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Surveillance of *Anaplasma phagocytophilum* Infection in Rodents on Nangan Island, Matsu

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Twenty-seven spleens from small rodents, including *Rattus norvegicus*, *Rattus losea* and *Mus musculus* captured between October 5 and 7, 2010 on Nangan Island, Matsu were examined. The DNA from spleen was extracted, detected by nested PCR, and compared with 16s ribosomal RNA(r RNA) genes. The results showed that seven samples were 100% homologous to *Anaplasma phgocytophilum* and one sample was 100% homologous to *Anaplasma bovis*. The infection rate of *Anaplasma spp*. in rodents in this area was 30% (8/27).

Key words: *Anaplasma phagocytophilum*, nested PCR, rodent, Matsu

INTRODUCTION

Anaplasma phagocytophilum is a Gram-negative obligate intracellular bacterium, which encompasses former *Ehrlichia phagocytophila*, *E. equi*, and the human granulocytic ehrlichiosis (human granulocytic anaplasmosis, HGA) agent according to phylogenetic analyses.[1-2] The bacterium was first identified in Wisconsin, USA, in 1994, from a patient who developed a severe febrile illness after a tick bite and subsequently died.[3] In humans, a febrile illness developed shortly after a tick bite. In addition, leukopenia, thrombocytopenia, and a mild to moderate elevation of hepatic enzymes are often present. Moreover, other factors such as age, neutrophilia, lymphopenia, anemia, and immunosuppression can increase the severity of illness and risk for hospitalization.[4] *A. phagocytophilum* has been detected from rodents in northeastern China[5] and dogs in Taiwan.[6] In the field, *A. phagocytophilum* is transmitted between ticks and rodents. So far, there were no prevalence data of *A. phagocytophilum* in rodents on Matsu Island. Hence, this study used spleen specimens of rodents captured in 2010 to identify the natural infection of *A. phagocytophilum* in rodents as previously reported[5] in order to understand the current prevalence of *A. phagocytophilum* in rodents in Matsu area as a reference for clinical follow-up.

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MATERIALS AND METHODS

Collection locations

Rodents were captured in a 3-day period between October 5 and 7, 2010, on Nangan island, Matsu from wild fields near the (1) Matsu Village, (2) Jhuluo Village and (3) Wujenpai in Shiwei Village, and (4) Sangyao San; residential areas in (5) Fuao, (6) Jeshou, (7) Renai, and (8) Chingshui; as well as and military camps at (9) Jinsha, (10) Renai and (11) Jhuluo (Fig. 1).

Sampling

Rodents were anesthetized via intraperitoneal injection by 0.05-1.0 ml Zoletil 50 (Virbac Lab. France, 10-fold dilution). Organs including liver, spleen, lung and kidney were harvested, kept in dry ice during transportation, and stored under -75 °C in laboratory.

Detection of *A. phagocytophilum*

DNA extraction

About 10 mg of spleen was cut and DNA was extracted according to the instructions of the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany).

Primers

Published sequences (ECCAPF, GE2r) were slightly modified[7] and used to selectively amplify *Anaplasma* spp. 16S rRNA genes. ECH16S-17APF, ECH16S-9-

