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Epidemiology of Tsutsugamushi Disease in Relation to the Serotypes of *Rickettsia tsutsugamushi* Isolated from Patients, Field Mice, and Unfed Chiggers on the Eastern Slope of Mount Fuji, Shizuoka Prefecture, Japan

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A total of 59 strains of *Rickettsia tsutsugamushi* were isolated from patients (24 isolates), *Apodemus speciosus* mice (30 isolates), and unfed larvae of *Leptotrombidium scutellare* (2 isolates) and *Leptotrombidium pallidum* (3 isolates) in the Gotenba-Oyama District, Shizuoka Prefecture, Japan. All these isolates were classified into the three serotypes Karp, Kawasaki, and Kuroki based on reactivity with strain-specific monoclonal antibodies. Kawasaki- and Karp-type rickettsiae were isolated from *L. scutellare* and *L. pallidum*, respectively, and the geographic distribution of patients and rodents infected with these two types of rickettsiae coincided with the areas densely populated by the respective chiggers. From these results, we conclude that Kawasaki-type rickettsiae are transmitted by *L. scutellare* and Karp-type ones are transmitted by *L. pallidum*. Kawasaki-type rickettsial infections were prevalent in early autumn, and Karp-type infections showed a peak of occurrence in the late autumn, reflecting the seasonal fluctuations of *L. scutellare* and *L. pallidum*. Isolates of Kuroki-type rickettsiae were obtained only from four patients in October and November, and the relationship between this type of rickettsia and its vector species could not be fully defined.

Tsutsugamushi disease is a febrile disease caused by chigger-borne *Rickettsia tsutsugamushi* that consists of several antigenic variants. Shishido (13) first defined the antigenic types of Gilliam, Karp, and Kato strains, and recent workers have shown the presence of other serotypes such as Shimokoshi, Kawasaki, and Kuroki, all of which were isolated from patients in Japan (10, 17, 18, 21, 22). Rickettsiae of the Kawasaki and Kuroki types are frequently isolated from patients in south Kyushu (21), while those of the Shimokoshi type are rarely isolated.

In Shizuoka Prefecture, since the first documentation of the disease in the area in 1948 (3), tsutsugamushi disease has been most heavily endemic in the Gotenba-Oyama District on the eastern slope of Mount Fuji (4). Recurrent outbreaks of the disease in Shizuoka Prefecture since 1976 as well in the other prefectures in Honshu and Kyushu led us to conduct an epidemiological study of scrub typhus in these areas. Our previous study (5) described the serological survey of patients and the distribution of infected wild rodents.

In the present study, we endeavored to isolate as many strains of rickettsiae as possible from patients, unfed chiggers, and wild rodents and to identify serotypes of the isolates by using the indirect immunofluorescence (IF) test with five strain-specific monoclonal antibodies against

Gilliam, Karp, Kato, Kawasaki, and Kuroki serotypes. The points reported here are (i) the relation between rickettsial serotype and the species of vector chiggers, (ii) the geographic distribution of serotypes of rickettsiae in our research area in relation to the distribution patterns of chiggers, and (iii) the correlation between the seasonal fluctuation of chiggers and the serotypes of rickettsiae isolated from patients.

MATERIALS AND METHODS

Rickettsiae and cells. Laboratory strains of *R. tsutsugamushi* used were Gilliam, Karp, Kato, Kawasaki, and Kuroki serotypes cultured in GMK or L929 cells. Culture methods for these rickettsiae were essentially the same as those reported previously (18).

Preparation of monoclonal antibodies against *R. tsutsugamushi*. To prepare strain-specific monoclonal antibodies against Gilliam, Karp, and Kato serotype strains, a BALB/c mouse received an intraperitoneal injection and an additional booster injection 2 weeks later of 0.5 ml of spleen emulsion from a mouse infected with one of these strains. For the preparation of anti-Kawasaki and anti-Kuroki antibodies, a mixture of homogenate of infected GMK cells and Freund's complete adjuvant was injected intraperitoneally into BALB/c mice as antigen and, 2 months later, intravenously for booster. All mice were sacrificed 3 days after the booster injection, and the spleen cells were hybridized with myeloma

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cells (P3X63Ag8U1) by using polyethylene glycol 1000 or 4000. The hybridomas were selected in HAT medium (RPMI 1640 medium containing 10% fetal calf serum, 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine), and antibody-producing cells were screened by IF test of the culture fluid. Antibody-positive clones were recloned twice by the limiting-dilution or single-cell-manipulation method. Then, 10^7 hybridomas and 0.5 ml of pristane (2,6,10,14-tetramethyl pentadecane) were inoculated intraperitoneally into BALB/c mice, and ascitic fluid was harvested on days 10 to 14. Other details were the same as for the method of Ando (1).

