ISSN 1021-366X

Isolation of a New Orientia Tsutsugamushi Strain in Hualien

Abstract

Background and purpose：Scrub typhus is an acute febrile disease caused by *Orientia tsutsugamushi*. The disease occurs mainly in the large triangular region extending from Japan in the north to Australia in the southwest and the South Pacific Islands in the southeast. Humans are infected by the bite of the larva of the trombiculid mite harbouring *O. tsutsugamushi*. This pathogen attacks endothelial cells resulting in vasculitis. The clinical manifestations are characterized by a papular rash, headache, fever, chills, and an eschar at the site of the chigger bite. The disease can be tested serologically. R. tustsugamushi can be subdivided into various serotypes and subtypes and the antigenic variation depends on a 56 Kda protein called major surface antigenic components on the cell surface. Furthermore there are great variations in virulence among the variants. Unfortunately the current vaccines are effective only against homologous strains and no single antigen that induces protection against all of the strains has been found. The purpose of this study is to investigate the specific serotype(s) of *O. tsutsugamushi* in Hualien. Better knowledge concerning *O. tsutsugamushi* in this part of our country is important in the process of classifying the possible variants and to ensure better preventive

medicine. Materials and Methods：Homogenate of mites from field rodents in Hualien region were inoculated onto L929 cell monolayer. Cultivation was continued until the growth of *O. tsutsugamushi* was established. Genotyping was performed using nested polymerase chain reaction（PCR）to amplify of a portion of the gene of the major surface antigenic components on the cell surface. Results：A strain with unique sequences named Hualien-A was found. Conclusions：due to geographic isolation, there may be unique strains with distinct pathogenicity and immunogenicity in this part of our country. Future work should include studies of infectivity, immunogenicity, and vaccine designs.

Keywords：*Orientia tsutsugamushi*, Serotype, Hualien

Introduction

Scrub typhus, also known as river or flood fever, is an acute febrile disease caused by a small obligate intracellular gram-negative micro-organism named *Orientia tsutsugamushi*. The disease occurs mainly in the large triangular region extending from Japan in the north to Australia in the southwest and South Pacific Islands in the southeast [1,2]

Humans are infected by the bite of the larva of trombiculid mite harbouring *O. tsutsugamushi*. The mites feed on rats and other small rodents and serve both as vector and reservoir of the etiologic agent. The mites transmit the rickettsiae to their offspring vertically via the ova [1,2].

In human, *O. tsutsugamushi* attacks endothelial cells resulting in vasculitis. The clinical manifestations are characterized by papular rash, headache, fever, chills, and an eschar at the site of the chigger bite. The disease can be tested serologically by the detection of significant increases of IFA(indirect fluorescent antibody)in paired serum drawn at the onset of disease and 2-3 weeks later.Traditionally,isolation of the rickettsia from the blood by inoculation

intraperitoneally into white mice is the definite confirmatory method [1-3].

R. tsutsugamushi can be subdivided into various serotypes and subtypes such as Gilliam, Karp, Kato, Shimokoshi, Kuroki, and Kawasaki [1,4] and there are great variations in virulence against mice among the variants [5]. Unfortunately, current vaccines are effective only against homologous strains and no single antigen that induces protection against all of the strains has been found. Current classification of *O. tsutsugamushi* in Hualien remains unknown. Moreover, whether there are other variants that could be important to us in this part of our country is unknown. Better understanding of specific *O. tsutsugamushi* strains in this area of Taiwan could be meaningful for both systemic arrangements of these possible variants and better preventive medicine [2].

Materials and Method

Cultivation and isolation of *O. tsutsugamushi*

Mites from field rodents（n=108）in the Hualien region were homogenized with a mortar and in MEM（Sigma, Missouri, USA）with 20 μg/ml of amphotericin B, 100 units/ml of penicillin, 150 μg/ml of streptomycin, and 2% fetal bovine serum. The homogenate was inoculated into a vial with L929 cell monolayer. The vial was centrifuged for 30 minutes at 600 X g to accelerate the adsorption of *R. tsutsugamushi* to the cell layer. After incubation at 37°C for 2 days, the cells were transferred into a new culture bottle. Cultivation was continued by replacing media every 3-4 days, until the growth of *O. tsutsugamushi* was recognized in cell smears stain with Giemsa [5].

