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Original Article

Investigation of *Ehrlichia chaffeensis* Infections in Rodents in Kinmen Area, 2012

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Abstract

The investigation of *Ehrlichia chaffeensis* infections in wild rodents was conducted on the main island of Kinmen on October 16-18, 2012. Livers and spleens were obtained from 108 rodents, including 2 *Rattus norvegicus*, 73 *Rattus losea exiguus*, and 33 *Suncus murinus*, followed by DNA extraction and nested PCR. *E. chaffeensis* DNA was detected in 16 rodents (14.8%), from liver in 4 rodents, from spleen in 10 rodents, and from both liver and spleen in 2 rodents. In terms of geographic distribution, rodents captured from 8 out of 10 field sites were positive for *E. chaffeensis* DNA, suggesting that *E. chaffeensis* could be widely present in wilderness areas of Kinmen. Therefore, when a patient who develops fever of unknown origin after tick bites is recognized in these areas, our investigation results can be used as a reference for diagnosis.

Key words: *Ehrlichia chaffeensis*, *Rattus losea exiguus*, nested PCR, liver, spleen

Introduction

Human monocytic ehrlichiosis (HME) is an infection caused by *Ehrlichia chaffeensis*. *E. chaffeensis*, which infects human and animals through tick bites, is an obligatory intracellular parasite, in the order *Rickettsiales* and the family *Anaplasmataceae* [1]. This microorganism was first discovered from the blood smear of a patient with unknown etiology after tick bites in Arkansas, USA, in 1986. It has been recognized as a pathogen for livestock in the United States [2]. In 1991, it was isolated by cell culture, and named *E. chaffeensis*. Nowadays, ehrlichiosis has become an emerging disease. The symptoms for human after infections ranged from asymptomatic seroconversion to fatal cases [3]. The incubation time for this microorganism is 1-2 weeks, and 9 days in average. Major symptoms include fever (＞95%), headache (60-75%), myalgia (40-60%), nausea (40-50%), arthralgia (30-35%), and malaise (30-80%) [4]. Other

symptoms include gastrointestinal discomfort, lymphadenopathy, splenomegaly, and skin rash in a few cases. Few cases developed serious conditions like renal failure, central nervous system manifestations, and respiratory failure [5].

Natural reservoir hosts for *E. chaffeensis* include canine, deer and goat. The major vector is *Amblyomma americanum* in the United States [6]. In addition to being distributed in America, Europe, Thailand and Northeast Asia [7], *E. chaffeensis* was detected from ticks in Kinmen area by our Institute in 2009 [8], as well as from rodent spleens in Korea, and in Fujian province of China [9-11]. In recent years, cross-Strait travel between Mainland China and Taiwan is fully open, where Kinmen is at the strategic point, and has the same rodent species as that in Fujian area [12, 13]. Currently, massive cuts to troop numbers in Kinmen area left behind large undeveloped areas or grazing grasslands at the empty military bases and training grounds. Under the circumstances of high potential risk for infections due to high density of wild rodents and ticks that carry pathogens [14], distribution of *E. chaffeensis* infection among rodents in Kinmen was not clear. The aim of the study was to investigate carrier status of *E. chaffeensis* among rodents in Kinmen after the discovery of ticks that carried *E. chaffeensis* in 2009, in order to provide a diagnosis reference for tick-borne infections with fever of unknown origin in this area.

Materials and methods

1. Location of field sites

Capturing of rodents was conducted on the main island of Kinmen on October 16-18, 2012. The field sites were wilderness at Shanhou Folk Village, Yangdi, Qionglin, Sihu, Dongsha, Gugan, Shuitou, and Xintang, as well as Shamei Red-Flag pig farm, and Yucun harbor (Figure 1).

