Research Note

Multiple *Rickettsia tsutsugamushi* types detected by polymerase chain reaction in an infected laboratory colony of *Leptotrombidium pallidum*

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Abstract: The *Rickettsia tsutsugamushi* (Rt)-DNA was detected and classified by polymerase chain reaction from larvae of an infected laboratory colony of *Leptotrombidium pallidum*. Rt-DNAs were detected in 6 of 14 engorged larvae (42.9%). Although Kawasaki, Karp·Kawasaki, Karp·Kawasaki·Kuroki, and unidentified Rt-DNA were detected from the infected larvae, only the Karp type was found by PCR in blood clots of a mouse attached with those mites used for Rt detection and in those of mice up to the 4th passage.

The type of *Rickettsia tsutsugamushi* (Rt) isolated by mouse passage method from an infected laboratory colony of *Leptotrombidium pallidum* in our laboratory has been always Karp type (Takahashi *et al.*, 1988). This infected colony was established from a single naturally infected larva detached from *Apodemus speciosus*, a wild rodent.

Usually, detection of Rt from mites has been performed by a sensitive modified method of mouse passage (Kawamura *et al.*, 1980), which took several weeks.

For rapid and simple detection of Rt, an attempt was made to use PCR for detecting Rt-DNA in mites (Furuya *et al.*, 1991, 1993) in our infected colony, instead of the time-consuming method of mouse passage. By the PCR method, we found multiple DNA types of Rt in mites besides the one detected by mouse passage from peritoneal smears by immunofluorescence (IF).

Materials and methods

Trombiculid mites: The infected *L. pallidum* larvae used for the present study were F₉ generation from an infected female (F₈), infection of which was proven by mouse passage.

Type identification of Rt: Rt-DNAs in mites and in clots of mouse blood were detected and classified by PCR, and Rt types in peritoneal smears were examined by indirect IF using specific monoclonal antibodies

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against 3 prototypes: Gilliam, Karp and Kato type strains.

In this study, the above-mentioned larvae were attached to a mouse, and the larvae after feeding and the mouse attached were subjected Rt detection and type identification.

Unfed larvae at F₀ were transferred by a fine painting brush into the ear lobe under a stereomicroscope. After about 2 weeks, the mouse was dissected out under anesthesia; changes in abdominal tissues by Rt were observed, and blood clots and peritoneal smears were examined for Rt by PCR and IF, respectively.

The liver and spleen of the mouse were taken out and ground in sucrose phosphate glutamate (SPG) solution of Bovarnick *et al.*, (1950) to make 10% emulsion. One fourth ml of supernatant of the emulsion was injected intraperitoneally (ip) into a female ddY mouse at age 4 weeks, and detection of Rt and antibodies was continued up to the 4th mouse passage.

PCR: A larval *L. pallidum* was crushed with the tip of a syringe needle in 10 μ l of distilled water. A 10 μ l emulsion was added with 1 μ l of 1 M NaOH to make a final concentration of 0.1 M, and this solution was incubated at 37°C for 60 min. Then it was centrifuged for 15 sec in a microcentrifuge and neutralized with 1 μ l of 1 M HCl. This solution was used as DNA template for PCR.

The following oligonucleotide primers used were preparations ordered from a maker (Bex, Tokyo, Japan).

Primer 34: 5'-TCAAGCTTATTGCTAGTGCAATGTCTGC-3'

Primer 55: 5'-AGGGATCCCTGCTGCTGTGCTTGCTGCG-3'

Primer 10: 5'-GATCAAGCTTCCTCAGCCTACTATAATGCC-3'

Primer 11: 5'-CTAGGGATCCCGACAGATGCACTATTAGGC-3'

Primer GL: 5'-CTTTATATCACTATATATCTT-3'

Primer KP: 5'-ACAATATCGGATTTATAACC-3'

Primer KT: 5'-GAATATTTAATAGCAC TGGA-3'

Primer KW: 5'-ATGCTGCTATTGATACAGGC-3'

Primer KR: 5'-CACCGGATTTACCATCATAT-3'

The primers were designed from the nucleotide sequence (Furuya *et al.*, 1993), corresponding to a mature 56 kD protein of Rt (Stover *et al.*, 1990; Ohashi *et al.*, 1990).