Polymerase chain reaction

Genomic DNA was extracted using the DNeasy Tissue Kit（QIAGEN, California,USA）according to the manufacturer's instructions. Genotyping was performed using nested polymerase chain reaction（PCR）amplification of a

portion of the gene of the major surface antigenic components on the cell surface. The first primer pair was RTS-8（5'-AGGATTAGAGTGTGGTCCTT-3'）and RTS-9（5'-ACAGATGCACTATTAGGCAA-3'）. The second primer pair was RTS-6 （ 5'-GTTGGAGGAATGATTACTGG-3' ） and RTS-7 （ 5'-AGCGCTAGGTTTATTAGCAT-3'）[4]. The PCR reaction mixture contained 0.25 mM of each dNTPs, 1 U of PfuTurbo DNA polymerase in 1x reaction buffer （Stratagene Corporation,California,USA）, 20 pmoles of each primer. The template was one microgram of the extracted DNA for the first step of the nested PCR. One-tenth volume of the first amplification products was used for the second amplification. PCR conditions were: 94°C for 1 minute, 55°C for 1.5 minutes, and 72°C for 2 minutes for the first step of the nested PCR and 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute for the second step of the nested PCR. Amplification was performed for 30 cycles for both of the first and second steps [4] using Perkin-Elmer GeneAmp PCR System 9600 DNA thermal cycler（Perkin-Elmer,New Jersey,USA）. The resulting DNA fragments were then cloned into the TOPO cloning site of pCR-Blunt II-TOPO vector（Invitrogen,California,USA）and analyzed with the dideoxy-mediated chain termination method of nucleic acid sequencing using the forward primer for the second step of the nested PCR as primer.

Results and Discussion

We have isolated a new strain of *O. tsutsugamushi* (called Hualien-A) with unique sequences (Fig. 1 and Table 1). It has been shown that *O. tsutsugamushi* can be subdivided into various serotypes and subtypes [1, 4] with varying virulence against mice [5], and such antigenic variation depends on a 56 Kda protein named major surface antigenic components on the cell surface [5-6]. It has been shown that the major surface antigenic components are composed of 521-534 amino acids with four variable domains and these

variable domains are thought to be related to serotype specificity [1]. Moreover, the PCR method based on this 56 Kda protein could be used for diagnostic and genotype determination [1, 4, 6-9].

In this research, we performed sequencing of the whole PCR product fragments and compared these with those of various prototypic strains. We found that the newly isolated *O. tsutsugamushi* strain Hualien-A has unique sequences with identities between this strain and various *O. tsutsugamushi* prototypes ranging between 74.8 and 78.2 % (Table 1).

Due to geographic isolation and evolutional selection, there could be unique strains with distinct pathogenicity and immunogenicity in this part of our country. Future work should include studies of immunogenicity, infectivity and vaccine designs.

Prepared by: JM Cherng[1], JH Wang[1], TH Kao[2], HY Lin[3], CR Wang[4], LS Wang[1]

[1]Department of Internal Medicine, Buddhist Tzu-Chi General Hospital

[2]Division of Rheumatology, Department of Internal Medicine, Cathay General Hospital

[3]Division of Allergy, Immunology and Rheumatology, Department of Internal Medicine, National Yang-Ming University

[4]Department of Internal Medicine, National Cheng-Kung University, Taiwan, Republic of China

References

1. Chang WH. Current status of tsutsugamushi disease in Korea. J Korean Med Sci 1995; 10:227-238.
2. Hornick RB. Rikettsial diseases. In: Bennett JC, Plum F, eds, Cecil

Textbook of Medicine, 20th ed. Philadelphia: W. B. Saunders, 1996:1726-1736.

3. Tamura A, Ohashi N, Urakami H, et al. Classification of Rickettsia tsutsugamushi in a new genus, Orientia gen. Nov., as Orientia tsutsugamushi comb. nov. Int J Syst Bacteriol 1995; 45:589-591.

4. Horinouchi H, Mural K, Okayama A, et al. Prevalence of genotypes of Orientia tsutsugamushi in patients with scrub typhus in Miyazaki Prefecture. Microbiol Immunol 1997; 41:503-507.

5. Ohashi N, Koyama Y, Urakami H, et al. Demonstration of antigenic and genotypic variation in Orientia tsutsugamushi which were isolated in Japan, and their classification into type and subtype. Microbiol Immunol 1996; 40:627-638.

