DEMONSTRATION OF THE NATURAL FOCI OF TSUTSUGAMUSHI DISEASE IN NAN PENG LIE ISLANDS IN CHINA

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Abstract. In recent years, the incidence of tsutsugamushi disease has increased in Nan Peng Lie Islands in China, and the disease has not been recorded in this region. The natural foci of tsutsugamushi disease were investigated in this paper. Isolation of *Orientia tsutsugamushi* and the study of preventive measures were also performed. The region was the island natural foci of south subtropical zone. The main host and vector were *Rattus norvegicus* and *Leptotrombidium (L.) deliens* respectively. The seasonal quantity trends of *Rattus norvegicus* and *Leptotrombidium (L.) deliens*e were consistent with the incidence of human infection in the region. The strains of *O. tsutsugamush*i were isolated from *Rattus norvegicus* and *Leptotrombidium (L.) deliense*. The identification showed that most strains were Karp. The seroepidemiology showed a high prevalence of antibody against *O. tsutsugamushi*. After preventive measures were implemented, the incidence was descent. So Nan Peng Lie Islands were the natural foci of tsutsugamushi disease.

INTRODUCTION

Tsutsugamushi disease is a febrile illness found in many areas, especially in Asia (Sugita *et al*,1992; Tay *et al*, 1996; Shieh *et al*, 1996), and is sometimes fatal. The causative agent of tsutsugamushi disease is *Orientia tsutsugamushi* and transmitted by infected mites. *O. tsutsugamush*i is a very small coccobacillus and an obligate intracellular parasite of infected human beings. The antigenic types of *O. tsutsugamush*i referred to as Gilliam, Karp and Kato strains are commonly recognized, and thus frequently called the prototype strains. Yet, additional antigenic types such as Shimokoshi (Tamura *et al*, 1984), Kawasaki (Yamamoto *et al*, 1986), Kuroki (Ohashi *et al*, 1990; Yamamoto *et al*, 1989), Boyong (Kim *et al*, 1993) and Yonchon (Seong *et al*, 1997) were also isolated. These variants are distinguishable from each other by serological cross-tests with strain-specific polyclonal or monoclonal antibodies. The serotype variation of *O. tsutsugamush*i depends on the antigenicity of an immunodominant 56 kDa major protein, which was demonstrated to be a type-specific antigen, located on *O. tsutsugamush*i outer membrane (Murata *et al*, 1986; Ohashi *et al*, 1992). In recent years, the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) have provided a valuable diagnostic approach in the diagnosis (Kelly *et al*, 1994; Tselentis *et al*, 1996; Horinouchi *et al*, 1996, 1997; Ohashi *et al*, 1996).

Nan Peng Lie Islands are located in the southeast along the coast of China. The islands belong to Nan Ao County, Guang Dong Province. Since 1991, the incidence of tsutsugamushi disease has increased, the health of people in this area has been affected. The biological characteristics of *O. tsutsugamushi*, the type of natural foci of the islands have not been reported. In order to prevent this disease and to develop control strategies we have investigated the natural foci since 1998.

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MATERIALS AND METHODS

Background of study sites

Nan Peng Lie Islands are located at north latitude 23°12′~23°19′, east longitude 117°13′ ~117°19′. Nan Peng Lie Islands consisted of four islands. The climate of the area is south subtropical type. The average temperature is 21.5ºC. The precipitation volume of yearly average is 1,331 mm. The relative humidity is 70-80%.

Investigation of hosts

The preponderant kind, seasonal variation, and ectoparasites of rats in the islands were investigated. Rats were captured in the east, west, south and north of the islands by mouse trapcages. The mouse trapcages were laid up at dusk and were taken back in the morning. The rats captured were killed, weighed, measured and species and sex identified. Each rat was searched thoroughly for ectoparasites and bled by intracardial puncture. The serum samples were stored at -20ºC. The collected ectoparasites from rats were placed in cryotubes and stored in liquid nitrogen. From May 1998 to April 1999, rats were captured monthly for seasonal variation. The infecting rate of *O. tsutsugamushi* of the preponderant rat was determined by the isolation of *O. tsutsugamushi*. The kinds of sea birds on the islands were investigated. Three to five of each kind of sea birds were captured and sera and ectoparasites were collected. Meanwhile the sera and ectoparasites of domestic animals and fowl were also collected.