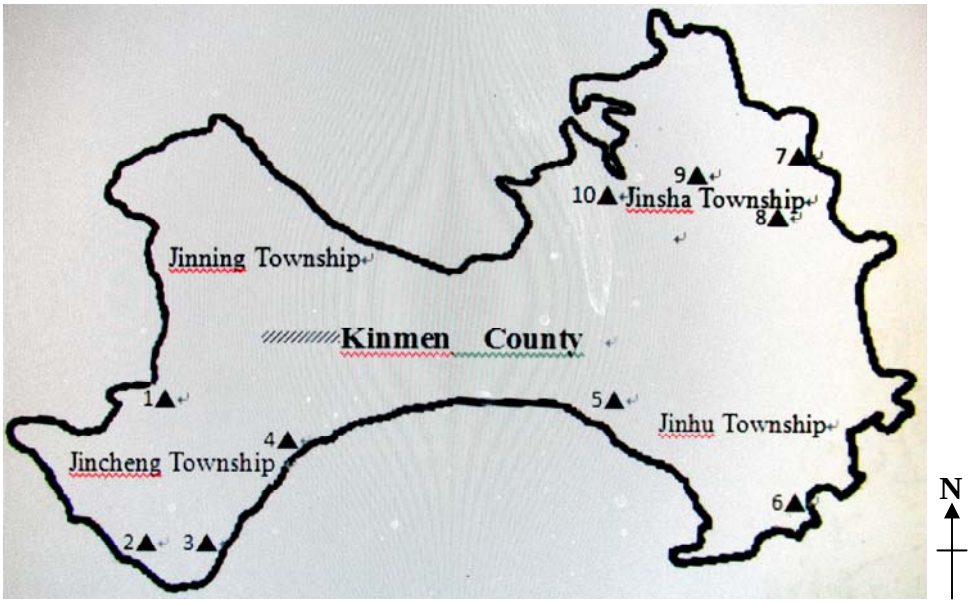

Figure 1. Locations of the field sites used for the investigation of *E. chaffeensis* in rodents in Kinmen area, 2012. 1-Shuitou, 2-Gugan, 3-Dongsha, 4-Sihu, 5-Yucun, 6-Xintang, 7-Shanhou Folk Village, 8-Yangdi, 9-Shamei Red-Flag pig farm, 10-Qionglin.

2. Capture of rodents

Mousetraps were set up in the afternoon and retrieved the next morning. Peanuts in shell were used as bates for rodents at wilderness. Pork jerky was used as bates for rodents at harbor and pig farm.

3. Collection of rodent organs

Rodent was fixed with a nylon net, followed by intraperitoneal injection of animal use, not a controlled substance, anesthetic Zoletil 50 (Virbac Lab, Carros, France). According to sizes and species, rodents were injected with 0.05-1.0 mL of 10-fold diluted anesthetic. Rodents were numbered. Species, gender and site of capture were recorded. After blood withdrawal and dissection, rodent organs, including liver and spleen, were collected. Organ specimens were placed into NUNC 2 mL freezing tubes, and capped tightly. The tubes were first immersed in dry ice-alcohol for 1 minute and then removed quickly to dry ice. The tubes were stored and shipped back to the laboratory in dry ice by express delivery. Specimens were stored in -70°C deep freezers for future pathogen detection.

4. Detection of *E. chaffeensis*

（1）**DNA extraction**：DNA was extracted from about 10mg of liver or spleen by using QIAamp DNA mini kit (QIAGEN GmbH, Hilden, Germany) according to protocols provided by the manufacturer.

（2）**Primers**：The primers used were the same as those for detection of *E. chaffeensis* in previous study [8]. Primers ECC and ECB were used to screen for 16S rRNA gene of *Ehrlichia* spp., and primers HE1, HE-3, ECH16S-17F and ECH16S-97R were used to screen for 16S rRNA gene of *E. chaffeensis*. The primers were prepared by Genomics BioSci & Tech (New Taipei, Taiwan), and the real time PCR probe, ECH16S-38PRO, was from ABI (Foster City, CA, USA) (Table 1). Positive control *E. chaffeensis* strain Arkansas 16S rRNA gene (479 bp) was prepared by Genomics BioSci & Tech.

