences were determined using an ABI PRISM 3100 DNA sequencer (Applied Biosystems). Identities and similarities of the nucleotide sequences were analyzed using BLASTN online at GenBank. The SeqMan and MEGA 6 programs were used to build the alignment of all selected sequences, and then a neighbor-joining phylogenetic tree was constructed using the MEGA 6 program. The confidence of internal branches was estimated by bootstrapping with 1,000 replications.

Statistical analysis: The results were analyzed by using the χ^2 test in IBM SPSS Statistics ver. 20 (Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Rodent population density in Nanchang between 2011 and 2015: Over the five-year trapping period, 944 (0.86%) rodents were caught at 58 sites in Nanchang.

The overall male-to-female sex ratio was 1:1.02 (467:477), and the rodent population density did not differ significantly between sexes ($\chi^2 = 0.105$, $df = 1$, $P > 0.05$). However, the rodent population density was significantly higher between 2011 and 2013 (0.92% to 0.99%) than from 2014 to 2015 (0.68% to 0.73%) ($\chi^2 = 22.14$, $df = 4$, $P < 0.001$; Table 1). The rodent population density showed a peak in March–April, followed by a gradual decline from May to a low level in October. This was followed by a substantial decrease in density from October to the lowest level in December (Fig. 2).

Rodents captured included sewer rats (*Rattus norvegicus*), house mice (*Mus musculus*), and *Rattus flavipectus*. The most frequently captured rodents were sewer rats, accounting for 34.75% (328 individuals); house mice consisted of 33.47% (316 individuals). 28.07% (265 rodents) of captures were *R. flavipectus*. Some rarely seen small wild mammals were also caught around residences during the trapping period, for example, rodents (*Apodemus agrarius* and *Rattus losea*) and the insectivore *Suncus murinus*. Rodent populations were at the highest density (3.65%) in Anyi County of Nanchang city. Anyi County had three-fold higher rodent population density than Nanchang County, which had the second highest (0.99%) rodent population density at the county level across Nanchang city. Sewer rats in Anyi County had undergone large population increases between 2012 and 2015 and consequently became the

Table 1. Average annual rodent density and composition at the county level in Nanchang city, China, between 2011 and 2015

Location	Species	2011	2012	2013	2014	2015	Whole period
City center	Sewer rats	0.21 (26) ¹⁾	0.36 (44)	0.36 (45)	0.17 (21)	0.24 (30)	0.27 (166)
	<i>Rattus flavipectus</i>	0.12 (15)	0.04 (5)	0.07 (9)	0.05 (6)	0.12 (15)	0.08 (50)
	House mice	0.08 (10)	0.03 (4)	0.04 (5)	0.03 (4)	0.05 (6)	0.05 (29)
	Other	0.04 (5)	0.02 (2)	0.02 (2)	0.00 (0)	0.00 (0)	0.01 (9)
	subtotal	0.46 (56)	0.45 (55)	0.49 (61)	0.26 (31)	0.41 (51)	0.41 (254)
Xinjian County	Sewer rats	0.16 (4)	0.28 (7)	0.12 (3)	0.13 (3)	0.20 (5)	0.18 (22)
	<i>Rattus flavipectus</i>	0.21 (5)	0.16 (4)	0.29 (7)	0.22 (5)	0.16 (4)	0.21 (25)
	House mice	0.45 (11)	0.52 (13)	0.41 (10)	0.04 (1)	0.16 (4)	0.32 (39)
	Other	0.29 (7)	0.00 (0)	0.00 (0)	0.09 (2)	0.04 (1)	0.08 (10)
	subtotal	1.11 (27)	0.97 (24)	0.81 (20)	0.49 (11)	0.57 (14)	0.79 (96)
Nanchang County	Sewer rats	0.16 (4)	0.28 (7)	0.04 (1)	0.00 (0)	0.13 (3)	0.12 (15)
	<i>Rattus flavipectus</i>	0.24 (6)	0.16 (4)	0.16 (4)	0.25 (6)	0.04 (1)	0.17 (21)
	House mice	0.73 (18)	1.19 (30)	0.83 (21)	0.25 (6)	0.39 (9)	0.69 (84)
	Other	0.00 (0)	0.00 (0)	0.00 (0)	0.04 (1)	0.00 (0)	0.01 (1)
	subtotal	1.13 (28)	1.63 (41)	1.03 (26)	0.54 (13)	0.57 (13)	0.99 (121)
Anyi County	Sewer rats	0.52 (13)	0.28 (7)	0.49 (12)	1.05 (26)	2.42 (60)	0.96 (118)
	<i>Rattus flavipectus</i>	1.17 (29)	2.03 (50)	1.91 (47)	0.97 (24)	0.40 (10)	1.30 (160)
	House mice	1.49 (37)	1.63 (40)	1.71 (42)	1.46 (36)	0.16 (4)	1.29 (159)
	Other	0.04 (1)	0.04 (1)	0.16 (4)	0.04 (1)	0.28 (7)	0.11 (14)
	subtotal	3.22 (80)	3.98 (98)	4.28 (105)	3.52 (87)	3.27 (81)	3.65 (451)
Jinxian County	Sewer rats	0.09 (2)	0.00 (0)	0.00 (0)	0.22 (5)	0.00 (0)	0.06 (7)
	<i>Rattus flavipectus</i>	0.17 (4)	0.00 (0)	0.16 (4)	0.00 (0)	0.04 (1)	0.08 (9)
	House mice	0.17 (4)	0.04 (1)	0.00 (0)	0.00 (0)	0.00 (0)	0.04 (5)
	Other	0.04 (1)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.01 (1)
	subtotal	0.47 (11)	0.04 (1)	0.16 (4)	0.22 (5)	0.04 (1)	0.19 (22)
Nanchang city	Sewer rats	0.22 (49)	0.29 (65)	0.27 (61)	0.26 (55)	0.45 (98)	0.30 (328)
	<i>Rattus flavipectus</i>	0.27 (59)	0.28 (63)	0.32 (71)	0.19 (41)	0.14 (31)	0.24 (265)
	House mice	0.36 (80)	0.40 (88)	0.35 (78)	0.22 (47)	0.10 (23)	0.29 (316)
	Other	0.06 (14)	0.01 (3)	0.03 (6)	0.02 (4)	0.04 (8)	0.03 (35)
	Total	0.92 (202)	0.99 (219)	0.97 (216)	0.68 (147)	0.73 (160)	0.86 (944)