Investigation of biological vectors

The ectoparasites of the host were biologically distinguished. The preponderant trombiculid mite was determined. *O. tsutsugamushi* were isolated from the vectors. The seasonal variation of preponderant trombiculid mites was recorded at each month from May 1998 to April 1999.

Isolation and identification of *O.tsutsugamushi*

The spleen, kidney and liver of rats captured were aseptically collected. 0.5g of each of the above organs of 1~3 rats was ground in sterilized grinding bowl, while adding 2 ml pH7.4 0.2 M sucrose buffer containing 100 units/ml of penicillin and 20 μg/ml of amphotericin B. Each of 3 athymic nude mice was inoculated intraperitoneally with 0.5 ml of the above grinding liquid. Each pool consisting of 50~100 mites was homogenized with 1.5 ml of the above buffer, then 1.5 ml homogeneous liquid was inoculated into 3 athymic nude mice, which were observed thereafter for 4 weeks. When the mice became sick, their spleens were aseptically harvested. The infection of *O. tsutsugamushi* was confirmed by Giemsa stain. The methods of identification were (1) complement fixation test, (2) virulence: LD_{50}, ID_{50}, and (3) immune index. The Gilliam, Karp and Kato prototype *O. tsutsugamushi* strains were from Beijing Institute of Biological Products.

Nested polymerase chain reaction (NPCR)

DNA was amplified by means of NPCR. Three primers were selected from the DNA sequence of the gene encoding the serotype-specific 56-kDa protein gene of Karp, primer 1,5′GACAAGCTTCCTCAGCCTACTAT AATGCC 3′ (nt 395-424), primer 2, 5′CTAGA AGTTATAGCGTACACCTGCACTTGC 3′ (nt 1598-1569), primer 3, 5′CTAGGGATCCCGA CAGATGCACTATTAGGC 3′ (nt 931-902).

0.5 g of spleen and abdominal fluid of sick mice were respectively added 1 ml lysis buffer (10 mM Tris,10 mM EDTA,150 mM NaCl, 1% SDS,100 μg/ml proteinase), then kept at 50ºC for 6 hours. DNA was extracted twice with phenol/chloroform, then precipitated in ethanol and used as template for NPCR. Briefly, the first amplification was carried out by primer 1 and primer 2. The second amplification was carried out by primer 1 and primer 3. All reactions were performed in a volume of 100 μl containing 10 x reaction buffer 10 μl (10 mM Tris-HCl, pH8.3, 2 mM $MgCl_2$, 50 mM KCl), template DNA, 2.5 units of thermus aquaticus DNA polymerase and 200 μM each of dATP, dCTP, dGTP, dTTP. The amount of primer added to the reaction mixture

was 0.1 μM . The reaction was respectively carried out for 35 cycles in both the first and second amplifications. Briefly, the amplification conditions were heat denaturation at 94ºC for 45 seconds, annealing at 58ºC for 1 minute and extension at 72ºC for 2 minutes. The PCR products were electrophoresed, then stained with ethidium bromide and observed under ultraviolet transillumination. When the 480~507 base pair specific band was detected, the sample was designated positive (Peng *et al*, 1999). Three prototype strains Gilliam, Karp, Kato were used as control.

Restriction fragment length polymorphism (RFLP)

The restriction endonucleases HincII and PstI were selected, referencing prototype *O. tsutsugamushi* strains Gilliam, Karp, Kato. When amplified products were digested with PstI, DNA of Karp became the fragments of 129 bp, 162 bp, 216 bp, DNA of Gilliam became the fragments of 123 bp, 357 bp.When amplified products were digested with HincII, DNA of Kato became the fragments of 173 bp, 307 bp (Peng *et al*, 1999).

