DETECTION OF SPOTTED FEVER GROUP RICKETTSIAE IN TICKS AND RODENTS BY POLYMERASE CHAIN REACTION TECHNIQUE IN PEOPLE'S REPUBLIC OF CHINA

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Summary. – The polymerase chain reaction (PCR) technique for amplification of genomic fragments of spotted fever group (SFG) rickettsiae directly from field samples of ticks, tick ova, tick larvae, tick faeces and organs of wild mice was employed for the first time in P. R. of China. Ticks and rodents were collected in Beijing and Heilongjiang, Hainan and Hebei Provinces. The PCR primers were designed from the DNA sequence encoding the 190 K protein of *R. rickettsii* for a 532 bp long product. Seven of ten tick samples, three of four tick ovum samples, one of two tick larva samples, four of seven tick faeces samples (the samples represented pools of several individuals), and two of twenty-seven wild mouse organs were found PCR-positive. In comparison with PCR assay, the haemolymph test gave similar but not so clear-cut results. PCR assay is recommended as a rapid, sensitive and convenient tool for the detection of SFG rickettsiae in endemic foci. The fact that tick faeces were found to certain extent PCR-positive for the presence of SFG rickettsiae is apparently the first report on this subject and contributes to the knowledge of the transmission of these microorganisms in the nature.

Key words: polymerase chain reaction; spotted fever group rickettsiae; ticks; rodents; haemolymph test

Introduction

The SFG is the most complex group among rickettsiae that is distributed all over the world (Walker, 1989). Because rickettsiae are obligatory intracellular microorganisms, their isolation and cultivation are rather difficult. Therefore the study of the SFG rickettsiae was not much developed in P. R. of China in the past. In 30 years, only a little more than 10 strains of SFG rickettsiae have been isolated in P. R. of China (Fan, 1992). Recently, the use of PCR technique as a screening method for detection of rickettsia-positive ticks in large-scale surveys has been proposed (Beati *et al.*, 1992). To test the applicability of this procedure as a new convenient detection method in epidemiological investigations in China, and to provide a clue for isolation of rickettsiae, the PCR technique was applied directly to ticks and their ova, larvae and faeces, as well as to rodent organs during epidemiological investigations in Beijing, Heilongijang, Hainan and Hebei Provinces in P. R. of China in 1993.

Abbreviations: PCR = polymerase chain reaction; SFG = spotted fever group

Materials and Methods

Ticks. Haemaphysalis concinna, Ixodes persulcatus and *Dermacentor silvarum* (732 ticks) were collected by flagging the vegetation in Hulin County, Raohe County and city of Suifenhe in Heilongjiang Province in April 1993. *Dermacentor sinicus* (3 ticks) was collected in Changping County of city of Beijing in May 1993. *Haemaphysalis longicornis* (1 tick) was collected from a tourist in Hebei Province. *Boophilus microplus* (1 tick) was collected in Hainan Province in 1986. Ticks were stored at –70 °C.

Wild mice. Apodemus agrarius, Mus musculus, Microtus fortis, Clethrionomys rufocanus, Clethrionomys rutilus, Eutamias sibiricus, Cricetus triton and Cricetus barabensis were collected in Hulin County, Raohe County, and cities of Mishan and Suifenhe in Heilongjiang Province, dissected on the spot, and their livers and spleens were taken and stored at –20 °C.

Tick disposal and haemolymph test. After classification, ticks were divided into groups according to area of collection and vari-

Table 1. Results of PCR assay of SFG rickettsiae in ticks, tick ova, tick larvae and tick faeces originating from various localities of P. R. of China

Sample	Locality					
	Hulin County	Raohe County	Suifenhe city	Beijing city	Hebei Prov.	Hainan Prov.
I. persulcatus						
Ticks	–		–			
H. concinna						
Ticks	+		+			
Tick ova	+		+			
Tick larvae			+			
Tick faeces	+		+			
D. silvarum						
Ticks	+	+	–			
Tick ova			–			
Tick larvae			–			
Tick faeces	+	+	–			
D. sinicus						
Ticks				+		
H. longicornis						
Ticks					+	
B. microplus						
Ticks						+

Result positive (+), negative (–).

