## II. LITERATURE REVIEW

### 1. Common characteristics

*O. tsutsugamushi* is an obligate intracellular bacterium that multiplies in the host cell cytoplasm by binary fission (9). The microorganism posses a cell wall similar to that of gram negative bacteria, and it is surrounded by a cytoplasmic membrane. The cytoplasm has a clear periplasmic space, where amorphous material are seen. In the cytoplasm, the electron-dense ribosome rich area and less dense network area with DNA fiber are distinctive. The Orientia are easily observed with a light microscope in specimens stained by Giemsa stain. Generally *O. tsutsugamushi* is approximately 0.5 μm wide and 1.2 to 3.0 μm long. This size, estimated by electron microscopic observation, is a little larger than the typhus and spotted fever group ricketsiae, which range from 0.2 to 0.5 μm in width and 0.5 to 2.5 μm in length (Table 1) (10). On light microscopic observations, however, *O. tsutsugamushi* seems to be smaller than the other ricktettsiae (11). This discrepancy may be due to the presence of capsule layers that are recognized by electron microscopy on the surface of typhus and spotted fever-group rickettsiae but not on *O. tsutsugamushi.*

There are morphological differences in the cell walls among species of the genus *Rickettsia. O. tsutsugamushi* is surrounded by a very soft and fragile outer envelope and disrupted easily by osmotic shock (12). The differences on the morphology and fragility of the cell wall between *O. tsutsugamushi* and other species of the genus *Rickettsia* may also reflect differences in the chemical structure among these rickettsiae. Recently, Tamura et al., (13) proposed that *O. tsutsugamushi* should be taxonomically transferred from the genus *Rickettsia* to the newly established *Orientia*, as *Orientia tsutsugamushi*, because of the existence of several distinguishing characteristics from other species in the genus. These differentiating characteristics include its lack of peptidoglycan and lipopolysaccharide (LPS) and differences in protein-constitution in the cell wall. In addition, Amano *et al.* (14) found that *O. tsutsugamushi* lacked muramic acid, glucosamine, heptose, 3-deoxy-D-mannooctulosonic acid, and hydroxy fatty acids, which are the

**Table 1.** Characteristics of Rickettsia and size of Rickettsia in ultrathin sections

| Species | Size (μm) | | Host cell | References |
|---|---|---|---|---|
| | Width | Length | | |
| *R. prowazekii* | 0.35 | 0.9 | L cells | Smirnova et al., (22) |
| | 0.45 | 1.5 | CE cells | Silverman et al., (23) |
| (in log phase) | 0.32 | 1.4 | CE cells | Wisseman et al., (24) |
| (in stationary phase) | 0.27 | 0.7 | CE cells | Wisseman et al., (24) |
| *R. typhi* | 0.4 | 2.0 | Louse | Ito et al., (25) |
| | 0.2-0.3 | <1.0 | Yolk sac | Ito et al., (25) |
| | 0.3-0.45 | <2.5 | Flea | Ito et al., (25) |
| *R. canada* | 0.4 | 1.6 | Tick | Lyle and Burdofer, (26) |
| *R. rickettsii* | 0.25-0.4 | | CE cells | Siverman and Wisseman, (27) |
| *R. rhipicephali* | 0.39-0.56 | 0.8-1.7 | Tick | Hayes and Burdorfer, (28) |
| *R. tsutsugamushi* | 0.5-0.6 | 1.5-1.6 | L cells | Wright et al., (29) |
| | 0.6-0.8 | 1.2-3.0 | BHK cells | Rikihisa and Ito., (30) |
| | 0.5-0.7 | 1.3-2.5 | L cells | Urakami et al., (31) |
| | 0.6 | 1.3-2.5 | Mite | Urakami et al., (32) |

general components of peptidoglycan or lipopolysaccharide. These lines of evidence lead to the conclusion that *O. tsutsugamushi* lacks both peptidoglycan and LPS in the cell wall. This conclusion coincides well with evidence that *O. tsutsugamushi* is completely resistant to the beta-lactam antibiotics (benzylpenicillin, ampicillin, carbenicillin and cefotaxime) (15), which inhibit bacterial peptidoglycan synthesis. Drugs with a specific action against *O. tsutsugamushi*, which consequently are especially effective against scrub typhus, include antibiotics of the tetracycline family such as tetracycline, methylchlortetracycline, deoxycycline and minocycline (16).