Table 1. Primers and probe used for detection of *E. chaffeensis* in rodent specimens in Kinmen area

Primer	Sequence (5'-3')	Target	Gene	Size,bp
ECC	AGAACGAACGCTGGCGGCAAGCC	*Ehrlichia.*	16S	479
ECB	CGTATTACCGCGGCTGCTGGC	spp.		
HE1	CAATTGCTTATAACCTTTTGGTTATAAAT	*E.*	16S	389
HE-3	TATAGGTACCGTCATTATCTTCCCTAT	*chaffeensis*		
ECH16S-17F	GCGGCAAGCCTAACACATG	*E.*	16S	81
ECH16S-97R	CCCGTCTGCCACTAACAATTATT	*chaffeensis*		
ECH16S-38 PRO	FAM-AGTCGAACGGACAATTGCTTATAACCTTTTGGT -TAMARA			

5. Nested PCR

The reaction protocol and method for *E. chaffeensis* nested PCR was published elsewhere [8]. For initial screening of 16S rRNA, 23 μL mixture of primers and DNA polymerase was prepared and well mixed, that included Platinum PCR SuperMix (Invitrogen, Carlsbad, CA, USA) 22.5 μL, and primers ECC and ECB each 0.5 μL, followed by addition of sample DNA 2 μL. The PCR reactions were performed in a MJ Research PTC-200 PCR thermal cycler (Bio-Rad, Hercules, CA, USA). The reaction protocol was: preheating at 94°C for 5 min to denature DNA to single stranded; followed by 40 cycles of 94°C for 1 min for DNA denaturation, 60°C for 1 min for DNA annealing, and 72°C for 1 min for DNA extension; and then a final extension at 72°C for 10 min. Afterwards, the reactions were stored at 4°C. For real time PCR quick screening, 9.5 μL mixture of primers and DNA polymerase was prepared, that included sterile Q water 4.0 μL, TaqMan Universal PCR Master Mix 5.0 μL (ABI), 10 μM primer ECH16S-17F 0.2 μL, 10 μM primer ECH16S-97R 0.2μL and probe ECH16S-38PRO 0.1 μL. After thorough mixing, 0.5 μL of the above 16S rRNA PCR product was added. The reactions were performed in an ABI Fast 7500 Real-Time PCR System thermal cycler (ABI, Foster City, CA, USA). The protocol was: preheating at 95°C for 10 min to denature DNA to single stranded; followed by 40 cycles of 95°C for 15 sec for DNA denaturation, and 52°C for 1 min for DNA annealing. For further confirmation by DNA sequencing, 49 μL mixture of primers and DNA polymerase was prepared, that included Platinum PCR SuperMix (Invitrogen) 48 μL, and primers HE1 and HE-3 each 0.5μL, followed by addition of the above 16S rRNA PCR product 1.0 μL and well mixing. The PCR reactions were performed in a MJ Research PTC-200 PCR thermal cycler. The protocol was: preheating at 94°C for 5 min to denature DNA to single stranded; followed by 40 cycles of 94°C for 1 min for DNA denaturation, 52°C for 1 min for DNA annealing, and 72°C for 1 min for DNA extension; and then a final extension at 72°C for 10 min. The finished reactions were stored at 4°C. The product of the nested PCR was analyzed by agarose gel electrophoresis using TAE buffer. The DNA in the agarose gel of the size 389 bp was cut out, extracted by using QIAquick gel extraction kit, confirmed by another agarose gel electrophoresis, and sent for DNA sequencing by Genomics BioSci & Tech. The obtained DNA sequence was compared to DNA sequences in NCBI database (http://www.ncbi.nlm.nih.gov).

Results

One hundred and eight rodents were collected from 10 field sites, including 2 *Rattus norvegicus*, 73 *Rattus losea exiguus*, and 33 *Suncus murinus*. Sixteen out of 108 rodents were positive for *E. chaffeensis* DNA, including 2 rodents, KM30-52 and KM30-111, positive in both liver and spleen. The prevalence rate was 14.8%. According to the result of real time PCR quick screening, positive rate was 11.1% for rodent spleen and 5.6% for

rodent liver (Tables 2, 3). In terms of geographical distribution, *E. chaffeensis* DNA was detected in rodents captured from 8 out of 10 field sites, including wilderness at Shanhou Folk Village, Yangdi, Sihu, Dongsha, Gugan, Shuitou, and Xintang, as well as Yucun harbor. Only Shamei Red-Flag pig farm (2 *R. norvegicus*) and Qionglin (6 *R. losea exiguus* and 2 *S. murinus*) was negative. As shown in Table 3, the prevalence rate for *E. chaffeensis* DNA ranged from 6.7% to 66.7% among all positive field sites. The prevalence rate at the field site Dongsha was 66.7%, the highest among field sites. In terms of rodent species, PCR was positive for 13 *R. losea exiguus*, followed by 3 *S. murinus*.