Table 2. Comparison of PCR assay with haemolymph test in detecting SFG rickettsiae in ticks originating from various localities of P. R. of China

Tick	Assay	Locality			
		Hulin County	Raohe County	Suifenhe city	Beijing city
I. persulcatus	HL	±		±	
	PCR	–	–		
H. concinna	HL	+		+	
	PCR	+		+	
D. silvarum	HL	±	±	±	
	PCR	+	+	–	
D. sinicus	HL				+
	PCR				+

HL = haemolymph assay.
Result positive (+), ambiguous (±), negative (–).

ety. Dead ticks were stored at –70 °C, while the living ones were left to suck blood on ears of rabbits separately. Tick faeces were collected separately and stored at –70 °C as well as at room temperature. The ticks fed on rabbit blood were disinfected by immersing in 70% ethanol for 10 mins and rinsed in distilled water for 10 mins. The ticks were first subjected to the haemolymph test: after cutting off one foreleg one drop of the haemolymph was collected (Burgdorfer, 1970), applied onto a microscope slide and stained by the Gimenez method (Gimenez, 1964). The ticks negative in the haemolymph test were used in PCR as controls, while the positive or suspect ones were divided into three parts. The first part was used for isolation of rickettsiae, the second one was stored at –70 °C for PCR assay, and the third one was kept at room temperature in humid air for laying eggs. After the latter were laid, they were collected separately according to the tick variety and divided into three parts. The first and second parts were used in the same way as those of positive haemolymph samples, while the third one was used for hatching larvae. The latter were collected and stored at –70 °C for PCR assay.

R. sibirica strain 246, employed for the positive control in PCR assay, was provided by the Chinese Military Academy of Medical Research, Beijing. It was cultivated in SPF embryonated eggs (Stoenner *et al.*, 1962), purified according to Hanson *et al.*, 1981), and its DNA extracted by standard procedures (Sambrook *et al.*, 1989).

DNA preparation from field samples. Ticks were washed with distilled water (3 x 10 mins) and disinfected by immersing in 75% ethanol for 10 mins. After separation into groups, these were ground and suspended in 0.3 ml of TE buffer (Sambrook *et al.*, 1989). DNA extraction was carried out according to Furuya *et al.* (1991). *Livers and spleens* of wild mice were disinfected by immersing in water solution of antibiotics (penicillin G 500 U/ml and streptomycin 500 µg/ml) for 30 mins and then washed in distilled water (3 x 10 mins). Organs were ground and their suspensions were subjected to DNA extraction. *Tick ova, larvae and faeces*: 0.25 g of each was employed for preparation of suspension and DNA extraction by the procedure mentioned above.

PCR. A pair of oligonucleotide primers (Rr 190.70p: 5'-ATG GCG AAT ATT TCT CCA AAA-3'; Rr 190.602n: 5'-AGT GCA GCA TTC GCT CCC CCT-3') used in PCR was derived from DNA sequence of a gene encoding the 190 K antigen of *R. rickettsii* (Anderson *et al.*, 1990; Regnery *et al.*, 1991).It was synthesized and provided by the Chinese Academy of Science, Beijing. The amplification was carried out in 50 µl volume in Gene ATAQ Controller (Pharmacia) in 30 cycles (95 °C for 40 secs, 48 °C for 40 secs, 66 °C for 80 secs, the last cycle extended to 5 mins). PCR assays included one positive control (*R. sibirica* strain 246 DNA) and two negative controls (no template DNA; non-infected yolk sac; normal mouse organ and normal rabbit blood samples). The presence of amplified PCR products was confirmed by electrophoresing 10 µl sample of PCR mixture in 1.2% agarose gel. *Hinf*I-digested pBR322 DNA was employed as marker.

Results and Discussion

The results of PCR assay of SFG rickettsiae, as monitored by the presence of the specific 532 bp amplification product in PAGE, in ticks, tick ova, tick larvae, tick faeces, and wild mouse organs originating from various localities of P. R. of China are demonstrated in Figs. 1-5 and summa-

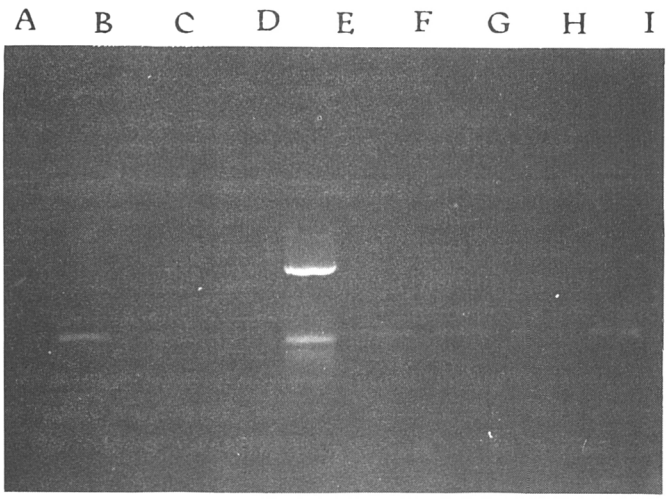

Fig. 1
PAGE of PCR products of tick samples
No template DNA, negative control (lane A), *R. sibirica* DNA, positive control (lane B), negative ticks control (lane C), negative rabbit blood control lane D), marker *Hinf*I-digested pBR322 DNA: 1631, 517, 506, 396 and 344 bp fragments (lane E), *H. concinna,* Hulin County (lane F), *D. silvarum,* Hulin County (lane G), *H. concinna,* Suifenhe city (lane H), *D. silvarum,* Raohe County (lane I).