The genomic size of the genus *Rickettsia* is in the range of $1.1\text{-}1.5\text{x}10^9$ Da which corresponds to about 2000-kb pairs (17, 18). Recently, pulse field gel electrophoresis analysis of genomic DNA of *O. tsutsugamushi* indicated that the DNA size for the various strains ranges

from 2400 to 2700-kb pairs (19). Sodium dodecyl sulfate (SDS)-PAGE of purified *O. tsutsugamushi* lysates (20) reveals as many as 30 polypeptide bands. The major polypeptides are designated as 110, 80, 70, 56, 46, 42, 35, 28, and 25 kDa from their mobility on the PAGE analysis. The 56-kDa polypeptide is present in the largest quantity. This polypeptide is an immunodominant antigen showing type-specific antigenicity (21), and is easily obtained by treatment of intact Orientia with trypsin (20), indicating that it is located in the outermost layer of the cell.

**2. Proliferation in animals**

When Orientia were injected intraperitoneally (i.p.) in mice, the organisms were observed in smears of peritoneal exudate, liver, spleen, kidney and lung stained by Giemsa stain (33) and direct immunofluorescence assay (34). After i.p. injection, the greatest numbers of Orientia were detected in the mesothelial and inflammatory cells of the peritoneal smears. On cryostat organ sections of mice, the growth of many Orientia were clearly observed in the inflammatory cells adhering around the capsule of liver, spleen and kidney

**3. Multiplication cycle in the host cell**

*Orientia tsutsugamushi* is an obligate intracellular parasite that propagates within the host cell cytoplasm by binary fission. Multiplication of this microorganism can be divided into three phases. These phases of multiplication have been observed under electron microscope ( 35, 36, 37).

(3.1) Adsorption and penetration

The first step in the penetration of Orientia into the host cell is the attachment of the Orientia to the host cell surface. This attachment may constitute a reaction between the attachment site on the oriential surface and receptors on the host cell membrane. The next step in penetration is the formation of projections at the site of oriential attachment to the cell surface, indicating the initiation of phagocytosis. The orientia is then taken into a phagosome of the host cell cytoplasm. In these phagosomes, the orientia are deformed and compressed and the oriential interior is heterogeneous and granular, indicating that the organisms are inactive. The process whereby the oriential penetrates the host cell cytoplasm takes 10-20 minutes.

(3.2) Intracellular multiplication

The intracellular multiplication is relatively slow. Twenty four hours after infection, a small number of Orientia are found in the cytoplasm. The site of Oriential multiplication is usually in the host cell cytoplasm. Many Orientia are seen under the host cell membrane in 72 hours.

(3.3) Release from the host cell

Seventy two hours after infection, many Orientia were seen under the host cell membrane and most of them seem to push the host cytoplasmic membrane from the inside to the outside. This stage of infection is referred to as budding. The Orientia detach from the cell surface at the site of the constricted cell membrane and are then released.

**4. Pathogenicity**

Many strains of *O. tsutsugamushi* have been isolated from patients and wild animals. Most isolates of Gilliam and Karp serotype in Japan are highly virulent in mice whereas isolates of Kawasaki and Kuroki serotype are not virulent. Such differences in pathogenicity are due to the differences in serotypes (5, 38). However, there are no reports of any relationship between the antigenic type and the severity of human illness. However, in the actual areas endemic for scrub typhus, heterogeneity in the causative *O. tsutsugamushi* strains is marked. Consequently, cellular immunity to *O. tsutsugamushi* does not function adequately, resulting in reinfections and multiple infections. Thus, in such areas endemic for scrub typhus, patients may exhibit typical symptoms at the time of initial infection, whereas they frequently show either mild or negligible symptoms at the time of reinfection. Further, the pathogenicity of *O. tsutsugamushi* varies by strain; there are *O. tsutsugamushi* strains that cause a range of clinical symptoms from typical to mild or abortive, even at the time of initial infection. The mildly pathogenic *O. tsutsugamushi* frequently results in a completely inapparent infection (39). The classical clinical symptoms are summarized by Saito et al (40).

