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Original article

Identification of *Orientia tsutsugamushi*, spotted fever group and typhus group *Rickettsia* by duplex and nested PCR methods

M-C Luan[1,2], D-Z Yu[1], L Tang[3], L-J Zhang[1]

[1]*Department of Rickettsiology, National Institute of Communicable Disease Control and Prevention, China CDC, Beijing* 102206, *China*
[2]*Department of Microorganism, Dalian Center for Disease Control and Prevention, Dalian* 116021, *China*
[3]*Department of Microecology, Dalian medical university, Dalian* 116044, *China*

Abstract

Objective: To identify members of genera of *rickettsia* and *O. tsutsugamushi* simultaneously. **Methods**: Rapid and duplex and nested PCR methods have been established by designing primers based on the conserved regions of heat shock protein *GroEL* gene. 345 mouse viscera samples including liver, spleen and kidney, 96 *Xenopsylla cheopis* and 32 chiggers collected from Hongta areas of Yuxi city, Yunnan province were tested by the new PCR methods. **Results**: The result of the study showed that the new PCR methods could identify most members of genera -*Rickettsia* and *Orientia* simultaneously with 100% specificity and its sensitivity could test one copy per microliter. The results of detection prevalence of rickettsioses in mouse, flea and mites DNA samples showed that the total rickettsia infection rate in mouse was 34.78% (120/345). The total infection rates in *R. typhi*, *O. t* Karp and *R. sibirica* of mouse samples were 28.12% (97/345), 19.71% (68/345) and 0.29% (1/345) respectively. Co-infection rates in *R. typhi* and *O. t* Karp of mouse samples were 13.33% (46/345). *O. t* Karp type has been the main epidemic strain in these areas. **Conclusion**: We concluded that this PCR method could be used to detect multi-genera *rickettsia* simultaneously. Molecular evidences provided in this and previous studies strongly support that Hongta areas of Yuxi city are a natural focus for typhus and scrub typhus with the common occurrence of their confection.

Keywords: *Rickettsia*, *GroEL* gene; Duplex and nested PCR

INTRODUCTION

Rickettsioses are worldwide known as zoonoses and emerging and reemerging human rickettsioses have dramatically increased throughout the world in the past 10 years[1-4]. Members of the family *Rickettsiaceae* have been mainly divided into two genera, *Rickettsia* and *Orientia tsutsugamushi*. The former has been subdivided into two genetically similar groups: the typhus group which consists of *R. typhi* and *R. prowazekii* and the spotted fever group which includes about more than 10 different pathogenic species. However, misdiagnosis and delayed treatment of *Rickettsioses* are a common practice because of nonspecific clinical manifestation and does not warrant proper and timely recovery. So, early diagnosis of *Rickettsioses* is very important. The serological tests are the most frequently used and widely available methods for its diagnosis, but they often delay recovery of illness. Isolation of *rickettsia* is done in an occupational laboratory and is time consuming. So, PCR and sequencing methods are regarded as sensitive and rapid tools to identify *rickettsia* throughout the world wherever these facilities are

Correspondence to: Zhang-lijuan, Changping P. O. Box5, Beijing (102206), China. Email: zhanglijuan@icdc.cn
Tel: 0086-10-61731692 Fax: 0086-10-61731692

available. Although several genes including 16S rRNA, *ompA*, *ompB*, *gltA* and gene D have been used to diagnose rickettsioses, single gene could not diagnose and differentiate between multi-genera *rickettsia* at one time[5,6]. Recently, *GroEL* gene has been used to identify many rickettsioses since it is more variable among different genera of the family *Rickettsiaceae*[7-9]. In this study, the duplex and nested PCR methods have been established based on *GroEL* gene for the simultaneous identification and differentiation of scrub typhus, spotted fever and typhus rickettsioses and its application on epidemiologic survey of wild mouse and vectors in farming areas of Hongta districts (Yuxi city, Yunnan province of China).