Table 2. Distribution of rodent livers and spleens positive for *E. chaffeensis* in Kinmen area

No	Specimen no	Source rodent	Specimen	Field site	Prevalence**,%
1*	KM30LI 52	*Suncus murinus*	liver	Xintang	6.7
2	KM30LI 96	*Rattus losea exiguus*	liver	Sihu	5.6
3	KM30LI 101	*Rattus losea exiguus*	liver	Dongsha	33.3
4	KM30LI 102				
5*	KM30LI 111	*Rattus losea exiguus*	liver	Gugan	5.9
6	KM30LI 126	*Rattus losea exiguus*	liver	Shuitou	7.1
7	KM30SP 36	*Suncus murinus*	spleen	Yucun harbor	14.3
8*	KM30SP 52	*Suncus murinus*	spleen	Xintang	6.7
9	KM30SP 57	*Rattus losea exiguus*	spleen	Yangdi	20
10	KM30SP 63				
11	KM30SP 71	*Rattus losea exiguus*	spleen	Shanhou Folk Village	10
12	KM30SP 87	*Rattus losea exiguus*	spleen	Sihu	11.1
13	KM30SP 93				
14	KM30SP 97	*Suncus murinus*	spleen	Dongsha	33.3
15	KM30SP 98	*Rattus losea exiguus*			
16	KM30SP 109	*Rattus losea exiguus*	spleen	Gugan	11.8
17*	KM30SP 111				
18	KM30SP 123	*Rattus losea exiguus*	spleen	Shuitou	7.1

***** Both liver and spleen specimens in rodents KM30-52 and KM30-111 were positive.

****** Prevalence rate = number of rodents with PCR positive liver or spleen/number of rodents captured at that field site

Table 3. Investigation of rodents positive for *E. chaffeensis* and ectoparasitic ticks in Kinmen area, 2012

Field site	Rate of rodent captured*, %	Prevalence of *E. chaffeensis***, %	Rate of ectoparasitic tick***, %
Dongsha	50	66.7	33
Yangdi	42	20	10
Gugan	53	11.8	53
Sihu	44	16.7	25
Shanhou Folk Village	37	10	30
Shuitou	58	14.3	36
Xintang	43	6.7	40
Yucun harbor	39	14.3	0
Qionglin	24	0	0
Shamei Red-Flag pig farm	33	0	0
Average	42	14.8	30

* Rate of rodent captured = number of rodents captured/number of mousetraps set-up

** Prevalence of *E. chaffeensis* = number of rodents with positive PCR/number of rodents captured at that field site

*** Rate of ectoparasitic tick = number of rodents with ectoparasitic ticks/number of rodents captured at that field site

DNA sequences of 16S rRNA from all 25 positive specimens, including those obtained in 2009 were compared. The sequence from 20 specimens was 100% identical to the sequence from the complete genome of *E. chaffeensis* str. Arkansas, and GQ499971 of Mainland China. The sequence from 5 specimens was 99% identical to EU111841 of Korea, and AF414399 of Mainland China. The difference of 1-2 nucleotides is located at different positions (Table 4).