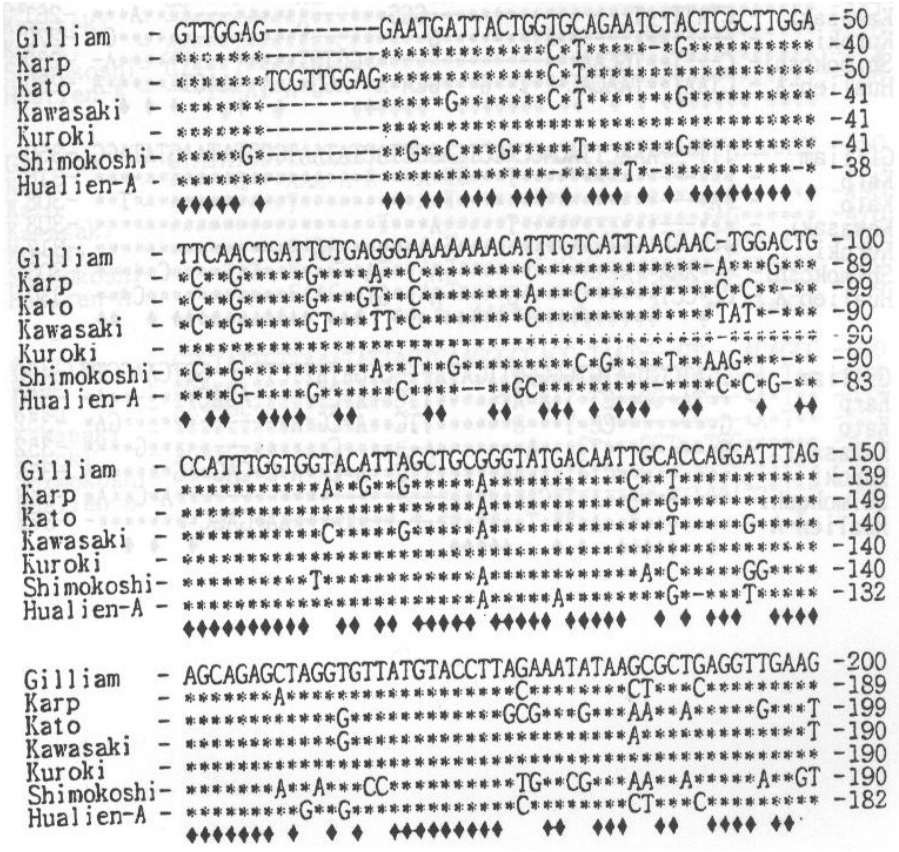
6. Song HI, Seong SY, Huh MS, et al. Molecular and serologic survey of Orientia tsutsugamushi infection among field rodents in southern Cholla Province, Korea. Am J Trop Med Hyg 1998; 58:513-518.

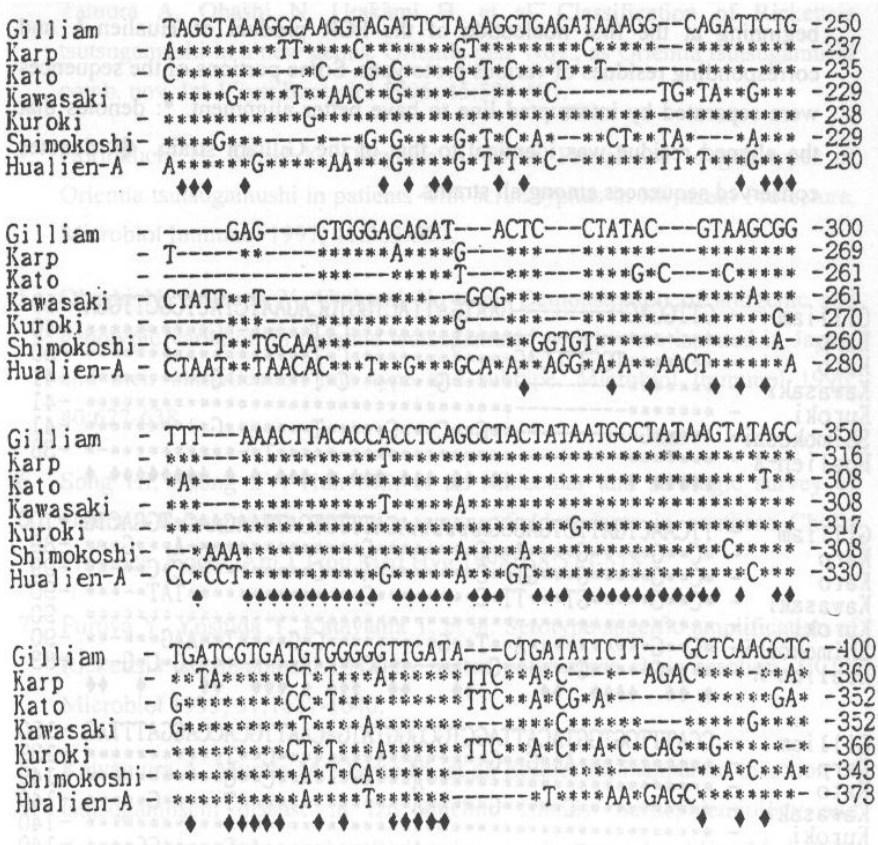
7. Furuya Y, Yoshida Y, Katayama T, et al. Serotype-specific amplification of Rickettsia tsutsugamushi DNA by nested polymerase chain reaction. J Clin Microbiol 1993; 31:1637-1640.