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study have been listed in Table 1. Members of genera *Rickettsia*, *Orientia tsutsugamushi* and *Bartonella* were kindly provided by WHO rickettsia collaboration center (Marseille, France), *E. chaffeensis* was supplied by US CDC and A. *phagocytophilum*, by Johns Hopkins University School of Medicine, Baltimore, USA. Members of genera *Coxiella burnetii* and spotted fever group rickettsia were conserved by our laboratory. Among them, *R. heilongjiangii* 054, *R. hulinii* HL-93 *and R. sibirica* BJ-90 were isolated and saved by the Chinese experts [10, 11]. Other strains were provided by departments of diarrhea disease, respiratory infections, tuberculosis, plague and anthrax respectively (National Institute of Communicable Disease Control and Prevention of China CDC). DNA was extracted by using QIAamp DNA blood and tissue mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Primer design and nested PCR amplification

The outer and inner primer sets of the *GroEL* have been listed in Table 2. The outer set of primers was derived from conserved regions based on a multiple-sequence alignment of *GroEL* gene sequences of different rickettsia obtained from GenBank: Gro-1 [5′-AAGAAGGA/CGTGATAAC-3′; from positions 603 to 618 in U96733] and Gro-2 [5′-ACTTCA/CGTAGCACC-3′; from positions 1251 to 1238 in U96733]. The inner primer sets were derived from literature[12]. Primers targeted for genera *Rickettsia* were: SF1: 5′-ATAGAAGAAAAGCAATGATG-3′ (from positions 938 to 958 in *R. rickettsia* U96733) and SR2: 5′-CAGCTATTTGAGATTTAATTTG-3′ (from 1154 to 1133 in U96733). Primers targeted for genera *O. tsutsugamushi*: TF1:5′-ATATATCACAGTACTTTGCAAC-3′ (from positions 1729 to 1750 in *O. t.* Karp M31887) and TR2: 5′-GTTCCTAACTTAGATGTATCAT-3′ (from 1092 to 1071 in M31887). DNA extracted from different bacteria as template (50 mg) was added to 20μL of PCR mixture, which contained 20 pmol of Gro-1 and Gro-2 primers, 250 μmol/L dNTPs, 1.5 U of *Taq* DNA polymerase (SBS Genentech), 2 μL of 10 × buffer and gel loading dye. The volume was adjusted to 20 μL with distilled water. For the first round, PCR mixtures were incubated at 95°C for 5 minutes and subjected to 30 cycles of 94°C for 40 s, 45°C for 40 s, and 72°C for 40 s and then to a final extension at 72°C for 5 minutes (PTC-100TM, MJ Research, Ltd.). In the second round, the PCR mixture contained 10 pmol of SF1, SR2, TF1 and TR2, 250μmol/L dNTPs, 2.5 U of Taq DNA polymerase, 2.5 μL of 10 × buffer and gel loading dye. The volume was adjusted to 25 μL with distilled water. The reaction was incubated at 94°C for 5 minutes and subjected to 30 cycles of 94°C for 35 s, 56°C for 35 s, and 72°C for 35 s and then to a final extension at 72°C for 4 minutes. The nested PCR products were electrophoresed on a 1.5% agarose gel and were sequenced by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd.

The specificity and sensitivity of the nested PCR

General primers for 16sRNA gene of bacteria (Roux V 1995), 16S1:5′-AGAGTTTGATCMTGGCTCAG-3′, 6S2: 5′-TACGGYTACCTTGTTACGACTT-3′ were used to amplify the rickettsial and non- rickettsial strains listed in the above Table 1. The products were sequenced by a commercial company and only the confirmed strains were tested by the duplex nested PCR established in this study so as to evaluate the specificity of the methods. The sensitivity test of the method was conducted as follows: The PCR products from *R. typhi*, W strain and *O. t* Karp strain (20 ng), were purified (Watson Biotechnologies, Inc.) and ligated with 50 ng of the pGM-T vector (Tiangen Biotech CO., Ltd.) at 16°C for 18 h and the obtained plasmid was transformed into

TOP10 (Tiangen) competent cells. Positive clones were selected by PCR using specific primers, and plasmid DNA was extracted with a High Pure Plasmid Isolation kit (Watson, Biotechnologies, Inc.). The concentration of the plasmids was determined by an optical density reading instrument at 260nm[13]. Serial 10-fold dilutions of plasmid of both strains in the distilled water were performed, resulting in final target concentrations of 10^9-10^0 copies/μL. Each concentration was amplified as standard template by using the prescribed methods in order to detect the sensitivity of the new PCR methods.