Table 4. Nucleotide difference in 16S rRNA from rodents and ectoparasitic ticks in Kinmen area

Isolate	Position of nucleotide difference									Similarity* , (%)
	78	81	87	95	160	213	215	218	326	
E. chaffeensis Arkansas	A	C	C	C	T	A	T	G	A	-
China(GQ499971)	A	C	C	C	T	A	T	G	A	100
KM30F58HME	A	C	C	C	T	A	T	G	A	100
KM30F88HME	A	C	C	C	T	A	T	G	A	100
KM29T35HME	A	C	C	C	T	A	T	G	A	100
KM30T104HME	A	C	C	C	T	A	T	G	A	100
KM30T114HME	A	C	C	C	T	A	T	G	A	100
KM30LI52HME	A	C	C	C	T	A	T	G	A	100
KM30LI101HME	A	C	C	C	T	A	T	G	A	100
KM30LI102HME	A	C	C	C	T	A	T	G	A	100
KM30LI111HME	A	C	C	C	T	A	T	G	A	100
KM30LI126HME	A	C	C	C	T	A	T	G	A	100
KM30SP36HME	A	C	C	C	T	A	T	G	A	100
KM30SP57HME	A	C	C	C	T	A	T	G	A	100
KM30SP63HME	A	C	C	C	T	A	T	G	A	100
KM 30SP71HME	A	C	C	C	T	A	T	G	A	100
KM30SP93HME	A	C	C	C	T	A	T	G	A	100
KM30SP97HME	A	C	C	C	T	A	T	G	A	100
KM30SP98HME	A	C	C	C	T	A	T	G	A	100
KM30SP109HME	A	C	C	C	T	A	T	G	A	100
KM30SP111HME	A	C	C	C	T	A	T	G	A	100
KM30SP123HME	A	C	C	C	T	A	T	G	A	100
KM30F85HME	A	C	C	C	T	A	C	G	A	99
KM30LI96HME	A	T	C	C	T	A	T	A	A	99
KM30SP52HME	A	C	C	C	T	G	T	G	A	99
KM30SP87HME	A	C	C	G	T	A	T	G	A	99
KM29T40HME	G	C	C	C	T	A	T	G	A	99
Korea(EU181141)	A	C	T	C	T	A	T	G	A	99
China(AF414399)	A	C	C	C	T	A	T	G	G	99

* Compared to 16S rRNA sequence of *E. chaffeensis* Arkansas

Discussion

The development of tourism in Kinmen has been greatly encouraged in recent years. However, there are still large undeveloped areas or grazing grasslands. The density of wild rodents is relatively high, and *R. losea exiguus* is the dominant species. In 2009, *E. chaffeensis* DNA was detected by PCR in ectoparasitic ticks, nymphs of *Ixodes granulatus* (KM29T40HME) and nymphs of *Rhipicephalus haemophysaloides* (KM29T35HME), of *R. losea exiguus* at Shanhou Folk Village [8]. In this study, ectoparasitic ticks, nymphs of *R. haemophysaloides* (KM30T104HME and KM30T114HME), from 2 *R. losea exiguus* at the Gugan grassland were positive for *E. chaffeensis* DNA. In addition, 3 *Nosopsyllus nicanus* (KM30F58HME, KM30F88HME and KM30F85HME) at the grasslands of Yangdi and Sihu were also PCR positive for *E. chaffeensis* DNA. Through DNA sequence comparison, the *E. chaffeensis* sequence from nymphs of *R. haemophysaloides* (KM29T35HME) obtained at Shanhou Folk Village in 2009 was 100% identical to the only PCR positive specimen (KM30SP71) obtained in the same field site in 2012, while the other one was 99% identical. In Gugan area, the *E. chaffeensis* sequence from nymphs of *R. haemophysaloides* collected from 2 *R. losea exiguus* was 100% identical to the sequences from all organ specimens from the two rodents, indicating that transmission between ticks and rodents was correlated in this area. However, the *E. chaffeensis* DNA sequence was more diverse among the three positive specimens in Sihu area. Only the sequence from specimen KM30SP93 was 100% identical to the control sequence, while the sequence from the other two specimens (KM30SP87 and KM30LI96) was 99% identical, with 3 nucleotide differences between them. Even the sequence from the two *N. nicanus* in this area was different; KM30F88HME was 100% identical to the control sequence while KM30F85HME was 99% identical (Table s2, 4). The role of *N. nicanus* in the transmission circle of *E. chaffeensis* was not clear due to lack of documented information. Current investigation of the prevalence of *E. chaffeensis* among rodents revealed that rodents infected with *E. chaffeensis* were widely present in wilderness, especially in the grasslands near ocean at the south of Kinmen island. The major rodent species was *R. losea exiguus*, and second by *S. murinus*. According to the literatures, transmission of *E. chaffeensis* depends on ticks and the chance of vertical transmission from generation to generation among ticks is very rare. Nymphs became infectious after sucking blood from rodents that carry *E. chaffeensis* [15]. Apparently, *R. losea exiguus* that carry *E. chaffeensis* plays a very important role in conservation of this pathogen in the tick-rodent pathogen transmission in nature.

