A Seroepidemiology of *Rickettsia tsutsugamushi* at Two Islands in Nagasaki

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Abstract: Serum specimens from 283 and 270 apparent healthy inhabitants aged from 6 to 79 years residing in Hirashima Island and in Enoshima Island of Nagasaki Prefecture, respectively, were examined to measure specific IgG antibody to *Rickettsia tsutsugamushi* by ELISA. It was found that the overall positive rate of *R. tsutsugamushi* infection was higher in Hirashima Island (33.2%) than in Enoshima Island (22.6%). While, antibody level of *R. tsutsugamushi* was higher in Enoshima Island (1.88±0.47) than in Hirashima Island (1.65±0.47). These differences were statistically significant. In Hirashima Island, the positive rate and the antibody level of *R. tsutsugamushi* at 19 years and younger were significantly higher than those at 40 years and older, while the positive rate and the antibody level at different age groups in Enoshima Island were not different. Furthermore, apparent *R. tsutsugamushi* infection was detected in only Hirashima Island. It was indicated that in Hirashima Island inhabitants below 19 years had more recent infection than 40 years and older and in Enoshima Island all inhabitants had infection at the same period, and virulent strain of *R. tsutsugamushi* existed in only Hirashima Island.

Key words: Rickettsia tsutsugamushi, Enzyme-linked immunosorbent assay, Antibody, Epidemiology

INTRODUCTION

Since 1977 cases with apparent type of *Rickettsia tsutsugamushi (R. tsutsugamushi)* infection have been increasing in number and this infection is widespread in Japan (Suto, 1982). The reason why this infection is increasing and spreading is not clear yet, though Suto (1982) pointed out reciprocal relation between the consumption of tetracycline and chloramphenicol and the number of cases with apparent *R. tsutsugamushi* infection. In

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Nagasaki prefecture first case of *R. tsutsugamushi* infection was recognized in Hirashima Island in 1982 (Annual report of health in Nagasaki Prefecture, 1986). After that, sporadic cases of *R. tsutsugamushi* infection have been reported from various places in Nagasaki prefecture (Annual report of health in Nagasaki Prefecture, 1986). Thus, seroepidemiological studies on *R. tsutsugamushi* infection were done in Nagasaki prefecture. It has been reported that the positive rate for *R. tsutsugamushi* infection is different from place to place (Suzuki *et al.*, 1985a; Suzuki *et al.*, 1985b), and accumulation of this infection is detected in family, and furthermore, the infection remains asymptomatic in many cases (Suzuki *et al.*, 1987). The difference of positive rate of *R. tsutsugamushi* in limited area (Kasuya *et al.*, 1985) and the diversity of *R. tsutusgamushi* infection (Shishido, 1962; Murata *et al.*, 1980) have been pointed out. In the present study, seroepidemiological investigation was performed in Hirashima Island where apparent cases of *R. tsutsugamushi* infection were detected every year and in Enoshima Island where no patient with *R. tsutsugamushi* infection has been reported (Annual report of health in Nagasaki Prefecture, 1986).

Materials and Methods

Samples source and structure. As shown in Fig. 1, the study was carried out in Hirashima and Enoshima Islands in Nagasaki prefecture. The area of Hirashima Island and Enoshima Island is 9.9km^2 and 2.8km^2, respectively. Structure of the population and