PCR-RFLP analysis

Restriction endonuclease, *RsaI* (SBS Genentech) was used to cleave the PCR products of genus *Rickettsia* to differentiate between the typhus and spotted fever group rickettsia, and the products of genus *Orientia tsutsugamushi* were digested with restriction endonucleases *HinfI* and *RsaI* so as to differentiate between different types of *Orientia tsutsugamushi*.

Investigation on natural focus of rickettsioses in Yuxi city of Yunnan Province

In 1999, North Asian tick borne spotted fever was first detected in Hongta district in Yuxi city by a seroepidemiologic survey [14]. However, unexplained febrile illnesses increased violently during summer and autumn every year in this region since 1990s. The patients were clinically diagnosed with scrub typhus by eschar on their bodies and experiential treatment effect by using chloramphenicol. A previous study had demonstrated that such kind of febrile cases were not only diagnosed as scrub typhus and typhus but also as the co-infection of scrub typhus and typhus in some patients [15]. In order to investigate the prevalence of these rickettsioses in hosts and vectors found in those areas, animal samples including mouse blood, viscera samples, fleas and mites were collected from Hongta districts in August 2005 and tested by the nested PCR method as described above.

Samples collection and DNA extraction

From 17th to 24th August 2005, a total of 345 *Rattus berkohout* (44) and *Rattus flavipectus* (301) were captured from LiQi, Da Yingjie, GaoCang, YanHe, LuoHe, ChunHe, Xiao Shiqiao, BeiCheng and Zhou Cheng towns in Yuxi city. 96 *Xenopsylla cheopis* and 32 chiggers were obtained from the surface of mouse. All samples used in this study were stored at 20°C until transferring to the Dept. of *Rickettsioses* of China CDC for DNA extraction. For each mouse, samples including liver, kidney and spleen were grinded to form a mixture by axenic glass sharpener with 200 μL of SPG buffer. 32 acarids and 32 fleas were grinded separately. 200 μL of grinded fluid of each sample was used to extract DNA by using the traditional method as described earlier.

PCR RFLP and phylogenetic analysis

10μL of extracted DNA from samples was amplified as template by the new PCR method described in this study. *R. typhi* W, *R. sibirica* and *O. t.* Karp strains were used as positive control while *E. coli* MG1655 and distilled water as negative control. All positive PCR products were divided into two parts. One part was cut by restriction endonucleases, *HinfI* and or *RsaI*, and the other part was sequenced by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. For the low concentration PCR products and products with double fragments, pGM-T vector (Tiangen Biotech CO. Ltd.) was used to clone according to the instructions of manufacturer. Positive clones containing inserts were identified by the PCR. Positive plasmids were sequenced. Sequences analyses were performed by NCBI and DNASTAR MegAlign software packages (version 10.0).

RESULTS

Specificity and Sensitivity of the Nested PCR

After the nested PCR had completed, 217-bp DNA fragments were amplified from the members of genera *Rickettsia* including the typhus and spotted fever group rickettsia, and 364-bp DNA fragments were amplified from the members of genera *O. tsutsugamushi* (Fig. 1 A). The specificity of the method was 100% and all rickettsial strains of genera *Rickettsia* and *Orientia tsutsugamushi* obtained in this study were positive amplifications and the PCR fragments were further confirmed by the sequence analysis. The negative control strains including members of genera *Ehrlichia*, *Coxiella*, *Bartonella* and non-*Rickettsia* were devoid of any PCR products (Fig. 1 B). The sensitivity of the method reached at one copy per microliter when PCR products were observed on ethidium bromide-stained gel (Fig. 1C).