8. Kawamura A, Murata M, Osono M, et al. Studies on inapparent infection of tsutsugamuschi disease in Izu Shichito Islands: Seroepidemiology and demonstration of an avirulent Rickettsia strain for mice. Jpn J Exp Med 1980; 50:91-105.

9. Seong SY, Park SG, Kim HR, et al. Isolation of a new *Orientia tsutsugamushi* serotype. *Microbiol Immunol* 1997; 41:437-443.

Fig. 1. Comparison of the partial nucleotide sequences of Hualien-A with those of various *O. tsutsugamushi* prototypes. The sequence was numbered beginning at the first nucleotide of the PCR product of Hualien-A and corresponding residues of various prototypes. Some portions of the sequences were separated by interrupted line to have better alignment. *: denotes that the aligned residue was identical to that of the Gilliam strain. ◆: denotes conserved sequences among all strains.




Gilliam
Karp
Kato
Kawasaki
Kuroki
Shimokoshi
Hualien-A


Gilliam - CT-GCT--------GG----GCAACCACA--G-CT--TACTGTTGAGCA- -450
Karp - *AA**ACA--------AGCC***G**T**---*G***--**A**A******A -398
Kato - G-A***AATCACCT**GTGAT-****T-------*GG*G**AA***TATT -393
Kawasaki - **-*T*---------**A----*****A***G---**--*********- -383
Kuroki - *AA***G----------*****--*C**CCGCT*AA***T**G -396
Shimokoshi- *----------A**---------***AG*A-----*A******---T -363
Hualien-A - *AA*---TACCACAA*CTCA*****----*AT*A----*C**C***TT*TT -410
Gilliam - --GCGGGCTGCAGATAGGATTGCTTGGTTGAAGAATTATGCTGGTATTGA -500
Karp - ---**T*******C******C********A*******G********** -445
Kato - CG***T****AC********CA**********G**** -443
Kawasaki - --*********A************************************ -431
Kuroki - AA***T*******C******C********A*******G********** -446
Shimokoshi- GT***T****T*C************************************ -413
Hualien-A - --*T**AT*A*G--***************A**AC*G********** -456
Gilliam - CTATATGGTCCCAGATCCTCAGAATCCTAATG---CTAGAGTTATAAATC -550
Karp - *****G***AAA*A*C***A*TG*********GGC***TG********* -495
Kato - *********T*********A*T******C*G*---*****A**G****** -490
Kawasaki - ***************T**************---********G****** -478
Kuroki - *********GAAG******A*T*******---*GGCA**TGA*GG****** -493
Shimokoshi- ****TAT**G**G***T**A*T******C*G*G---*******G****** -460
Hualien-A - *********GAAG******A*T*********-*GGCAG*TGA*GG****** -503
Gilliam - CTGTATTGTTAAATATTACTCAAGGGCCACC-TAAT---GT---ACAGCC -600
KARP - *GA**********C*A**-***TA***C****CCT**TGG*A*T** -544
Kato - *A**GC*A*********C*******T**G**-****GC-------*A*T** -533
Kawasaki - **************************G****-*********----*A*T** -521
Kuroki - *G**G**********C*A**-****A***C****CCT**TGG****** -542
Shimokoshi- ***G**********C*******AA-***C****C--------*T**T -501
Hualien-A - **************C*******T**G**-----*GC--*AAT*-*T** -546