4.1 Eschar

The detection of eschar, which is found even at early stage of illness, is essential for clinical differentiation from other febrile eruptive diseases. The eschar is sometimes found at the sites where a tick is biting and frequently occurred in difficult-to-find spots, and the reported differences are possibly due to differences in thoroughness with which physicians investigate the eschar. In regard to eschar in patients, the sites favored by mites for infestation are frequently soft regions and are difficult to find unless the patient's body is thoroughly searched. There may occasionally be more than one site. Although there are variations in eschar characteristics according to the species of mite causing the dermatitis, studies of eschar sites from the very early stage revealed bright red papules with induration around the site of attachment. The peripheral area of the papule is not always well demarcated. Subsequently, the blister, developed in the center, becomes pustular, the red circle around it is enlarged and a small ulcer with a blackish-brown scab occurs in the center. The surrounding red circle becomes darker, the scab enlarges and the red circle disappears, followed by the dropping off the scab.

4.2 Fever

The body temperature usually rises suddenly to 38 °C or above 39 °C and this persists for about 10 days, exhibiting a remittent. This is frequently accompanied by headache, low back pain, chill and sweats.

4.3 Lymphadenopathy

Enlargement of the lymph nodes accompanied by tenderness is sometimes limited to local lymph nodes of the eschar site, but generally leads to systemic lymphadenopathy.

4.4 Other findings

The liver and spleen are enlarged by approximately 30% and a hemorrhagic tendency becomes marked in serious cases. The patients who do not receive appropriate treatment due to undetermined diagnosis, a hemorrhagic

tendency and coagulation disorders are exhibited during the course of the disease. The hemorrhagic tendency that occurs in severe case has long attracted attention, as thrombocytopenia. The causative orientia show such biological characteristics as acute toxicity and hemolytic activity due to the oriential toxins which are harboured only by live orientia. The components of oriential toxins have not been identified but they are related to vascular endothelial cell permeability factors.

**5. Antigenic variants of *Orientia tsutsugamushi***

Antigenic heterogeneity among strains of *O. tsutsugamushi* has been recognized by complement fixation test, neutralization test and cross-protection test (41, 42, 43, 44, 45) In early studies, *O. tsutsugamushi* were classified into two groups Gilliam and Karp. Kawamura (46) introduced highly specific direct and indirect immunofluorescence assay to classify many isolates from patients, rodents and chiggers and then classified *O. tsutsugamushi* into three groups; Gilliam, Karp and Kato prototypes.

New antigenic types, differ from the three prototype, have been found in many countries. For example, the Kawasaki and Kuroki strains, isolated from patients in Miyasaki Prefecture, Japan. These two strains were shown to be distinct from the earlier prototype strains (47). Typing of newly isolated strains were performed by using three serotype specific monoclonal antibodies. Prior to these findings, Kobayashi, et al., had isolated the Irie and Hirano strains from patients in Miyazaki Prefecture the same area in which the Kawasaki and Kuroki strains were isolated but the identification of Irie and Hirano strains has not yet been confirmed (48). In Korea, the presence of another antigenic type was demonstrated in isolates from patients. The Boryong strains has recently been classified by Kim, et al.,(49). The identification was demonstrated in isolates from patient by using murine polyclonal and monoclonal antibodies. However, this serological method dose not always provide clear identification because there are new antigenic types that contain a mosaic of antigenic determinants reactive with multiple antisera.

**6. Geographic distribution**

Scrub typhus is an apparent or inapparent *O. tsutsugamushi* infection caused by the bite of mites which harbour *O. tsutsugamushi* and bite humans. The occurrence of the disease thus requires a point of contact between humans and the mites. Since mild or inapparent *O. tsutsugamushi* infection are confirmed, the mere tracking of patients is inadequate to comprehend the geographic distribution of *O. tsutsugamushi.* Consequently, it is necessary to study the cycle of *O. tsutsugamushi* in vertebrate animals centered around its main hosts, rodents, to elucidate the distribution of *O. tsutsugamushi.* In addition, various antigenic variants are recognized in *O .tsutsugamushi.* In Southeast Asia, different antigenic types of isolates, besides Gilliam, Karp and Kato prototypes have been identified. The *O. tsutsugamushi* strains isolated in Korea have been found in three types, using monoclonal antibodies. The Karp and Gilliam types were found mostly in the central part of Korea. The Boryong type, which seems to be a new local type closely related to the Karp type was distributed widely in the south. It has been distinguished immunologically from the former prototypes (50). In Taiwan, ten isolates were classified by IFA, which showed Gilliam and Karp serotype(51) whereas in Jiangsu, The People's Republic of China, the prevalent infection was due to Gilliam serotype (520). In Japan, Gilliam and Karp serotypes were predominantly isolated from patients (53). The Kawasaki and Kuroki types were also recognized in Shizuoka, Kanagawa and Chiba Prefecture, which are located along the west coast of the Pacific Ocean on Honshu Island, although Karp and Gilliam serotype also found in these areas (38, 54). In southeast Asia, Shirai (55, 56, 57) found a mixed infection of TA 716, TA763. TA686, Gilliam, Karp and Kato in chiggers. Unfortunately, Shirai identified the serotype by using polyclonal antibodies which was not definitive clear because of cross reaction with some heterologous types. It is obvious that in-depth studies are necessary using modern technique to clearly identify, such as the type-specific monoclonal antibodies and genetic analysis by PCR.