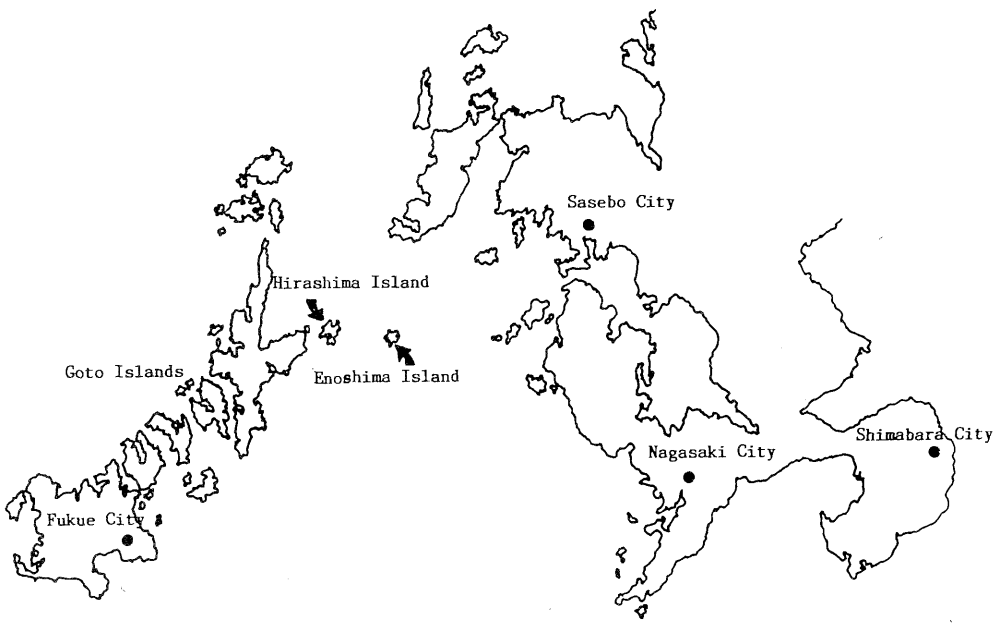

Fig. 1 Map of Nagasaki Prefecture.

sample source in both islands are shown in Table 1. In August 1985, serum samples were collected from apparently healthy inhabitants of the 2 islands. As shown in Table 1, the number of serum samples randomly selected for the above tests were 283 in Hirashima Island which corresponded to 55% of all inhabitants and 270 in Enoshima Island which corresponded to 61% of all inhabitants. The sera were stored at −20℃ until use. The subjects aged from 6 to 79 years in both islands and the sex ratio of male to female was 1:1.5 in Hirashima Island and 1:1.6 in Enoshima Island. Inhabitants lived at 7 areas in Hirashima Island and at 3 areas in Enoshima Island. Major occupation of inhabitants in both islands were fishing and farming.

Control serum. Serum with an antibody titer of 1:512 to *R. tsutsugamushi* by the immune peroxidase assay (IPA) (Suto, 1983) was used as positive control. Negative control serum to *R. tsutsugamushi* was prepared by the absorption method. Briefly, the serum with negative IPA was incubated with purified *R. tsutsugamushi* suspension (Dash *et al.*, 1979) at 37℃ for 1hr and then at 5℃ for 24hr. The serum was then centrifuged at 12,000rpm at 5℃ for 30min. The supernatant was filtered through a membrane filter with a pore size of 0.45μm and was stored at −20℃ until use.

ELISA procedure. For the determination of IgG antibody to *R. tsutsugamushi* (*Gilliam strain*), the microELISA technique by Dash *et al.* (1979) was used with some modification. The test was performed as described below: *R. tsutsugamushi* antigen for ELISA prepared according to the method of Dash *et al.* (1979) was diluted in 0.05M carbonate buffer at pH 9.6. After pouring 100μl of diluted antigen into the wells of a flat-bottomed microELISA plate (Immunolon of Dynatech MicroELISA System), the microtiter plate was kept at 5℃ overnight. The plate was washed with phosphate buffered saline (PBS)-Tween 20 3 times for 3min each. The plate was then shaken to dry and used soon or stored at −20℃ until use. 100μl of the test serum diluted in PBS-Tween 20 containing

Table 1 Sample source and structure of the population in Hirashima and Enoshima Islands of Nagasaki Prefecture

	Hirashima Island			Enoshima Island	
Area	No. of population	No. of specimens	Area	No. of population	No. of specimens
Oda	59	30 (51)	Higashi	132	69 (52)
Tomari	87	47 (54)	Hama	156	98 (63)
Kurosaki	57	44 (77)	Nishi	154	103 (67)
Ura	108	44 (41)			
Haedomari	79	50 (63)			
Miyazaki	72	33 (46)			
Yatsubo	50	35 (70)			
Total	512	283 (55)		442	270 (61)

Numbers in parenthesis indicate percentage.