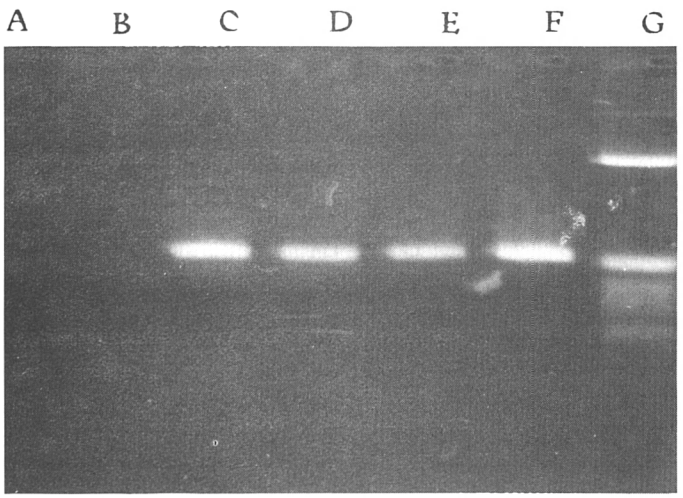

Fig. 2
PAGE of PCR products of tick ovum samples
No template DNA, negative control (lane A), negative ticks control (lane B), *R. sibirica* DNA, positive control (lane C), *D. silvarum,* Hulin County (lane D), *H. concinna,* Hulin County (lane E), *H. concinna*, Suifenhe city (lane F), marker *Hinf*I-digested pBR322 DNA (lane G).

rized in Table 1. It can be seen that 7 of 10 tick samples, 3 of 4 tick ovum samples, 1 of 2 tick larva samples, and 4 of 7 tick faeces samples were found positive (the results with wild mouse organs are not summarized in Table 1).

The results of the PCR assay were compared with those of the haemolymph test. Some of these comparisons made on tick samples originating from various localities are summarized in Table 2. While the PCR assay gave clear-cut results (positive or negative), the haemolymph test gave often ambiguous (±) results. In general, both the tests yielded roughly similar results.

Some of the samples tested by PCR were used also for isolation of rickettsiae by standard procedure. Results of these experiments showed that the isolations gave a lower

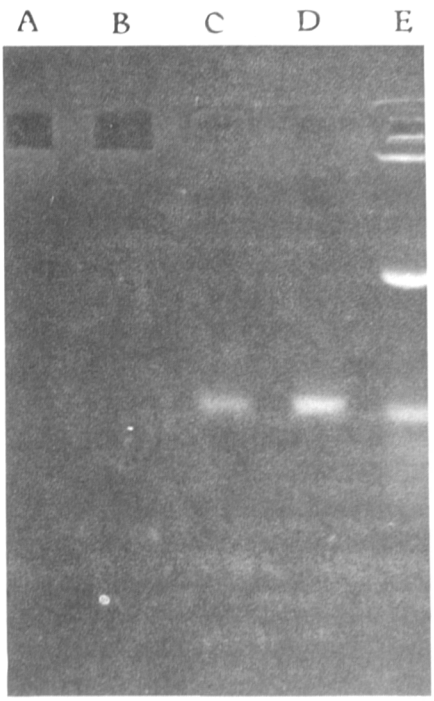

Fig. 3
PAGE of PCR products of tick larva sample
No template DNA, negative control (lane A), negative ticks control (lane B), *H. concinna,* Suifenhe city (lane C), *R. sibirica* DNA, positive control (lane D), marker *Hinf*I-digested pBR322 DNA (lane E).