**7. Antigenic structure of *Orientia tsutsugamushi***

Eisemann and Osterman (58) were the first to demonstrate that several components on the Oriential surface showed antigenicity reactive with antibodies against homologous or heterologous strains of *O. tsutsugamushi.*

7.1 The 56-kDa polypeptide, which is present in the largest amount on the Oriential surface, reacts strongly with homologous antiserum, but faintly or moderately with heterologous antisera, indicating that this polypeptide has strain or type specific antigenicity. The antigenic type of *O. tsutsugamushi* depends on the 56-kDa polypetide, which is the major polypeptide on the Oriential surface. When the orientia are solubilized at 80 °C or lower in SDS-containing buffer without 2-mercaptoethanol (2-ME), this antigenic polypeptide forms a band at 110 kDa or higher positions on PAGE analysis, suggesting that it is present as a dimer or oligomer in rickettsia. The 56-kDa polypeptide has been purified and the N-terminal amino acid sequence has been determined (59). Comparison of 35 amino acid sequence at the N-terminal side of the polypeptide in the Gilliam, Karp, and Kato strains revealed that isoleucine was the N-terminal amino acid in all cases. However, differences were observed for several amino acids and there was about 70% sequence homology among strains. The 56-kDa protein is located on the Oriential surface and shows type specific antigenicity. Genes of the type specific protein from the Gilliam, Kato (60) and Karp strains (61) have been cloned and sequenced (60, 61). These strains show distinct antigenicity. All the genes encode proteins consisting of 521-533 amino acids. New antigenic types differ from the three prototypes have been identified. In Japan, the Shimokoshi strain, isolated from a patient, was identified as a new type that showed low cross reactivity with hyperimmune sera against the Gilliam, Karp and Kato serotype (62). In the signal peptide of the Shimokoshi strain, two amino acids differed from those of the other strains. The amino acid sequence of signal peptides was identical in the six strains, Gilliam, Karp, Kato, Kawasaki, Kuroki, and Boryong. All the signal peptide of *O. tsutsugamushi* strains consist of a basic region and a hydrophobic core, as seen in those characteristic of other prokaryotic cells (63). In the comparative analysis of amino acid sequence alignments of the 56-kDa proteins in antigenic variants of the Gilliam, Karp, Kato, substitutions or deletions of one or several contiguous amino acid residues are recognized throughout the molecules; however, there are significant differences in sequences among strains in the variable domain (VD) regions. In the variable domains, there are both diversity and similarity among strains, and the relationships are complicated. It is not yet known how the polymorphism in the variable domains evolved, but this diversity appears to be due to point mutation

7.2 The 46-47 kDa and 70-kDa polypeptides react not only with homologous antiserum but also with heterologous antisera, suggesting that these polypeptides have characteristics of group specific antigenicity (64).

7.3 The 22-kDa polypeptide has been sequenced by Hickman et al. (65). The recombinant 22-kDa antigen was also found to elicit a proliferative response in helper T cell line, suggesting the presence of the stimulatory T cell epitopes in the 22-kDa protein as well as in the 47-kDa protein.

However, on the immunoblotting test, sera from patients varied (21). Most sera reacted with the 56-kDa polypeptide, suggesting that this polypeptide is the dominant antigen in the course of infection. The other polypeptides, the 60, the 50-52, the 46-47, the 21-25 kDa were reactive with some sera, but not all. The antigenic components in *O. tsutsugamushi* have been examined with monoclonal antibodies by various investigators (7, 66, 67). Many strain-specific monoclonal antibodies show a reaction with the 56 kDa polypeptide (66, 67).