¹⁾: Numbers before the round brackets represent rodent density (in %), and numbers in the parentheses are the total number of species-specific rodents in different years.

dominant rodent species in 2015 (Table 1).

Occurrence of *A. phagocytophilum* in rodents: A total of 19 live rodents were captured including 17 sewer rats, 1 *R. flavipectus*, and 1 home mouse in Anyi County. The distribution of rodents according to sex, stage, and infection status is shown in Table 2. The *Anaplasma* genus was detected in 6 (31.58%) rodents based on 16S rRNA PCR, and in 1 (5.26%) rodent by *gltA* amplification.

Four sewer rats and 1 *R. flavipectus* that were positive for *Anaplasma* genus infection by nested PCR of the partial 16S rRNA sequence were selected for sequence analyses. A 335-base pair nucleotide sequence was obtained from 5 individual clones of each sample. These sequences showed 94.53–99.40% identity with the published sequences of the HGA agent (KT454992).

Table 2. Presence of *A. phagocytophilum* in live rodent captures by using wire cages

No.	Species	Sex	DS	Result
1	SR	F	adult	
2	SR	F	adult	
3	SR	M	adult	
4	SR	M	adult	++ KY024480, KX757024 ¹⁾
5	SR	M	adult	
6	SR	F	immature	
7	HM	M	adult	
8	SR	M	adult	
9	SR	F	immature	
10	SR	F	immature	
11	SR	F	adult	+ KX757023
12	SR	M	adult	+ KX757023
13	SR	M	adult	+
14	SR	F	adult	
15	SR	M	adult	+ KX757023
16	SR	M	adult	
17	SR	F	adult	
18	SR	M	adult	
19	Rf	M	adult	+ KY024479

DS, developmental stage; SR, sewer rat; HM, house mouse; Rf, *Rattus flavipectus*; F, female; M, male; +, the rodents found to be positive for *Anaplasma phagocytophilum* based on the amplification of 16S rRNA and DNA sequencing. ++, the rodent found to be positive based on both 16S rRNA and *gltA* PCR. Letter/number combinations are accession No. of target 16S rRNA and *gltA* sequence deposited in GenBank.

¹⁾: The former is accession No. of *gltA* sequence, and the latter is accession No. of 16S rRNA.

Fig. 3. Phylogenetic analysis based on *Anaplasma* genus 16S rRNA fragment. ●, sequences were obtained in this study. The *Ehrlichia ruminantium* homolog gene (CR925678) was used as the outgroup. The number of each branch was provided as bootstrap value at the node.

Sequence analysis of the 25 plasmid clones revealed 3 variants of *A. phagocytophilum*. One (KX757023) was found only in sewer rats and was highly identical to the sequence from the HGA agent, with only 2 nucleotide differences at positions of 773 and 899 and formed a clade with the sequence of the HGA agent in the phylogenetic tree (Fig. 3). In the phylogenetic tree, the other variants (KY024479 and KY024480) formed distinct branches. These variants differed from the HGA agent by 7 and 18 base-pairs, respectively. The sequences of the 3 variants obtained in the study were not 100% identical to the sequences from non-rodent hosts (HQ872465, KU321304, and KX115422), or even from rodent hosts (HM439430 and KC470064) in China. However, the fragments (HM439430 and KC470064) from rodents in China fully constructed a subclade (Fig. 3).