0.6% bovine serum albumin was added into the wells and the plate was incubated at 37℃ for 1hr in a humid chamber. After the incubation, the plate was washed as above. Then, 100μl of alkaline phosphatase-labeled goat IgG fraction against human immunoglobulins (Tago Inc., Surlingame, CA., USA), diluted to 1:1,000 with PBS-Tween 20, was added into each well and the plate was incubated again at 37℃ for 1hr in a humid chamber. After the incubation, the plate was washed with PBS-Tween 20 as above. One-tenth μl of substrate solution prepared by adding 1mg of p-nitrophenylphosphate to 1ml of diethanolamine buffer (pH 9.8) was added to each well and the plate was incubated at room temperature for 30min and the absorbance of each well was read by MicroELISA Auto Reader (Dynatech Instruments Inc., Santa Monica, CA., USA). The appropriate concentration of antigen was determined by the checkerboard titration using positive and negative sera. The antigen of 0.4μg/100μl protein was used in a well (Lowry *et al.*, 1951).

Antibody level. Two-fold serial dilutions of serumsamples beginning from 1:20 were prepared using PBS-Tween 20 containing 0.6% bovine serum albumin. Optical density values greater than 3 times that of the negative control for corresponding dilution were considered as significant. The end point titer of the test showing positive reaction was expressed by the reciprocal of the highest dilution.

Detection of cases with R. tsutsugamushi infection. Before this study, inhabitants in Hirashima Island were educated about symptoms and mode of transmission of *R. tsutsugamushi* infection by our members. When an inhabitant developed any symptoms and signs, he was clinically examined by a doctor who is one of our colleagues. The diagnosis of *R. tsutsugamushi* infection was confirmed by ELISA using *Gilliam* strain, *Karp* strain and *Kato* strain. In case that a patient visited another hospital out of the island, diagnosis and outcome of the disease were followed. On the other hand, no cases of *R. tsutsugamushi* infection from Enoshima Island were obtained from reports to health department of Nagasaki prefectural government.

Statistical calculation. In order to check statistic difference, chi-square test, Student's t-test and Wilcoxon test were used. Furthermore, statistical analysis for antibody level was performed, after logarithmic conversion of the data.

RESULTS

Positive rate.

As shown in Table 2, overall positive rate of *R. tsutsugamushi* in all the inhabitants subjected to this study was higher in Hirashima Island (33.2%) than in Enoshima Island (22.6%) by using ELISA system and the difference was statistically significant. In Hirashima Island the positive rate at different age groups ranged from 22.5 to 64.5% and positive rate at 19 years and younger was significantly higher than that at 40 years older. On the other hand, in Enoshima Island the positive rate at each age group ranged from 18.6 to 42.9% and difference of the positive rate among age groups was not detected. Comparison of the positive rate at different areas in both islands is shown in Table 3.

Among 7 areas in Hirashima Island, 5 areas had the same age distribution, except 2 areas namely, Miyazaki and Yatsubo. Among 5 areas with the same age distribution the positive rate at Haedomari (38.6%) and Ura (38.0%) was significantly higher than that at Oda (16.7%). On the other hand, among 3 areas in Enoshima Island the positive rate at Higashi area (31.9%) was significantly higher than that at Nishi area (16.5%).

Table 2 Positive rate of *Rickettsia tsutsugamushi* in Hirashima and Enoshima Islands of Nagasaki Prefecture

Age in years	Hirashima Island		Enoshima Island	
	No. of specimens	No. of postive cases (%)	No. of specimens	No. of positive cases (%)
6−9	14	9 (64.5)	7	3 (42.9)
10−19	42	22 (52.4)	23	7 (30.4)
20−29	5	2 (40.0)	7	3 (42.9)
30−39	16	6 (37.5)	10	3 (30.0)
40−49	40	9 (22.5)	33	8 (24.2)
50−59	87	26 (29.9)	62	12 (19.4)
60−69	53	13 (24.5)	69	14 (20.3)
70−79	26	7 (26.9)	59	11 (18.6)
Total	283	94 (33.2)	270	61 (22.6)

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Satistical calculation was done by chi-square test. *: $p<0.05$, **: $p>0.01$.