**8. Genetic analysis of Oriential proteins**

Genes of Oriential proteins that have been cloned are for 110, 72, and 60 kDa and 56, 49, 47 and 22 kDa proteins ( 64, 65, 67, 68 ). These seven proteins are all antigenic and react with hyperimmune sera against *O. tsutsugamushi.* The nucleotide sequences of genes encoding the 60-, 56 and 22 kDa proteins and their amino acid sequences have been determined (47, 60, 61. 65). The 56- kDa protein is the major type specific antigen that determines the antigenic type of rickettsia and the recombinant 56-kDa protein is used as an antigen for serodiagnosis (49). On the other hand, 72, 60 and 47 kDa protein show cross reactive antigenicity among strains (69).

**9. Laboratory diagnosis**

**9.1. Isolation of *O. tsutsugamushi*** The isolation of *O. tsutsugamushi* from patients and wild rodents has been performed by inoculating laboratory mice and cultures of tissue cells. The majority of isolates are frequently highly pathogenic to mice. The mouse strains that are naturally susceptible to *O. tsutsugamushi* is controlled by *Ric* gene (70). Consequently, susceptible murine strains have been used to isolate *O. tsutsugamushi.* The growth of *O. tsutsugamushi* can be observed in various cultured cells. Yamamoto (71) used the cell lines

L-929 (derived from mouse fibroblast cells) and BS-C40 (dirived from African green monkey kidney cells) for rickettsia isolation from patients. The efficiency of isolating *O. tsutsugamushi* by the cultured cell method has not been favored because of its time consuming and complicated procedure.

**9.2. Detection of antibody** Diagnosis of scrub typhus has usually been determined simply by serological methods, by measuring significant increase of specific antibodies in the sera of patients. The procedures are examination of paired serum samples, differentiation of IgM and IgG, and the involvement of local types of *O. tsutsugamushi* as antigens. In scrub typhus , an increased antibody titer is usually observed 10-14 days after infection. The latent period before the appearance of clinical manifestations is 7- 10 days. However, the antibody titer at that time is usually low. For serodiagnosis, the second serum sample should be taken 7 days later. The increase of antibody titer between these paired sera should be examined to ensure a correct diagnosis. Changes in IgM and IgG titers along the time course of scrub typhus are similar to the other infectious disease. The results of paired serum examinations can be interpreted in three ways.

a) Absence of both IgG and IgM indicates no exposure to *O.tsutsugamushi* or a very early stage of infection.

b) Low IgM positivity and negative IgG indicates an early stage of infection. Both will become clearly positive after a few days. If there is no increase in titer of the second sample sera, the presence of rheumatoid factor should be considered.

c) High titer of both IgG and IgM indicate active infection

The diagnosis of scrub typhus in humans has usually been determined simply and rapidly by serological method, by measuring significant increase of specific antibodies in the sera of patients as follows:

(1) Weil-Felix test (WF): It is not a serological reaction between a specific antibody and antigen, but a reaction between *O. tsutsugamushi* specific antibody and some component of Proteus OX-K that is common to *O. tsutsugamushi.* The low specificity and sensitivity are thus a problem (72). The antibody is usually detected around day 12. Since

the test procedures are simple and can be performed in any clinical laboratory with commercial Proteus OX-K products, the test is still used in many laboratories, even though other, more specific, methods are now available.

(2) Complement Fixation test (CF): This test is serological reaction between specific antibody and *O. tsutsugamushi* antigen but no progress in diagnosis has been made because of technological complexity of the procedure. The antigen of *O. tsutsugamushi* are difficult to prepare from microorganism proliferated in the yolk sacs of embryonated chicken eggs. In addition, CF test does not precisely establish amounts of *O. tsutsugamushi* specific IgM and IgG antibodies individually (73).

(3) Indirect immunofluorescent assay (IFA): The IFA technique is now used worldwide and it is a gold standard method for the antibody detection of *O. tsutsugamushi* infections. Its advantages are that it has a higher sensitivity for IgM and IgG antibodies individually, so that even in the acute phase of infections, the antibody can be detected and diagnosis can be made. Antigens on glass slide are allowed to react with patient's serum in serial two-fold dilutions and Fluorescein isothiocyanate (FITC) conjugated anti-human IgG or IgM. A greenish color is developed at the antibody-binding sites by antigen-antibody reaction. However, *O. tsutsugamushi* strains show heterogeneity (74,75,76) so detection is sometimes inconclusive.