Isolation of *R. tsutsugamushi*. Blood samples were obtained from 32 febrile patients in the Gotenba-Oyama District from 1985 to 1990. All blood samples were drawn within 1 week after the onset of illness and prior to antibiotic treatment. Each blood clot obtained from the blood samples was triturated in a sterilized petri dish and homogenized in an equal amount of SPG buffer (3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, 4.9 mM L-glutamic acid, 218 mM sucrose, pH 7.0) containing 200 U of penicillin G per ml with a glass homogenizer (Tephron pestle; Iuchi, Osaka, Japan).

Sixty-nine *Apodemus speciosus* were captured with wire cage traps or Sherman box traps at 11 sites in the Gotenba-Oyama District from 1986 through 1990. The mice were sacrificed with ether, and the spleen of each mouse was triturated and emulsified in 1.5 ml of SPG buffer in a mortar.

A total of 3,947 and 1,545 unfed larvae of *Leptotrombidium scutellare* and *Leptotrombidium pallidum*, respectively, were collected by Tullgren funnel or black plate method as reported previously (11, 20) at 17 sites in the same district from 1988 through 1990. The larvae were divided into pools of 4 to 125 individuals and homogenized in 1 ml of SPG buffer with a glass homogenizer.

Each preparation obtained as described above was inoculated intraperitoneally into 5- to 8-week-old ddY female mice. The amount of inoculation for each mouse was 1.0 ml of the patients' blood clot homogenate, 0.5 ml of mouse spleen emulsion, or 1.0 ml of larval homogenate. In these cases, both immunosuppressant-treated and untreated mice were used. For the immunosuppressant treatment, a mouse received an injection of 0.25 mg of cyclophosphamide (CY) per g of body weight three times on days 0, 4 to 5, and 9 to 10 after inoculation. The mice were sacrificed on days 12 to 14 after inoculation, and smears of peritoneum were tested by indirect IF test with patient serum as the primary antibody. The serum showed cross-reactivity to different prototype strains of *R. tsutsugamushi*, with an IF test titer of more than 1,280, and was used at a 50-fold dilution. If rickettsiae were not seen in the smear, 0.5 ml of a 10% emulsion of mouse spleen in SPG buffer was inoculated into other mice for blind passages that were repeated five times at 2-week intervals for CY-treated mice and 3 times at 3-week intervals for untreated mice.

Preparation of antigens for serotyping of rickettsial isolates by IF test. All stocked preparations of rickettsial strains were passaged once in CY-treated ddY mice. The mice that showed piloerection and crouching were sacrificed with ether, and 5 ml of phosphate-buffered saline (Nissui Co., Tokyo, Japan) was injected into the peritoneal cavity of each mouse. After the abdomen was kneaded, fluid in the peritoneal cavity was harvested into a centrifuge tube containing heparin and centrifuged at $200 \times g$ for 10 min. The pellets obtained were homogenized with a Dounce stainless steel homogenizer (Kontes Glass Co., Vineland, N.J.) in a small amount of Eagle's minimum essential medium (Nissui Co.,

Tokyo, Japan) supplemented with 1% fetal calf serum (1% S-MEM). The homogenate (0.5 ml) was inoculated onto a monolayer of L929 cells in a 25-cm^2 tissue culture flask (Costar, Cambridge, Mass.), and after 2 h of incubation at 37°C to allow rickettsiae to be absorbed to the cells, the inoculum was removed and the cell layer was cultivated in 5 ml of 1% S-MEM at 37°C by renewing the culture medium at 3- to 4-day intervals. When sufficient growth of rickettsiae was observed in the specimens by the IF method, the cells were removed from the culture flask with a cell scraper (Costar) and used as antigens for IF tests.

IF test. All the antigens were smeared individually on glass slides (Cel-Line, Newfield, N.J.), fixed with acetone for 10 min, and kept in a −80°C freezer until use. The method for the IF test is as described previously (4).

Observation of seasonal fluctuation of chiggers on field rodents. Wild mice (*A. speciosus* and *Apodemus argenteus*) and voles (*Microtus montebelli*) captured at 16 sites in our research field from 1983 through 1990 were sacrificed, and their carcasses were hanged individually over petri dishes filled with water. Chiggers that fell off the rodents were mounted on slides in modified Berlese solution (8 g of arabic gum, 30 g of chloral hydrate, 10 ml of H_2O, 1 ml of acetic acid, 2 ml of glycerol) and identified microscopically by referring to published descriptions of chiggers (12).