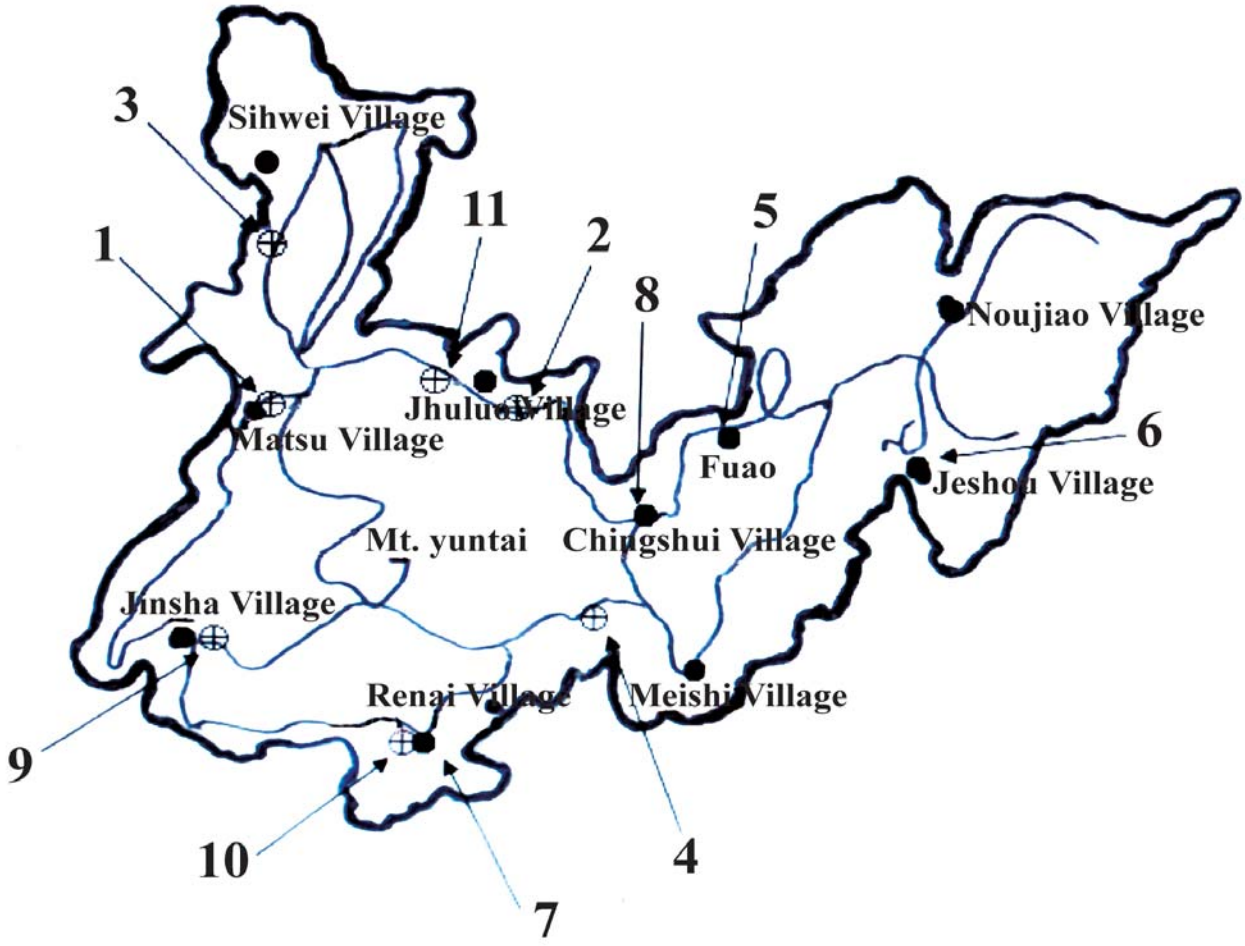

Fig.1 Rodent trapping locations on Nangan island.

APR[8], GE9Fand GE2r [9]were employed to amplify *A. phagocytophilium* genes. Primers were synthesized by Genomics Corp. ECH16S-3APPRO is a real-time PCR probe synthesized by ABI Corp. (Table 1).

PCR amplification

A. phagocytophilium strain HN was used as the positive control and Q H_2O was used as the negative control. 16S rRNA was first tested by 25 μl of mixture of Platinum® PCR SuperMix (invitrogen) 22.5 μl, 10 μM primer (ECCAPF) 0.25 μl, 10 μM primer (GE2r) 0.25 μl, plus 2 μl of DNA. PCR reaction was performed under the following condition: 94 ^{o}C 2 min, and 40 cycles of 94 ^{o}C 1 min, 52 ^{o}C 1 min, and 72 ^{o}C 1 min, followed by 72 ^{o}C 10 min and ended at 4 ^{o}C.

Nested PCR amplification

Real-time PCR amplification was carried out using 9.5 μl of primer and enzyme mixture (4 μl sterile Q water, TaqMan® Fast Universal PCR Master Mix (2X) 5 μl (Roche, USA), 10 μM primer (ECH16S-17APF) 0.2 μl, 10 μM primer (ECH16S-9APR) 0.2 μl, probe (ECH16S-3APPRO) 0.1 μl and 0.5 μl of products from the PCR mentioned above. The reaction was conducted with an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, Calif. USA) under the following condition: 95 ^{o}C 0.2 min -- 95 ^{o}C 0.03 min -- 55 ^{o}C 0.3 min$\times$40 cycles. The results were shown as averages of Ct values from triplicates. To further confirm the sequence of amplicons, 1 μl of the PCR products was added to 49 μl of mixtures of Platinum® PCR SuperMix (invitrogen) 48 μl, 10 μM primer (Ge9F) 0.5 μl, 10 μM primer (GE2r) 0.5 μl amplified under the following condition: 94 ^{o}C 2 min, 30 cycles of 94 ^{o}C 30 sec, 50 ^{o}C 30

Table 1 Primers for detecting 16S r RNA of *Anaplasma phagocytophilum* in rodents on Nangan Island, Matsu.