PCR was performed with "nested" primer pairs. For the first PCR, a pair of primers 34 and 55 was used.

At the 1st PCR, the PCR amplification mixture (total volume: 50 μ l) contained 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.001 w/v % gelatin, 200 μ M each of dATP, dCTP, dGTP and dTTP, 0.2 μ M of primer 34 and 55, 1.25 units of AmpliTaq polymerase (Cetus, Norwalk, CT), and 5 μ l of template DNA. The mixture was denatured at 94°C for 30 sec, annealed at 57°C for 2 min, then chain-extended at 70°C for 2 min in a thermal cycler (Perkin-Elmer Cetus). This thermal cycle was repeated 30 times.

For the second PCR amplification for determining types of Rt, 5 μ l of the first PCR product was amplified by the same procedures as described above except for using internal primer pairs as follows.

For detecting Rt species specific component irrespective of the types, primers 10 and 11 were used.

For Gilliam type, 407 bp of nucleotides were amplified with primers 10 and GL; for Karp type, 230 bp with primers 10 and KP; for Kato type, 242 bp with primers 10 and KT; for Kawasaki type, 523 bp with primers 11 and KW; and for Kuroki type, 220 bp with primers, 10 and KR.

The amplified samples (5 μ l) were electrophoresed in a 1.5% agarose gel and the DNA bands were stained with 0.5 μ g/ml ethidium bromide.

Antibody detection in mice: Antibodies in mouse sera were detected by indirect IF. Antigens used for antibody detection were L-929 cells infected with Gilliam, Karp or Kato type strain of Rt. For the secondary antibody for IF, the appropriate concentrations of anti-mouse IgG rabbit serum labeled

Fig. 1 Agarose gel electrophoresis of amplified DNA by nested PCR with the serotype-specific primers for Gilliam (lane 2), Karp (lane 3), Kato (lane 4), Kawasaki (lane 5), Kuroki (lane 6) using template DNA from engorged larvae and mouse.

A: Engorged larva No. 3; B: Engorged larva No. 4; C: Engorged larva No. 6; D: Blood clot of passage mouse (I). Lane 1, 1 kb DNA ladder as size markers (Bethesda Research Laboratories Life Technologies, Inc., MD, USA). The sizes of the fragments were 12,216, 11,198, 10,180, 9,162, 8,144, 6,108, 5,090, 4,072, 3,054, 2,036, 1,636, 1,018, 517, 506, 396, 344, 298, 220, 201, 154, 134 and 75 bp. The numbers in the figure are sizes (in base pairs).

with FITC (fluorescein isothiocyanate, IBS) were used for sera from mice.

Results and discussion

Fourteen engorged larvae were individually examined for Rt-DNA by PCR. Rt-DNAs were detected in 6 of 14 (42.9%). In 6 larvae, 1 to 3 Rt-DNA types were detected in each mite (Fig. 1, Table 1).

Even though a mouse was attached with larvae harboring at least 4 strains of Rt, Rt detected by the Rt-DNA method in blood clots of the attached mouse and those up to the 4th passage was only Karp type, and sera of all mice in 4 passages showed IF titers at 1:160 or higher to Karp type (Table 2).

Some papers similar or related to our results have been published. Shirai *et al.* (1981a, b, 1982a, b, c) reported that the identification of rickettsiae in chiggers collected in the field was performed by direct IF using 8 type strains of Rt, and 1 to 5 types of Rt from wild chiggers were detected.