Table 3 Postive rate of *Rickettsia tsutsugamushi* at different areas in Hirashima and Enoshima Islands of Nagasaki Prefecture

Area	No. of samples	Years in age (m±s. d.)	No. of positive samples (%) #	Antibody level in positive samples## (mean±s. d.)
Hirashima Island				
Oda	30	41±22	5 (16.7)	1.66±0.54
Tomari	47	46±20	13 (27.7)	1.78±0.70
Kurosaki	44	46±23	13 (29.5)	1.67±0.35
Haedomari	50	47±23	19 (38.0)	1.63±0.37
Ura	44	47±23	17 (38.6)	1.76±0.46
Miyazaki	33	51±17	13 (39.4)	1.51±0.38
Yatsubo	35	50±14	14 (40.0)	1.51±0.34
Enoshima Island				
Nishi	103	56±18	17 (16.5)	1.90±0.33
Hama	98	53±21	22 (22.5)	1.87±0.43
Higashi	69	54±18	22 (31.9)	1.87±0.60

★, *

★: In Hirashima Island age distribution of the samples at Miyazaki and Yatsubo was different from that of other 5 areas. # and ##: Statistical calculation was performed by chi-square test and Wilcoxon test, respectively. * : $p<0.05$.

Antibody level. As shown in Table 4, antibody level of *R. tsutsugamushi* of the inhabitants in both islands was higher in Enoshima Island (1.88±0.47) than in Hirashima Island (1.65±0.45) and the difference was statistically significant ($p<0.01$). In comparison of the antibody level among different age groups the level in Hirashima Island was higher at 6 to 19 years than at 40 to 79 years old. While, in Enoshima Island there was no difference of antibody levels among different age groups. On the other hand, the antibody level at different areas in each island was not significantly different (Table 3).

Apparent case with R. tsutsugamushi infection.

As showin in Table 5, 5 cases with clinically apparent *R. tsutsugamushi* infection were detected only in Hirashima Island since 1982 to 1985. Their age ranged from 31 to 57 years old and the time of infection were from May to September.

Table 4 Antibody level of *Rickettsia tsutsugamushi* in Hirashima and Enoshima Islands of Nagasaki Prefecture

Age in years	Hirashima Island			Enoshima Island		
	No. of samples	No. of positive samples(%)	Antibody level (mean±s. d.)#	No. of samples(%)	No. of positive samples(%)	Antibody level (mean±s. d.)#
6−19	56	31 (55.4)	1.90±0.50	30	10 (33.3)	1.78±0.47
20−39	21	8 (38.0) *1)	1.68±1.55*2)	17	6 (35.3)	2.05±0.59
40−79	206	55 (26.7)	1.51±0.33	223	45 (20.2)	1.88±0.46
Total	283	94 (33.2)	1.65±0.45	270	61 (22.6)	1.88±0.47

*1)

**3)

#: After logarithmic conversion of data, statistical analysis was performed.
1): chi-square test, 2): Wilcoxon test, 3): t-test, *$p<0.05$, **: $P<0.01$.

Table 5 Apparent case of *Rickettsia tsutsugamushi* in Hirashima Island

Year	No. of cases with apparent infection	Month of development	Age in years	Sex
1982	2	August	31	F
	(couple)	September	42	M
1983	1	September	45	F
1984	1	May	46	F
1985	1	June	57	F
Total	5			

Discussion

Our present data is comparable to the seroepidemiological studies done so far in Nagasaki (Suzuki *et al.*, 1985 a & b), the positive rates in Hirashima Island (33.2%) and in Enoshima Island (22.6%) were higher than those in Shimabara city (2%), Fukue city (4%), Wakamatsu town (4.5%), Nagasaki city (5.8%) and Oseto town (8%). While, the positive rates in both islands were lower than those in Arikawa town (49%) and Shinuonome town (60%) (Suzuki *et al.*, 1985b). Furthermore, when our results were compared to those reported from any other areas in Japan the positive rates in our Islands were similar to those reported for Toshima Island (23.5%), Miyake Island (26.1%) and Yamanashi prefecture (36.5%) (Otsuru, 1984). However, the positive rates in both islands were lower than that of Hachijo Island (56.3%) (Otsuru, 1984). It has already been reported that positive rate of *R. tsutsugamushi* infection was different from area to area in a small location in Nagasaki (Suzuki *et al.*, 1985a). Of 7 areas in Hirashima Island and 3 areas in Enoshima Island the positive rates of some areas were significantly higher than those of other areas. Therefore, it was again suspected that invasion of *R. tsutsugamushi* has been limited at special areas in the same locality. This might be implicated that rodents with mites infected with *R. tsutsugamushi* are localized in limited areas (Takada, 1982). Furthermore, the positive rate at each age group was significantly higher at 19 years or younger than 40 or older in Hirashima Island. This is in contrast with the positive rate of *R. tsutsugamushi* in Hachijo Island which was reported to increase according to age (Shishido *et al.*, 1937). Nevertheless, in Enoshima Island difference of the positive rate among age groups was not detected.