Primer	Sequence (5'-3')	Target	Gene	Size(bp)
ECCAPF	5'-AGAACGAACGCTGGCG GCAAGCT-3'	*Anaplasma.spp*	16S	585
GE2r	5-GGCAGTATTAAAAGCAG CTCCAGG-3'			
GE2r	5-GGCAGTATTAAAAGCAG CTCCAGG-3'	*Anaplasma phagocytophilum*	16S	546
GE9F	5-AACGGATTATTCTTTATA GCTTGCT -3'			
ECH16S-17APF	5'–GCGGCAAGCTTAACAC ATG-3'	*Anaplasma phagocytophilum* (real-time PCR)	16S	81
ECH16S-9APR	5'-TCACCC GTCTGCCACTA ACTATT -3			
ECH16S-3APPRO	FAM-AGTCGAACGGATTATTCTT TATAGCTTGCT -TAMARA			

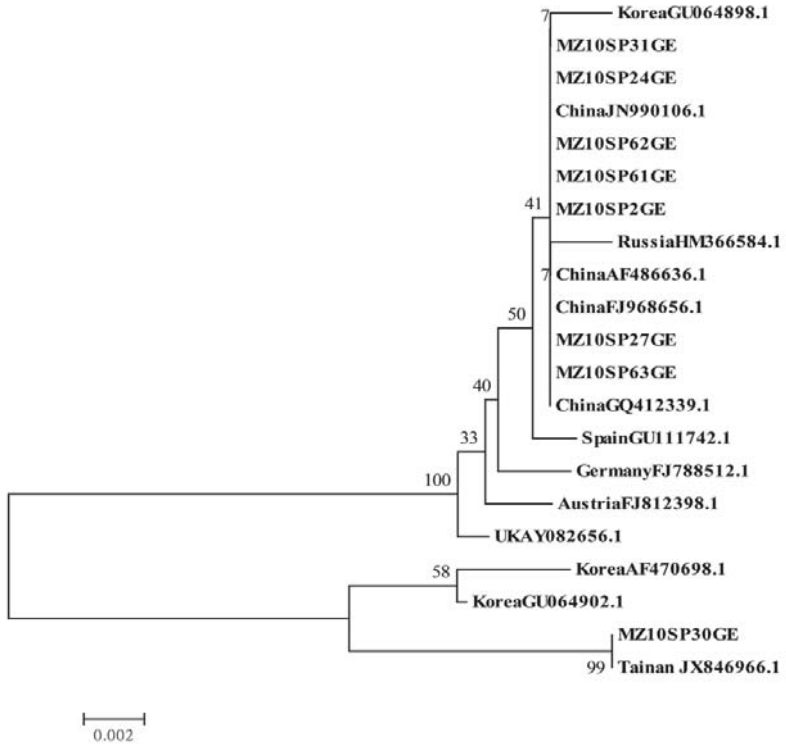

Fig. 2 Phylogenetic relationship of all positive samples analyzed using MEGA4.

sec, 72 °C 1 min, followed by 72 °C 10 min and ended at 4 °C. Amplicons of the secondary PCR were analyzed by electrophoresis in the TAE buffer. Products of 546 bp were extracted by the QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany), confirmed again by electrophoresis, and sequenced by the Genomics Corp.

Phylogenic analysis

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 496 positions in the final dataset.

RESULTS

Twenty-seven spleens from small rodents including *Rattus norvegicus, Rattus losea* and *Mus musculus* trapped between October 5 and 7, 2010 on Nangan island were examined. The sequences of 7 samples were 100% identical to *Anaplasma phgocytophilum* with China JN990106, China AF486636, China GQ412339 and China FJ968656 and 1 sample was 100% identical to *Anaplasma bovis* with Tainan JX846966 (Fig. 2 and Table 2). Among the positive samples, one pool was from *Rattus norvegicus* and seven pools were from *Rattus losea* captured at five places on Nangan island (Fig. 1 and Table 3). The infection rate of *Anaplasma spp*. in rodents on Nangan Island, Matsu was 30% (8/27).

Table 2 Differences in 16S rRNA genes between *A. phgocytophilum* and *A. bovis* in rodents on Nangan Island, Matsu.