RESULTS

Isolation and serotyping of *R. tsutsugamushi*. The results of isolation of *R. tsutsugamushi* from patients and natural hosts and of serotyping of the isolates are summarized in Table 1. From 248 samples, 59 isolates were obtained. The rate of successful isolations from patients' blood samples was high (24 isolates from 32 samples), especially in CY-treated mice (22 from 27), but the rate of isolation from chiggers was low (only 5 from 147 pools, which consisted of 5,492 chiggers).

All strain-specific monoclonal antibodies used for serotyping of the isolates were highly specific to the homologous strain and did not show any cross-reactivity with other strains. From their reactivities with these monoclonal antibodies, each of the 59 isolates was clearly classified into one of the three groups, i.e., Karp, Kawasaki, or Kuroki. Karp-type isolates were dominant (35 among 59 isolates) and were isolated not only from patients but also from *L. pallidum* and *A. speciosus*. Kawasaki-type rickettsiae were isolated frequently from patients (13 among 24 isolates) and from *L. scutellare* and *A. speciosus*. Rickettsiae of the Kuroki type were isolated only from patients. All isolates of the Kawasaki and Kuroki types were recovered only from CY-treated mice.

Geographic distribution of rickettsiae. Figure 1 shows the geographic distribution of sites at which the 59 isolates were obtained. All 13 patients who yielded Kawasaki-type rickettsiae were either self-defense personnel working on the Eastern Fuji Maneuver Ground at more than 500 m above sea level or residents near the maneuver ground. Coincidentally, the same type of rickettsia was recovered from two pools of unfed *L. scutellare* larvae and from five *A. speciosus* mice in the same area. On the other hand, the four Kuroki-type strains were isolated from patients in the eastern and northeastern parts of the district below an altitude of 500 m. Positive sites for Karp-type rickettsiae were scattered over the entire district except the maneuver ground, and patients with this type of rickettsia were found in the central part of the district at or lower than an altitude of 500 m. Among the 25 Karp-type isolates from *A. speciosus*, 16 were

TABLE 1. Isolation of *R. tsutsugamushi* from patients, chigger mites, and rodents and serotyping of isolates by indirect
IF test with monoclonal antibodies

Source of rickettsiae	Type of mouse	No. of samples	No. of successful isolations of rickettsiae	No. of isolates				
				Gilliam	Karp	Kato	Kawasaki	Kuroki
Patient's blood	CY treated	27	22		5		13	4
	Untreated	5	2		2			
L. scutellare	CY treated	83[a] (3,645[b])	2				2	
	Untreated	11[a] (302[b])						
L. pallidum	CY treated	24[a] (744[b])						
	Untreated	29[a] (801[b])	3		3			
A. speciosus spleen	CY treated	33	10		5		5	
	Untreated	36	20		20			
Total		248	59	0	35	0	20	4

[a] Number of pools.
[b] Total number of chigger mites.

obtained from rodents captured at the foot of eastern mountains.

Seasonal distribution of patients and chigger mites. Most tsutsugamushi disease cases occurred from September to November in the Gotenba-Oyama District, and all 24 isolates from patients were obtained during this period (Fig. 2). A few patients appeared in March and April, but rickettsiae could not be isolated from them. Serotyping of the isolates revealed that infection by Kawasaki-type rickettsiae was dominant in October and infection by Karp-type rickettsiae was dominant in November (Fig. 2).

The 113 captured rodents consisted of 95 *A. speciosus* mice, 12 *A. argenteus* mice, and 6 *M. montebelli* voles. A total of 10,481 chiggers collected from those rodents were classified into 4 genera and 11 species (Table 2). The dominant species were *Leptotrombidium fuji*, *L. scutellare*, and *L. pallidum*. The seasonal fluctuations of *L. scutellare*,

L. pallidum, and *Neotrombicula japonica* recovered from *A. speciosus* are shown in Fig. 3. The larvae of *L. scutellare* were seen in September through December, with a peak of occurrence in October. The larvae of *L. pallidum* were recovered from rodents for a longer period than those of *L. scutellare*, with a peak of occurrence in December to February.