Antibody level was significantly higher in Enoshima Island than in Hirashima Island. It was speculated that infection caused by *R. tsutsugamushi* was more recent in Enoshima than in Hirashima, if the strain of *R. tsutsugamushi* was the same strain in both islands. Furthermore, in both islands difference of the antibody level among different areas was not demonstrated. Therefore, it was assumed that *R. tsutsugamushi* infection was caused at similar period at each area in both islands. In Hirashima Island the antibody level of younger inhabitants whose age ranged from 6 to 19 years was higher than that of 40 years and older. It is suspected that younger inhabitants in Hirashima Island had either more recent *R. tsutsugamushi* infection or had higher sensitivity to the strain than 40 years and older people. Furthermore, there might be a possibility that in Hirashima Island younger inhabitants were infected with a different strain from that of inhabitants whose age was 40 years and loder.

Only in Hirashima Island 5 apparent cases with *R. tsutsugamushi* infection were detected since 1982 to 1986. These cases were mainly from inhabitants aged more than 31 years. No apparent case was detected from younger inhabitants belowe 19 years with higher positive rate and higher antibody level of *R. tsutsugamushi*. Correlation between the positive rate and antibody level and the incidence of apparent *R. tsutsugamushi* infection was not found out, although in Miyazaki and Aomori prefectures patients with apparent *R.*

tsutsugamushi infection ranged from 5 years to 71 and older years (Tachibana *et al.*, 1982; Takada., 1982). The reason for low incidence of apparent *R. tsutsugamushi* infection in Hirashima Island is unknown. Kawamura *et al.* (1980) have reported inapparent infection caused by avirulent strain of *R. tsutsugamushi*. Therefore, in Hirashima Island inhabitants with antibody to *R. tsutsugamushi* might have been infected by avirulent strain of *R. tsutsugamushi* except for cases with apparent infection. In their study antibody level to avirulent strain was up to 1:160 by immunofluorescence assay (Kawamura *et al.*, 1980). While, in our study higher level of antibody was detected. Therefore, it is difficult to explain higher antibody only by avirulent strain. On the other hand, in Enoshima Island apparent *R. tsutsugamushi* infection was neither detected in inhabitants with low level antibody nor with high level antibody to *R. tsutsugamushi*. In both islands it is difficult to explain the pathogen of *R. tsutsugamushi* of the higher antibody. Furthermore, it was pointed out that strains of *R. tsutsugamushi* (Tachibana *et al.*, 1982; Tamura *et al.*, 1984; Yamamoto *et al.*, 1986) immunologically differ from the prototype strains in the same district. Therefore, it was assumed that strains of *R. tsutsugamushi* with different pathogenicity existed in these island.

To resolve the above problems further investigations for pathogenicity of *R. tsutsugamushi* strains in the field has to be analyzed in more detail.

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長崎県の2島におけるリケッチア・ツツガムシ感染症に関する血清疫学的研究

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長崎県において，つつが虫病が発生している平島（283名）と発生していない江ノ島（270名）の住民を対象として，リケッチア・ツツガムシ感染症に関する血清疫学的研究を行った．IgG抗体は抗原としてギリアム株を用い，酵素抗体法により測定した．全住民に対する抗体陽性率は平島（33.2%）が江ノ島（22.6%）より高値であったが，抗体レベルは平島（1.65±0.47）より江ノ島（1.88±0.47）で高値であった．年齢別抗体の陽性率とレベルの比較では，平島では19才以下が40才以上より高値であったが，江ノ島では差異を見い出し得なかった．これらの成績は平島では年齢による感染時期の差，つまり若年者が比較的最近感染し，江ノ島では全住民が同じ時期に感染していること，更に，つつが虫病患者が発生していない江ノ島においても弱毒性リケッチア・ツツガムシが存在していることを示唆した．

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