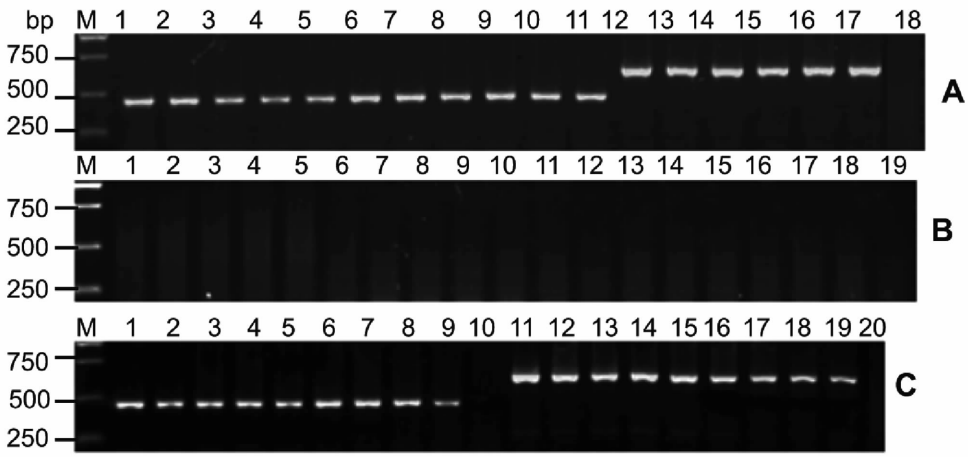

Fig. 1. The results of the nested PCR for some members of genera *Rickettsia* and *Orientia tsutsugamushi* (A), some non-rickettsial strains (B) and the sensitivity test for the members of genera *Rickettsia* and *Orientia tsutsugamushi* (C).
A M: DL 2000 Marker; 1: *R. prowazekii* B strain; 2: *R. typhi* W; 3: *R. conorii*; 4: *R. sibirica*; 5: *R. akari*; 6: *R. africae*; 7: *R. australi*; 8: *R. parkeri*; 9: *R. heilongjiangii* 054; 10: *R. hulinii* HL-93; 11: BJ-90; 12: *O. t* Karp; 13: *O. t* Kato; 14: *O. t* Gilliam; 15: *O. t* Kawasaki; 16: TA763; 17: TA1817; 18: distilled water. B M: DL 2000 Marker; 1: *E. chaffeensis*; 2: *A. phagocytophilum*; 3: *B. henselae*; 4: *B. elizabethae*; 5: *B. birtlesii*; 6: *B. quintana*; 7: *B. vinsonii*; 8: *C. burnettii*; 9: *S. flexneri* 2a 301; 10: *E. coli* MG1655; 11: *S. typhi* CT18; 12: *V. cholera* N16961; 13: *Legionella pneumophila*1 Lp1; 14: *N. meningitidis*; 15: *Haemophilus influenzae type b*, Hib; 16: *S. pneumoniae*; 17: *Mycobacterium tuberculosis* H37Rv; 18: *Y. pestis*; 19: *Bacillus anthracis*. C M: DL 2000 Marker; From 1 to 9 lanes: Copies of *R. typhi* W strains form $10^8/\mu L$ to $10^0/\mu L$; 10 lanes: distilled water; From 11 to 19 lanes: Copies of *O. t* Karp from $10^8/\mu L$ to $10^0/\mu L$; 20: distilled water.

PCR-RFLP

HinfI and *RsaI* digestions were easily distinguished among the common strains of *O. tsutsugamushi*. Kato, and Kawasaki types of *O. tsutsugamushi* had the same restriction pattern (322 and 42bp) when PCR products were digested by *HinfI* but Karp strain was cut into three fragments of 299bp, 42bp and 23bp. Gilliam strain had not been digested by *HinfI* (Fig. 2 A). Kato and Karp strains digested by *RsaI* had the same restriction pattern (resulting 352bp and 12bp). Kawasaki and Gilliam strains resulted in three fragments of 270bp, 82bp and 12bp (Fig. 2 B). Typhus group rickettsia was easily distinguished from spotted fever group rickettsia by *RsaI* digestion. Most members of the typhus group rickettsia were divided into two fragments of 108bp and 109bp but spotted fever group rickettsia could not be digested except *R. australis* (Fig. 2C).