Robinson *et al.* (1977), Dohany *et al.* (1978) and Shirai *et al.* (1982d) examined a large number of mites in infected laboratory colonies of *L. fletcheri* and *L. arenicola* for Rt types by IF. Both species were found to carry rickettsiae antigenically related to Karp and other types, and some of the *L. fletcheri* contained Gilliam type only.

In experimental infections of mice with *Rickettsia* strains, Gotoh (1968) prepared 3 combinations of 2 prototypes, *i.e.*, Gilliam-Karp, Kato-Gilliam and Kato-Karp, injected them to mice and followed up rickettsiae by mouse passage. He reported that from passage mice with a Gilliam-Karp mixture, rickettsiae found were Kato type, and in the mice with a Kato-Gilliam mixture, Karp type began to appear at about 4th passage.

In Japan, many strains of Rt had previously been isolated from patients and other natural hosts by the mouse passage method. As regards the affinity of rickettsial types to the chigger species, Karp and Gilliam types

Table 1 DNA types of *R. tsutsugamushi* detected in the infected colony of *L. pallidum* by PCR typing method.

No. of engorged larvae (F ₉)	Nested PCR primers for					
	Common*	Gilliam	Karp	Kato	Kawasaki	Kuroki
1	+	—	—	—	—	—
2	+	—	—	—	—	—
3	+	—	—	—	+	—
4	+	—	+	—	+	—
5	+	—	+	—	+	—
6	+	—	+	—	+	+
7	—	ND**	ND	ND	ND	ND
8	—	ND	ND	ND	ND	ND
9	—	ND	ND	ND	ND	ND
10	—	ND	ND	ND	ND	ND
11	—	ND	ND	ND	ND	ND
12	—	ND	ND	ND	ND	ND
13	—	ND	ND	ND	ND	ND
14	—	ND	ND	ND	ND	ND

* Common: *Rickettsia tsutsugamushi* species specific and type common primers. ** ND: not done.

Table 2 Detection and identification of *R. tsutsugamushi* types in blood clots by PCR, and peritoneal smears of mice by direct IF, and reactivity of mouse sera to Rt types by indirect IF in a ddY mouse attached by the infected larvae of *L. pallidum*, and in passage mice.

Mouse passage	Blood clots by PCR	Peritoneal smears of mice by IF	Antibody titers in mice		
			Kato	Karp	Gilliam
i	Karp	Karp	160	640	40
ii	Karp	Karp	160	640	40
iii	Karp	Karp	640	640	640
iv	Karp	Karp	40	160	40

are connected with *L. pallidum*, Kawasaki and Kuroki types with *L. scutellare*, and Kato type with *L. akamushi* (Tamiya, 1962; Murata *et al.*, 1986; Yamamoto *et al.*, 1986, 1989; Kawamori *et al.*, 1992).

In the present study with PCR, when mites apparently harboring 4 Rt-DNA types were fed on a mouse, only Karp type out of the 4 was detected in blood clots of the mouse and following passage mice by PCR. Since ddY mouse is not sensitive to Kawasaki and Kuroki types, Rt found in the passage mice would be only Karp. In our results with PCR typing, mites in an infected colony likely

harbored varied types in accordance with the results of immunofluorescence by Shirai *et al.* (1981a, b, 1982d).

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摘 要

フトゲツツガムシのつつか虫病リケッチア
陽性コロニーにおける PCR 法に
よるリケッチアの検出

フトゲツツガムシのつつか虫病リケッチア (Rt) 陽
性コロニーにおいて, PCR 法によりダニから直接 Rt

の検出を行った. 1 個体の Rt 陽性雌の次世代の14個
体の満腹幼虫のうち6 個体 (42.9%) が Rt 陽性であ
った. PCR による血清型別により, 個々のダニ体内
には Kawasaki, Karp・Kawasaki, Karp・Kawasaki・
Kuroki, および同定できなかった Rt-DNA が認めら
れた. それにもかかわらず, それらが吸着したマウス
およびその脾臓接種 マウス 4 代までの おのおの血餅
の Rt-DNA は常に Karp 型であった.