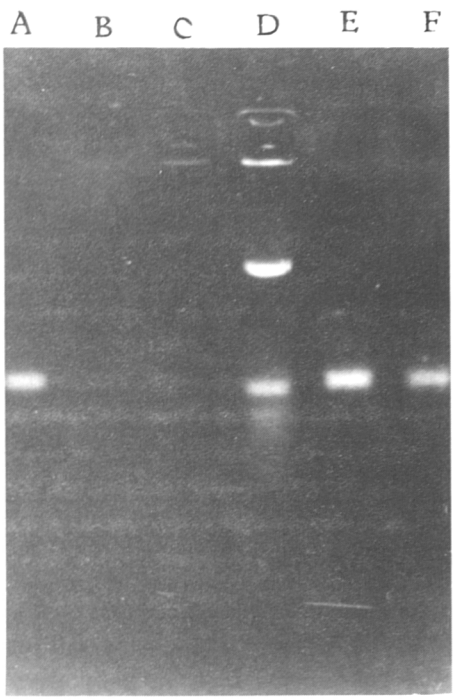

Fig. 5
PAGE of PCR products of wild mouse organs samples
R. sibirica DNA, positive control (lane A), no template DNA, negative control (lane B), negative mouse organs control (lane C), marker *Hinf*I-digested pBR322 (lane D), *M. fortis*, Hulin County (lane E), *M. musculus*, Hulin County (lane F).

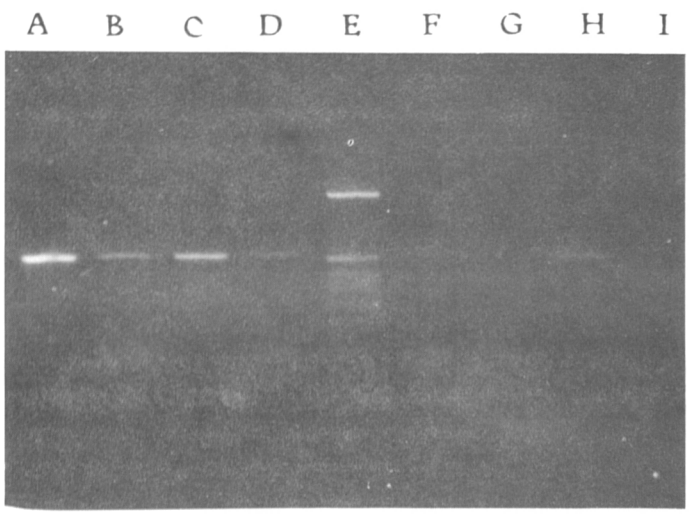

Fig. 4
PAGE of PCR products of tick faeces samples
H. concinna, Suifenhe city, sample stored at –70 °C (lane A), *D. silvarum*, Raohe County, –70 °C (lane B), *H. concinna*, Hulin County, –70 °C (lane C), *D. silvarum,* Hulin County, –70 °C (lane D), marker *Hinf*I-digested pBR322 DNA (lane E), *D. silvarum* Hulin County, room temperature (lane F), negative control (lane G), *R. sibirica* DNA, positive control (lane H), no template DNA, negative control (lane I).

incidence of positivity as compared to the PCR or haemolymph assays (data not shown).

To identify the amplified 532 bp PCR product with the corresponding sequence of the 190 K gene of SFG rickettsiae, we digested the PCR products of some tick samples with restriction endonucleases *Pst*I and *Rsa*I and analyzed them by PAGE. The restriction profiles of the tick samples were the same as those of the reference SFG rickettsiae (isolates HL-93 and BJ-93; data not shown).

Since Beati *et al.* (1992) suggested that PCR combined with the restriction fragment length polymorphism (RFLP) analysis could be employed for identification of rickettsiae in large scale epidemiological studies, a few researchers had used this approach in detecting rickettsiae directly in naturally infected ticks or in identifying previously unrecognized rickettsial serotypes in isolates (Gage *et al.*, 1994; Beati *et al.*, 1994; Bacellar *et al.*, 1995). In this study we attempted to use the PCR technique in rapid screening of SFG rickettsiae in naturally infected ticks and rodents during epidemiological investigation in Heilongjiang Province and the city of Beijing in 1993. Using a specific primer pair, we succeeded in amplification of a DNA fragment of a predicted size of 532 bp, corresponding to a part of the gene encoding the 190 K protein of SFG rickettsiae, directly from naturally infected ticks, tick ova, tick larvae, tick faeces, and rodent organs. We consider the PCR assay more sensitive than the isolation experiment, and more precise than the haemolymph test.

As far as we know, the finding of PCR-positivity of tick faeces for SFG rickettsiae was not yet reported before. Anyway, it contributes to our knowledge of the spread of rickettsiae in the nature. Altogether with a partial positivity of tick ova, tick larvae and wild mice these data support the views that ticks can transmit these microorganisms also vertically, and that ticks are not only their vectors but also their reservoirs.

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