Sequencing

According to results of RFLP, 4 isolated strains from rats and mites were sequenced. 500 μl of NPCR amplified products followed by phenol/chloroform extraction and ethanol precipitation. DNA products were cloned to PGEM-T by T4 DNA ligase. Cloned bacteria were selected, lysised and DNA was extracted for template. DNA of the template was amplified with primer l and primer 3. Amplified products were then identified by RFLP. The positive clone was rocked at 37ºC for 8-12 hours, and extracted with wizard plus sv minipreps DNA purification system (Promega). The extracted DNA was sequenced by fluorescence automatic sequencing equipment (Ohashi *et al*, 1992; Seong *et al*, 1997).

Serum

The sera of army personnel and inhabitants in the area were collected. The sera of domestic animals, domestic fowls, sea birds, rats were also collected. The samples were preserved at -20ºC for detection of antibodies against *O. tsutsugamushi*.

RESULTS

Host

Two hundred and twelve rats were captured in 5 months in the area in 1998. *Rattus norvegicu*s was the preponderant kind. The infecting rate of *O. tsutsugamushi* was 33.75%. The ectoparasites of rats were mite, gamasid and, flea. 20,994 mites were obtained. The number of isolated gamasid was 468, 387 of which was *L. huttalli*, 81 was *Laelaps echidninus*. Ninety-two fleas were captured and identified as *X. cheopis*. From May 1998 to April 1999 seasonal variation of rats was observed (Fig l).

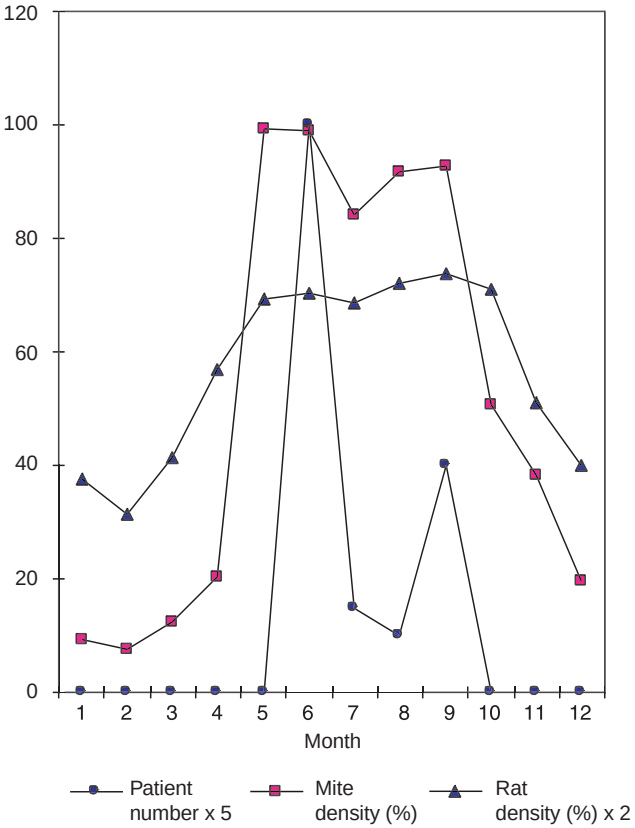

Fig 1–Incidence of tsutsugamushi disease and seasonal trens of rats and mites on Nan Peng Lie Islands.

Vector

Twenty thousand, nine hundred and ninty mites were obtained from 212 rats, 20,700 (98.60%) of which was *Leptotrombidium (L.) deliense*, 202 (0.96%) was *Walchia chinensis*, 5 was *Odontacarus majesticus*. The infecting rate of *O. tsutsugamushi* was 75.0%. The seasonal variation of mites was observed (Fig l).