Five clones of the *A. phagocytophilum gltA* sequence were obtained from a sewer rat in our study. Four clones (numbered 1, 3, 4, and 5) had identical sequences to each other and differed from clone 2 by a single nucleotide mutation. Here, we used clone 1 to represent *A. phagocytophilum gltA* sequence (KX757024) extracted from the sewer rat. Our sequence was well separated from other *A. phagocytophilum* corresponding sequences from dog (KP861637) and sheep (KP861639) hosts, and tick (KP861638 and KP276595) vectors in the phylogenetic tree. However, the sequence (KX757024) shared a clade with *A. phagocytophilum* previously described in rodents from south-eastern China (DQ458809) and showed 99% identity to each other (Fig. 4).

DISCUSSION

Rodents are suspected to act as natural reservoirs for *A. phagocytophilum*. The prevalence of *A. phagocytophilum* was detected in *Apodemus sylvaticus*, *Apodemus flavicollis*, and *Myodes glareolus* in France (7). The first wild animals observed to be positive for *A. phagocytophilum* in east China were *Apodemus peninsulae*, *A. agrarius*, and *Eutamias sibiricus* (15). *A. agrarius* was subsequently found to be infected with *A. phagocytophilum* in west China (9). This evidence appeared to support the role of the genus *Apodemus* as reservoirs in the natural life cycle of *A. phagocytophilum*.

Fig. 4. Phylogenetic analysis based on *A. phagocytophilum gltA* fragment. ●, sequence was obtained in this study. The *Anaplasma bovis* homolog gene (KU586317) was used as the outgroup. The number of each branch was provided as bootstrap value at the node.

The fragment of 16S rRNA from *A. phagocytophilum* was detected in blood samples from 6 rodents by semi-nested PCR and DNA sequence analysis. However, the *gltA* gene of *A. phagocytophilum* was determined in only 1 rodent. The difference in detection rates of the same samples was likely explained by the failure of our primers to bind to highly variable regions of *gltA* gene. A recent study in France (7) showed that 6.6–22.8% of rodents can carry *A. phagocytophilum* bacteria, similar to those (5.26% for *gltA* amplification and 31.57% for 16S rRNA PCR and DNA sequencing) described in our study.

Molecular typing based on single nucleotide polymorphisms (SNPs) in genes is the most frequently used method to analyze *A. phagocytophilum* genetic diversity. The most intensively used markers are the *groESL* operon, 16S rRNA locus, and the *ankA* and *msp2* genes. One previous study of species-specific 16S rRNA in the USA isolated the human pathogenic variant AP-ha (EP-ha, *Ehrlichia phagocytophila*-human agent) from other non-pathogenic variants (16). In Europe, the use of the species-specific 16S rRNA also clearly discriminated variants infecting red deer from those infecting roe deer (17,18). However, the conclusions were challenged by other studies in which this marker showed poor resolution and did not confirm any host species segregation for *A. phagocytophilum* variants (19). Finally, species-specific 16S rRNA data is unable to distinguish variants according to their geographical origins (20). In the present study, in Anyi County, we used PCR amplification of genus-specific 16S rRNA fragments extracted from individuals of different rodent species and DNA sequencing to classify *A. phagocytophilum* into 3 variants. Despite the identical geographical origins of the microorganisms in the same host species, they were dispersedly distributed in the phylogenetic tree.

The *gltA* gene encodes citrate synthase which catalyzes the condensation of acetyl coenzyme A with oxaloacetate to form citrate and has been assumed to be an important control point for determining the metabolic rate of the cell. The *A. phagocytophilum gltA* gene has multiple gene polymorphisms. *A. phagocytophilum* isolated from different dog samples showed diverse SNPs in the *gltA* gene and could be sub-grouped by alpha and beta variants (21). Analysis of the *gltA* sequences shows that our strain, in combination with that of a rodent from south-eastern China, was unique compared with all other variants, branching out from the group of sequences isolated from dog and sheep hosts in Spain, and tick vectors in the USA and Spain. Our study indicated that polymorphisms in the *A. phagocytophilum gltA* gene are probably related to the hosts and their geographical distribution.

Taken together, our results suggested that commensal rodents are the reservoirs for *A. phagocytophilum*, and controlling the rodent population would make a great contribution to preventing HGA in Nanchang, China.

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Conflict of interest None to declare.

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