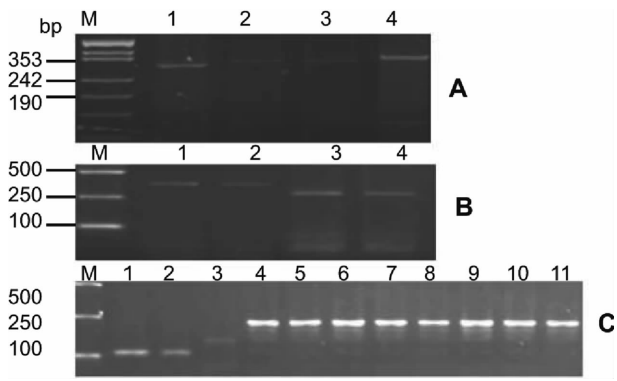

Fig. 2. The results of digestion of members of genera *O. tsutsugamushi* by *HinfI* (A) and *RsaI* (B) and the results of digestion of members of genera Rickettsia by *RsaI* (C).
A M: PUC18 DNA/MspI Marker; 1: *O. t* Karp; 2: *O. t* Kato; 3: *O. t* Kawasaki; 4: *O. t* Gilliam B M: DL2000 Marker; 1: *O. t* Karp; 2: *O. t* Kato; 3: *O. t* Gilliam; 4: *O. t* Kawasaki C M: DL2000 Marker; 1: *R. prowazekii B*; 2: *R. typhi W*; 3: *R. australis*; 4: *R. conorii*; 5: *R. sibirica*; 6: *R. akari*; 7: *R. africae*; 8: *R. parkeri*; 9: *R. heilongjiangii* 054; 10: *R. hulinii* HL-93; 11: BJ-90.

Results of epidemiologic survey of wild mouse and vectors in Hongta Areas of Yuxi City

A total of 345 (*Rattus berkohout* 44 and *Rattus flavipectus* 301) mouse viscera were tested by the nested PCR method in this study. The general positive rate was 34.78% (120/345). RFLP analysis showed that the positive rate of *R. typhi* was 28.12% (97/345), the positive rate of *O. t* Karp was 19.71% (68/345), and the positive rate of *R. sibirica* was 0.29% (1/345). It was observed that 13.33% (46/345) of tested samples were confirmed to be coinfected by *R. typhi* and *O. t* Karp. One positive

result was found for the tested three flea DNA samples and identified to be *R. typhi* by the sequences analysis. *O. t* Karp sequence was obtained from only one sample of acarids. The infection rates of *Rattus berkohout* and *Rattus flavipectus* have been listed in Table 3.

Table 1 Strains used in the study

Genera	Group	Strain
Rickettsia	Spotted fever	*R. conorii*
		R. sibirica
		R. akari
		R. australis
		R. africae
		R. parkeri
		R. heilongjiangii 054
		R. hulinii HL-93
		R. sibirica BJ-90
	typhus	*R. prowazekii* B
		R. *typhi* W
O. tsutsugamushi		*O. t Karp*
		O. t Kato
		O. t Gilliam
		O. t Kawasaki
		TA763
		TA1817
Ehrlichia		*E. chaffeensis*
Anaplasma		*Anaplasma phagocytosis*
Bartonella		*B. henselae*
		B. elizabethae
		B. quintana
		B. vinsonii
		B. birtlesii
Coxiella		*C. burnettii*
Other strains		*S. flexneri* 2a 301
		E. coli MG1655
		S. typhi CT18
		V. cholera N16961
		Legionella pneumophila 1 Lp1
		N. meningitidis
		Haemophilus influenzae type b, Hib
		S. pneumoniae
		Mycobacterium tuberculosis
		H37Rv
		Y. pestis
		Bacillus anthracis