Isolation and identification of *O. tsutsugamushi*

Thirty-five strains of *O. tsutsugamushi* were from rats and mites. After mice were injected, at days 13-20, they became symptomatic and were killed. Results of Giemsa stain were positive. Seven strains were identified, 5 of which were Karp, LD_{50} was 10^{-5}-$10^{-5.25}$, ID_{50} was 10^{-5}-$10^{-5.25}$, 2 strains showed LD_{50} 10^{-1}, ID_{50} 10^{-1}. Further immunological identification was performed. After mice were respectively immunological with the above 2 strains with LD_{50} 10^{-4} and $10^{-3.25}$, the mice were attacked with Karp LD_{50} 10^{-5}. The immune index was respectively $10^{-1.5}$ and $10^{-2.25}$.

NPCR and RFLP

Eight isolates were detected by NPCR, 7 of which were positive. DNA of positive strains detected by NPCR were analyzed by RFLP with PstI and HincII. The Karp, Kato, Gilliam Prototype *O. tsutsugamushi* strains were used as control. A part of DNA of the 3 strains can be digested by PstI and was consistent with Karp, another part of DNA was digested by HincII and was consistent with Kato. The 3 strains were thus infections of Karp and Kato. DNA of 2 strains can not be digested by the 2 enzymes, so the 2 strains might be a new strain. A part of DNA of 1 strain can be digested by HincII, another part can not be digested by 2 enzymes, so the strain might be infection of a new strain and Kato. DNA of 1 isolated strain can be digested by Pst I, so the strain was Karp.

Clone sequencing

Seven clones were obtained from 7 positive strains by RFLP. The results of sequencing were compared with the sequence of Karp, Kato, Gilliam, Yonchon, Shimokoshi, TA 763,TA 686, Boryong, Karoki, Kawasaki in DDBJ gene bank. The homology of 2 clones and Yonchon was 99%. That of 3 clones and Karp was 92%. That of 2 clones and Kato was 98%. So the types of *O. tsutsugamushi* in the area were Karp, Kato, Yonchon.

Seroepidemiology

The positive rate of antibody against *O. tsutsugamushi* in fishermen was 100%, that of army personal was 4.0%. The antibodies of calf and sheep in the area were positive. The positive rate of antibody in *Rattus norvegicus* was 68.1%. The main serotype was Karp. The patients appeared in June~September, the peak of sickness was in June. The symptomatic of the patients was typical.

DISCUSSION

Since 1991, patients suffering from fever with headache and rash have been observed in Nan Peng Lie Islands in China. The clinical cases could easily be misdiagnosed due to lack of specificity of presenting symptoms. On the other hand, the symptoms of tsutsugamushi disease appeared after 1 to 2 weeks-latent period. Because the antibody titer against *O. tsatsugamushi* was not high enough to be detected at the initial stage, the first definitive diagnosis of tsutsugamushi disease in this area was not established by IFA untill 1998. This natural foci have not been recorded previously. This is the first successful confirmation of Nan Peng Lie Islands as natural foci. The area belonged to the south subtropical island natural foci. The main host was *Rattus norvegicus*. The vector was *Leptotrombidium (L.) deliense*. The seasonal variation of host and vector was correlated with seasonal incidence of human infection. A high prevalence of antibody against *O. tsutsugamushi* has been demonstrated. In the area, health education were extensively performed to increased knowledge of preventing tsutsugamushi disease. Rats and mites were

extensively destroyed. Meanwhile preventing measures were performed. Before the epidemic season in 1999 the above measures were used, no cases appeared in the year.

In our study, serotypes of most isolates were Karp. We combined the nested PCR, RFLP and sequencing for genotypic identification of *O. tsutsugamushi.* The genotypic identification in this report was the classification based on the difference of the 56-kDa protein gene among the isolates. The results suggested that Karp, Kato, Yonchon strains existed in the area. It might be that genetic variation existed within serotype. The reasons why serotypes are not perfectly correlated with genotypes are unknown. The existed cross-reaction of the monoclonal antibody of Karp strain with Yonchon (Seong *et al*, 1997) might also be a reason.

Although the area was small, but types of *O. tsutsugamushi* were complicated. The merchant ships of other nations such as Korea sheltered from the wind in this area might also be an important reason.

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