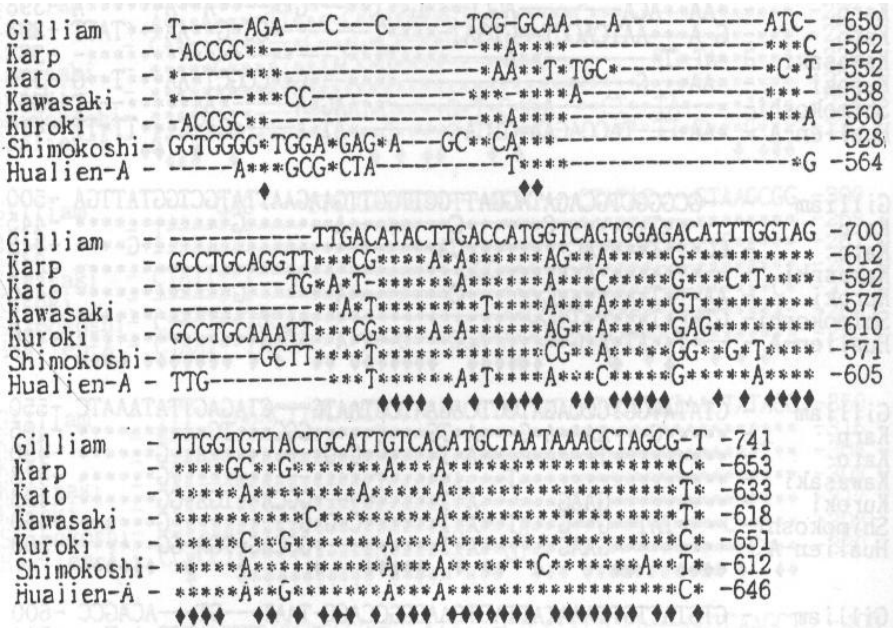

Gilliam - T-----AGA---C---C------TCG-GCAA---A----------ATC- -650
Karp - -ACCGC**----------**A****----------***C -562
Kato - *------***----------*AA**T*TGC*----------*C*T -552
Kawasaki - *------***CC----------***-****A----------***- -538
Kuroki - -ACCGC**----------**A****----------***A -560
Shimokoshi- GGTGGGG*TGGA*GAG*A---GC**CA***---------- -528
Hualien-A - -----A***GCG*CTA---------T****----------*G -564
Gilliam - ----------TTGACATACTTGACCATGGTCAGTGGAGACATTTGGTAG -700
Karp - GCCTGCAGGTT***CG****A*A******AG**A*****G********* -612
Kato - ---------TG*A*T-******A******A***C*****G***C*T**** -592
Kawasaki - ----------**A*T*******T****A***A*****GT******** -577
Kuroki - GCCTGCAAATT***CG****A*A******AG**A*****GAG******* -610
Shimokoshi- -------GCTT****T**********CG**A*****GG**G*T**** -571
Hualien-A - TTG---------***T******A*T****A***C*****G*****A**** -605
Gilliam - TTGGTGTTACTGCATTGTCACATGCTAATAAACCTAGCG-T -741
Karp - ****GC**G*******A***A*****************C* -653
Kato - *****A********A*****A*****************T* -633
Kawasaki - **********C********A*****************T* -618
Kuroki - *****C**G*******A***A*****************C* -651
Shimokoshi- *****A**********A***A******C******A**T* -612
Hualien-A - *****A**G*******A***A*****************C* -646

Table 1. Relationships between Hualien-A and various *O. tsutsugamushi* prototypes. Identities between Hualien-A and various *O. tsutsugamushi* prototypes were denoted as percentages of numbers of identical nucleotides between Hualien-A with those of the indicated strains. Numbers of gaps inserted were calculated from Fig. 1

Strain	Identity（%）	Number of gaps inserted
Hualien-A		25
Gilliam	76.4	31
Karp	76.3	21
Kato	76.3	24
Kawasaki	78.2	26
Kuroki	77.4	17
Shumokoshu	74.8	21