Sequence analysis based on *GroEL* gene in the study suggested that nucleotide sequences of 217bp from 47 mouse samples showed similarity to *R. typhi* W (AY191590) of these sequences, 42 sequences had 100% similarity to *R. typhi* W strain while 5 of them had 99% similarity with *R. typhi* W strain. Among 47 samples, 45 of deduced amino acid sequences had 100% similarity to *R. typhi* W while 2 of them were 98% homologous to *R. typhi* W. The nucleotide sequences of 364bp from the successfully sequenced 25 mouse samples showed high similarity to *O. t* Karp(M31887), 10 samples had 100% similarity to *O. t Karp* and 15 samples had 99%, For their deduced amino acid sequences, 21 samples had 100% similarity to *O. t* Karp strain while 4 samples, 99%. Only one mouse sample was identified with spotted fever group *rickettsia*, and its nucleotide sequence and deduced amino acid sequenced indicated 100% similarity to *R. sibirica* (AY059014). One of the three tested samples of flea, amplified by the sequence of *R. typhi* and its nucleotide and deduced amino acid sequences had 100% similarity to *R. typhi* W. The results of sequence analysis were fully consistent with those of RFLP analysis.

DISCUSSION

Rickettsioses are known as zoonoses worldwide and impose a big threat to public health. Emerging and reemerging rickettsial diseases have been identified frequently in recent years. However, the challenges to the prevention and control of Rickettsioses in China have been universal misdiagnosis and delayed treatment because there is no advanced diagnostic technology available in most clinical laboratories. With the improved economic and living conditions in China, epidemic typhus has been controlled largely, but endemic typhus still continues to prevail in the countryside. Natural focus of scrub typhus had begun to distract south areas of China from the whole country[16]. In addition, some studies indicated that nucleotide sequences of *R. africae*, *R. honei*, *R. marseille* and *R. heilongjiangii* were identified in ticks collected in southern provinces by a molecular epidemiological survey [2, 3]. Regular or nested PCR method based on the single and specific gene amplification including 16S rRNA gene, *gltA* gene, *ompA* gene, *ompB* and 17kD gene, has broadly been used to diagnose rickettsial diseases , but it usually

Table 2 The primer sequences used to perform the nested PCR in the study and its variation in different strains

Primer	Strain (accession No)	Forward	Reverse
Gro1 603-618 in U96733	*R. rickettsia* U96733	AAGAAGGCGTGATAAC·····	GGTGCTACGGAAGT
Gro2 1251-1238 in U96733	*R. prowazekii* (Y15783)	AAGAAGGCGTGATCAC·····	GGTGCTACCGAAGT
	R. typhi W (AY191590)	AAGAAGGTGTGATCAC·····	GGTGCTACAGAAGT
	R. conorii (AE008650)	AAGAAGGCGTGATAAC·····	GGTGCTACGGAAGT
	R. felis (CP000053)	AAGAAGGCGTGATAAC·····	GGTGCTACAGAAGT
	R. helvetica (DQ442911)	AAGAAGGCGTGATAAC·····	GGTGCTACGGAAGT
	O. t Kato (AY191586)	AAGAAGGAGTGATAAC·····	GGTGCTACTGAAGT
SF1 938-958 in U96733	*R. rickettsia* U96733	GATAGAAGAAAAGCAATGATG	····· CAAATTAAATCTCAAAT-AGCTG
SR2 1154-1133 in U96733	*R. prowazekii* (Y15783)	GATAGAAGAAAAGCAATGATG	····· CAAATTAAATCTCAAAT-AGCTG
	R. typhi W (AY191590)	GATAGAAGAAAAGCAATGATG	····· CAAATTAAATCTCAAAT-AGCTG
	R. conorii (AE008650)	GATAGAAGAAAAGCAATGATG	····· CAAATTAAATCTCAAAT-AGCTG
	R. felis (CP000053)	GATAGAAGAAAAGCAATGATG	····· CAAATTAAATCTCAAAT-AGCTG
	R. helvetica (DQ442911)	GATAGAAGAAAAGCAATGATG	····· CAAATTAAATCTCAAAT-AGCTG
TF1 1729-1750 M31887	*O. t* Karp (M31887)	ATATATCACAGTACTTTGCAAC	····· ATGATACATCTAAGTTAG-GAAC
TR2 1092-1071 M31887	*O. t* Kato (AY191586)	ATATATCACAGTACTTTGCAAC	····· ATGATACATCTAAGTTAG-GAAC
	O. t Gilliam (AY191585)	ATATATCACAGTACTTTGCAAC	····· ATGATACATCTAAGTTAG-GAAC

Sequences with lines are the sequences of primers; red part is different base pair.