DISCUSSION

Many strains of *R. tsutsugamushi* had previously been isolated from patients and other natural hosts by inoculating samples into haired mice. The existence of strains weakly virulent to haired mice has been demonstrated (7), and for the isolation of these strains, the use of nude mice, CY-treated haired mice, or cultured cells has been recommended (6, 7, 15). Indeed, isolates with rickettsial serotypes such as Shimokoshi, Kawasaki, and Kuroki were isolated from patients by using CY-treated haired mice (18, 21, 22). In this study, we used both CY-treated and untreated mice for the isolation of rickettsiae and found that rickettsiae of the Kawasaki and Kuroki types were recovered only from the CY-treated mice, while those of the Karp type were isolated from both treated and untreated mice. These results support our previous contention that all Kawasaki-, Kuroki-, and probably Shimokoshi-type strains are less virulent to haired mice than are the Gilliam-, Karp-, and Kato-type strains (17).
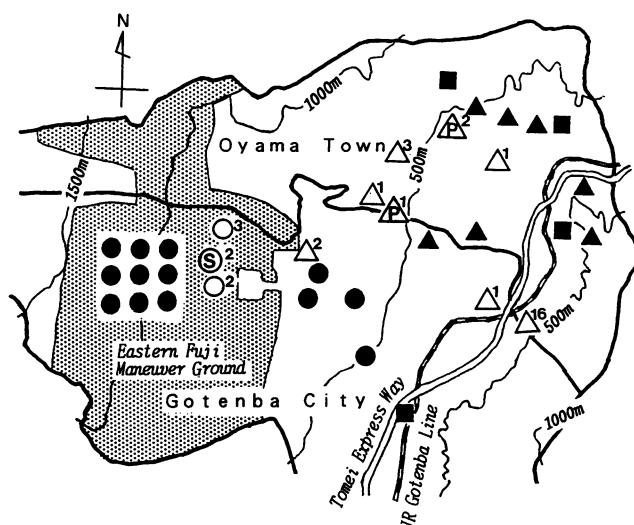

FIG. 1. Geographic distribution in the Gotenba-Oyama District of sites positive for all 59 strains. Symbols ●, ▲, and ■ indicate the rickettsial serotypes Kawasaki, Karp, and Kuroki, respectively, from individual patients. Symbols ○, and △ indicate Kawasaki- and Karp-type rickettsiae, respectively, from *A. speciosus*. Symbols Ⓢ and Ⓐ indicate Kawasaki- and Karp-type rickettsiae isolated from *L. scutellare* and *L. pallidum*, respectively. For cases of isolation from *A. speciosus* and chiggers, the number of successful isolations at the same site is indicated by a superscript.
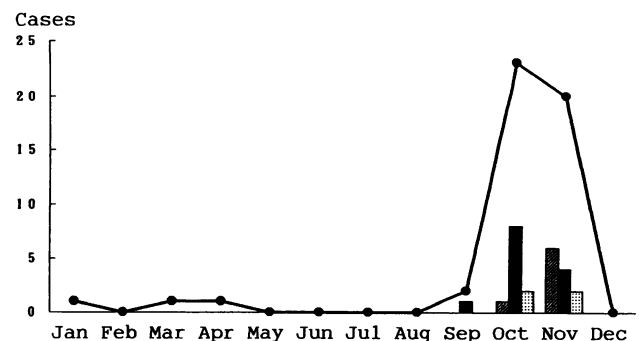

FIG. 2. Seasonal incidence of tsutsugamushi disease and serotypes of rickettsiae isolated from patients in the Gotenba-Oyama District (1985 to 1990). Symbols: ●, number of patients; ▨, Karp; ■, Kawasaki; ▦, Kuroki.

TABLE 2. Trombiculid mites taken from field rodents (1983 through 1990)

Trombiculid species	No. on rodents (chigger index[a])			Total (n = 113)
	A. speciosus (n = 95)	A. argenteus (n = 12)	M. montebelli (n = 6)	
Leptotrombidium scutellare	2,574 (27.1)	117 (9.8)	656 (109.3)	3,347 (29.6)
Leptotrombidium pallidum	1,841 (19.4)	12 (1.0)	18 (3.0)	1,871 (16.6)
Leptotrombidium intermedium	1 (0.01)		131 (21.8)	132 (1.2)
Leptotrombidium palpale	22 (0.2)		1 (0.2)	23 (0.2)
Leptotrombidium fuji	3,379 (35.6)	326 (27.2)		3,705 (32.8)
Leptotrombidium kitasatoi	41 (0.4)			41 (0.4)
Leptotrombidium miyajimai	102 (1.1)	15 (1.3)		117 (1.0)
Neotrombicula japonica	459 (4.8)	22 (1.8)	48 (8.0)	529 (4.7)
Neotrombicula mitamurai	77 (0.8)	3 (0.3)		80 (0.7)
Cheladonta ikaoensis	34 (0.4)	2 (0.2)	12 (2.0)	48 (0.4)
Gahrliepia saduski	479 (5.0)	61 (5.1)	48 (8.0)	588 (5.2)
Total	9,009 (94.8)	558 (46.5)	914 (152.3)	10,481 (92.8)

[a] The chigger index is the average number of chiggers per rodent.