Strain	Nucleotide Position																									Homology
	3	4	6	7	11	59	75	123	124	125	149	156	163	177	178	227	239	332	343	346	364	460	465	466	469	
MZ 10SP2GE	G	G	G	G	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	100%
MZ 10SP24GE	G	G	G	G	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	100%
MZ 10SP27GE	G	G	G	G	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	100%
MZ 10SP31GE	G	G	G	G	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	100%
MZ 10SP61GE	G	G	G	G	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	100%
MZ 10SP62GE	G	G	G	G	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	100%
MZ 10SP63GE	G	G	G	G	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	100%
China JN990106	G	G	G	G	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	100%
China AF486636	G	G	G	G	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	100%
China GQ412339	G	G	G	G	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	100%
China FJ968656	G	G	G	G	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	100%
Russia HM366584	G	G	G	G	A	G	A	T	T	A	G	A	A	G	A	T	T	A	A	G	T	C	C	G	A	99%
Spain GU111742	G	G	G	G	A	G	A	T	T	A	A	A	T	G	A	T	T	A	A	G	T	C	C	G	A	99%
Austria FJ812398	G	G	G	A	G	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	99%
Korea GU064898	G	G	G	G	A	G	A	T	T	A	A	A	A	G	A	T	C	A	A	G	T	C	C	G	A	99%
Germany FJ788511	A	A	G	A	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	99%
UK AY082656	A	G	G	A	A	G	A	T	T	A	A	A	A	G	A	T	T	A	A	G	T	C	C	G	A	99%
Korea AF470698	A	A	G	A	A	A	G	C	A	T	A	G	T	A	G	C	T	A	G	G	C	T	T	G	T	97%
Korea GU064902	A	A	G	A	A	A	A	C	A	T	A	G	T	A	G	C	T	A	G	G	C	T	T	G	A	97%
Tainan JX846966	G	G	--	A	A	A	G	C	A	T	A	G	A	A	G	C	T	G	G	A	C	T	T	A	T	96%
MZ 10SP30GE	G	G	--	A	A	A	G	C	A	T	A	G	A	A	G	C	T	G	G	A	C	T	T	A	T	96%

Table 3 Locations of *A. phgocytophilum*-infected rodents trapped

Locations of rodents trapped	Rodent species	Infected rodent No.	Location code*
Military camps at Jinsha Village	*Rattus losea*	MZ10-02	9
Wild fields near Jhuluo Village	*Rattus losea*	MZ10-24	2
Wild fields near Matsu Village	*Rattus norvegicus*	MZ10-27	1
Wild fields near Shiwei Village	*Rattus losea*	MZ10 -30	3
		MZ10 -31	
Wild fields near Sangyao San	*Rattus losea*	MZ10 -61	4
		MZ10 -62	
		MZ10 -63	

* See Fig. 1 for location codes

DISCUSSION

This study used spleen specimens to identify the natural infection of *A. phagocytophilum* in rodents with reference to previously reported results. In mice that were experimentally infected with the HGA agent, spleen infection was obvious and persistent[5]. The results show that DNA sequences of *A. phagocytophilum* from seven infected spleens were 100% identical to the variants of *A. phagocytophilum* found at a place only 10 km from the coast of southeastern China (Fig. 1 and Table 2), thus implying that *A. phagocytophilum* could be transported across the sea. According to the result in this study, *A. phagocytophilum* is obviously prevalent in wild fields outside villages and *R. losea* is the reservoir host in that area and the previous report from China stated that *A. phagocytophilum* is present in wild animals from southeastern China, which implies that wild animals may play a role in the enzootic maintenance of *A. phagocytophilum* in the region.[10]

Telford *et al.* (1996)[11] and Castro *et al.* (2001)[12] reported persistent infections with HGA variants in mice and rats under natural conditions. In BALB/c mice experimentally infected with HGA variants, bacteraemia was reported to persist for 9 and 12 weeks[13] and wood rats were shown to be persistently infected with HGA variants for up to 14 months, suggesting that they could serve as reservoirs of infection for prolonged periods.[12] In nature, *A. phgocytophilum* was transmitted and maintained between mice and rats by ticks with the bacteria transmitted through the moulting process in ticks and infecting new hosts during the next feeding. However, vertical transmission is reported to be non-existent or inefficient.[14-15] Rodents are the known important reservoirs of *A. phagocytophilum*. Although two dominant tick species, *Ixodes granulatus and Rhipicephalus haemaphysaloides*, were prevalent in Matsu area (unpublished data), rodent pest control is still the efficient way to block the tick-rodent transmission cycle in wild fields for HGA disease control in Matsu area.

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DISCL OSURE

All authors declare that this study has no conflict of interest.

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