Table 3 The infection rates of *Rattus berkohout* and *Rattus flavipectus*

Agents	Prevalence (%)		Total
	Rattus berkohout	*Rattus flavipectus*	
R. typhi	34.09% (15/44)	27.24% (82/301)	28.12% (97/345)
O. t Karp	18.18% (8/44)	19.93% (60/301)	19.71% (68/345)
R. sibirica	0(0/44)	0.33% (1/301)	0.29% (1/345)

consumed a lot of time to identify different agents of rickettsioses. Since the sequences of 16S rRNA gene are highly conserved, it is difficult to differentially diagnose them. Although sequence comparison of *gltA* gene is more sensitive than that of 16S rRNA gene, reliable phylogenetic interrelationships could be found only among those rickettsial strains, which diverged early from the common ancestor of spotted fever group *reckettsiae*[17]. Although differences of *ompA* gene, *ompB* and 17KD genes are greater than those of 16S rRNA and *gltA* genes, the single gene amplification could not detect multi-agents simultaneously[18]. To overcome these problems, the nested and duplex PCR primers targeted for multi-genera in-

cluding *Rickettsia* and *Orientia tsutsugamushi* were developed base on *GroEL* gene in the study. This method not only does identify and differentiate between the genera *Rickettsia* and *Orientia tsutsugamushi* by their sizes of PCR fragments but also differentiate between species by sequencing PCR products. The evaluation of the methodology proved that the specificity of the methods was 100% and the analysis of sensitivity conformed that they could detect at the lowest concentration of 1×10^0 copy.

Since 90s in the last century, the unknown febrile patients increased violently in the summer every year in Yuxi city of Yunnan province. These patients principally had a high fever, headache, myalgia and an eschar on their body but no rash was observed. The average incidence rate of these patients was $222.1/10^5$, $204.3/10^5$ and $109.6/10^5$ in four town of Chunhe, Da yingjie, Gaocang and Beicheng respectively, from 2002 to 2004[15]. Although laboratory evidence was absent, a clinical empirical diagnosis of scrub typhus was made based on symptoms and valid antibiotic therapy. Laboratory's affirmative diagnosis was made in 2005 in which co-infection of typhus and scrub typhus was confirmed except diagnoses of scrub typhus and typhus themselves. Based on these data, the further investigation of the natural focus was conducted by using the duplex and nested PCR methods established in is study. The studies indicated that the infection rates of *R. typhi* and *O. tsutsugamushi* of mouse were 28.12% and 19.13% respectively. Co-infection rate of typhus and scrub typhus was 13.33%. The diagnosis was further supported by the detection of vector samples of fleas and acarids collected from mouse. This survey and the previous study provided a strong evidence that natural focus of typhus and scrub typhus existed in Yuxi city (Yunnan province, China), and furthermore, co-infection of typhus and scrub typhus was very common. The predominant strain in these areas was *O. t.* Karp type; a result which coincides with the previous study [19, 20].

The vector-associated infections severely impact on the public health in Yunnan province located in subtropial areas of China. Based on the documented data, nearly all kinds of *Rickettsioses* found in China have once been reported in Yunnan province. Mouse is abundant in these areas, so the mouse- associated infections are prevalent severely. Since new China was founded, there have been three times bigger gene outbreaks of typhus in China, which attacked more than ten thousand people every time and the prevalences were inevitably associated with Yunnan province. In the second outbreak, the number of cases in only Yunnan province was half of the total cases reported in the whole country. Scrub typhus is an important rickettsial disease in southeastern Asia. At present, it is considered as one of the principal rickettsial diseases, threatening human health especially tourists. Since Yunnan province is the centre of tourism, close monitoring and prevention of vector born infectious diseases especially rickettsioses in these areas would be a significant contribution to the public health safety goal.

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