In the literatures, spleen was most often used for detection of *E. chaffeensis* during investigation of wild rodents infected with *E. chaffeensis* [7, 16]. By using animal models for rodent infections in the laboratory, 9 days after inoculation with *E. chaffeensis*, the major infected organ is spleen, followed by liver, lung and bone marrow by using immunochemistry method to analyze infected organs [17]. It was comparable to the result of this investigation in Kinmen area in which the prevalence rate among spleen was much higher than that among liver,

11.1% versus 5.6%. In this investigation, most rodents were infected in a single organ. Some rodents were infected in both liver and spleen. However, the rate was small, only 2 out of 16 positive rodents (KM30-52 and KM30-111) were infected in both organs. Detection performed on both liver and spleen, nevertheless, will provide a more accurate status of geographic distribution *E. chaffeensis* infections among wild rodents.

Rodents are reservoir hosts for *E. chaffeensis* in this area. Ticks transmit *E. chaffeensis* by sucking blood from rodents that carry *E. chaffeensis*. Therefore, the prevalence rate of rodents that carry this pathogen and the rate of rodents that are ectoparasitized by ticks greatly affect transmission of *E. chaffeensis*. In this investigation, no adult tick was found among ectoparasitic ticks, and only 2 ectoparasitic nymphs from rodents in Gugan grassland were positive for *E. chaffeensis* DNA. However, rodents carrying *E. chaffeensis* were all over wilderness areas in Kinmen (Table 3). Except Qionglin, rodents from all field sites were ectoparasitized by ticks, especially in Dongsha area (the wilderness near the former Dongsha hospital) where the prevalence rate for *E. chaffeensis* among captured rodents was the highest, 66.7%, and 33% rodents were ectoparasitized by ticks. The tick species for *E. chaffeensis* in Kinmen was different from the typical vector documented in the literatures, *Amblyomma americanu* [6], therefore, susceptibility and competence for *E. chaffeensis* is not clear among ticks in Kinmen. Since rodents that carry *E. chaffeensis* are widespread in Kinmen, likely resulted from rodent-tick transmission mechanism, potential risk for *E. chaffeensis* infection does exist epidemiologically.

In addition to HME, ticks detected in Kinmen area might transmit other diseases. The more important ones include Lyme disease, caused by *Borrelia burgdorfei* [18], and Spotted fever, caused by spotted fever group rickettsiae [19]. Dual infection with *E. chaffeensis* and a spotted fever group rickettsiae was reported in the United States [20]. All this information can be used as a reference for diagnosis of tick-borne fever of unknown origin in Kinmen area.

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Outbreak Investigation Express

An Influenza Cluster in a Nursing Institution in Tainan, 2013

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Abstract

On November 15 of 2013, 5 residents who had fever, cough and runny nose were reported by a nursing institution in Tainan. It also was reported as an influenza cluster event through Symptom Notification System. As for the testing results, four residents were identified as influenza AH3; the investigation and disease prevention measures have been adopted by the local health authorities. During November 8-28, a total of 91 residents and 3 staffs appeared influenza-like symptoms; the student attack rate was 20.2%. The institution did not promptly inform at the beginning of the outbreak, even the health authorities had administered the antiviral prophylaxis, and the institution did not do well on isolation and infection control. As the result, the outbreak was once lost controlled. By reviewing this case, we hope to prompt densely populated institutions to strengthen the knowledge of infection control, and to implement the investigation, notification as well as isolation functions; meanwhile, the health authorities have to supervise properly and timely intervention in order to avoid the spread of the outbreak.

Keywords: influenza cluster, epidemic notification, isolation, vaccination

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