Of the species of vector mites of *R. tsutsugamushi*, Asanuma (2) listed 13 infected naturally with rickettsiae, including *L. scutellare*, *L. pallidum*, *Leptotrombidium akamushi*, and *N. japonica*. Researchers in the USSR isolated rickettsiae from *Neotrombicula mitamurai* and *Neotrombicula pomeranzevi* (8, 14). Concerning the relationship between the serotypes of rickettsiae and these species of vector mites, several lines of evidence have been presented. All isolates from patients and wild rodents in Niigata Prefecture, where *L. pallidum* was the main vector in recent outbreaks of tsutsugamushi disease, were classified into two serotypes, Karp and Gilliam (9). Isolates from patients in Miyazaki Prefecture, where *L. scutellare* is abundant as vector, were all classified into the other two serotypes, Kawasaki and Kuroki (21). These data suggest that rickettsiae of the Karp and Gilliam types are transmitted by *L. pallidum* and that those of the Kawasaki and Kuroki types are transmitted by *L. scutellare*. Murata et al. (9) identified two isolates from fed *L. pallidum* as Karp type, and Takahashi (16) demonstrated Karp-type rickettsiae in the naturally infected colony of *L. pallidum* which had been maintained in his laboratory. In the present study, we isolated Karp-type rickettsiae from unfed larvae of *L. pallidum* collected in the field. Furthermore, Kawasaki-type rickettsiae were isolated from *L. scutellare*. This is the first demonstration of the relation between Kawasaki-type rickettsiae and species of vector chiggers. However, we could not define the species of vector of the Kuroki type in the

present study because this type of rickettsia was isolated only from patients and not from chiggers.

Our previous study (19) showed that in the Gotenba-Oyama District, *L. scutellare* was abundant on the sparsely covered *Miscanthus* grassland, on well-drained granular soil on the maneuver ground, and sporadically in other mountainous areas higher than 500 m. The distribution pattern of *L. scutellare* coincides well with the sites at which rickettsiae of the Kawasaki type were isolated from patients, rodents, and chiggers (Fig. 1). No isolates of the Karp type were recovered from rodents in the maneuver ground area. On the other hand, *L. pallidum* was distributed widely in the district, with the heaviest distribution on large paddy banks located lower than the specific habitat of *L. scutellare* (19). This area coincides with the area where the Karp type rickettsiae were isolated in the present study. Geographic studies of both patients and chiggers suggest no relation between Kuroki-type rickettsiae and species of vector chiggers, because patients with this type of rickettsia were found in areas where both *L. scutellare* and *L. pallidum* occurred.

According to the data on seasonal fluctuations of chiggers (Fig. 3), the number of *L. pallidum* in autumn was lower than that of *L. scutellare*, but Karp-type rickettsiae were isolated from patients (Fig. 2). This may suggest that the ratio of infected chiggers to total population of *L. pallidum* is higher than that for *L. scutellare*. In winter, numbers of *L. pallidum* were seen, but only a few patients appeared. This may be due to the lessened opportunity for human contact with chiggers because there is little field work in the cold season.

In our previous study of patient sera from the Gotenba-Oyama District, many sera reacted strongly with Gilliam strain (5). At that time, only three prototype strains, Gilliam, Karp, and Kato, were used as antigens for diagnosis of the disease. A recent report (22) demonstrates that Kawasaki strain shows some degree of cross-reaction with Gilliam strain when polyclonal antisera are used. It is presumed, therefore, that the sera of patients infected with Kawasaki-type rickettsiae showed high reactivity with Gilliam strain.
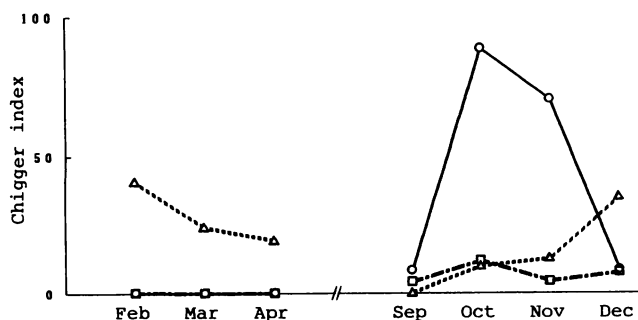

FIG. 3. Seasonal fluctuations of the larvae of *L. scutellare* (O), *L. pallidum* (△), and *N. japonica* (□) on *A. speciosus* in the Gotenba-Oyama District (1983 to 1990).

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