(4) Indirect immunoperoxidase test (IIP): The principle of IIP technique is similar to IF. Antigens on glass slide are allowed to react with patient's serum in serial two-fold dilutions and peroxidase conjugated anti-human IgG or IgM. A brown color is developed by the reaction of the enzyme and substrate. The IP test has shortcomings, such as the necessity for an experienced observer to interpret which reactions are specific or non specific, due to the great extent of nonspecific reactions with peroxidases contained in the tissues (77).

(5) Enzyme linked immunosorbent assay (ELISA): The ELISA is employed to detect specific antibodies of *O. tsutsugamushi* and established to increase the sensitivity of IF. A technique based on IgG and IgM capture. Anti- human IgG or IgM is adsorbed on the solid phase on a microplate. Reactions are followed in the order of test serum, *O. tsutsugamushi* antigens and peroxidase labeled anti- *O. tsutsugamushi* monoclonal antibody. Color development is performed. The degree of colour development represented by absorbance and is read with spectrophotometer (58). A dot-immunoblot method (78) also based on ELISA and performed on a nitrocellulose membrane is available (79). The principle of the procedure is the detection of anti- *O. tsutsugamushi* antibodies in test sera by reaction with antigen adhering to a solid membrane and the combined human antibodies are then detected by further reactions with peroxidase conjugated anti-human IgM or IgG rabbit serum, with diaminobenzidine serving as the enzyme substrate. The reactions are discriminated by reading the color intensity of the dots on the membrane. The advantage of this method is that no special instruments are required.

**9.3 Detection of Oriential DNA** The nucleotide sequence of DNA fragment encoding the serotype specific antigen polypeptide (56 kDa) of *O. tsutsugamushi* was determined by Stover (61) and Ohashi et al., (68). On the basis of these studies, polymerase chain reaction (PCR) was developed to be used in practice for the diagnosis of scrub typhus in humans and for the detection of rickettsiae in mites ( 80, 81, 82). PCR is effective, being particularly useful for the diagnosis at a very early stage of the disease before the antibody titer has increased. The increased sensitivity permitted by nested PCR can lead to increases in false positivity due to the contamination in some steps of the procedure. Thus, this technique should be performed very carefully.

**10. Genetic polymorphism of 56-kDa protein gene**

The sequencing of the PCR amplified DNA fragment was applied to study the molecular epidemiology of *O. tsutsugamushi.* At least 3 serotypes, Gilliam, Karp and Kato, were among the clinical isolates of *O. tsutsugamushi* and then the 56 kDa protein gene sequence comparisons among serotypes were revealed. The prototype of *O. tsutsugamushi*, 3 seroypes, have been sequenced by Ohashi *et al.* ( 60, 66) and Stover *et al.*, (61). Their reference nucleotide sequences were obtained from the GenBank database. The comparative analysis of nucleotide sequence homologies of three serotypes were carried out on the basis of the 56 kDa protein gene diversity among *O. tsutsugamushi* serotypes. The clustered nucleotide substitution was found to be closely related to serotypes, while the insertions or deletions were observed among distantly related serotypes. The new *O. tsutsugamushi* serotypes, Kawasaki, Kuroki and Boryong, were found by the comparing the nucleotide sequences to prototype serotype sequences. It is not yet known how the polymorphism in variable domains of *O. tsutsugamushi* evolved. The nucleotide sequences of 56 kDa protein gene of *O. tsutsugamushi* reference serotypes are available in the GenBank database and they were used as a prototype sequences for the comparative analysis of the 56 kDa protein gene of serotypes from clinical samples. These nucleotide sequences are shown in Figure 1. This nucleotide sequencing method will be useful in the future for identification of *O. tsutsugamushi* genotypes.

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          501                                                       550
Gilliam AGTACTATTA AATATTAGTA TACTAAGTAA TAGTTTTTTG ATATAAAAGT.
Karp    AGTACTATTA AATATTAGTA TACTAAATAA TAGTTTTTTG ATATAAAACT
Kato    AGTACTATTA AATATTAGTA TACTAAATAA TAGTTTTTTG ATATAAAACT
          601                                                       650
Gilliam AAATTTAATT CTTTTAAGGA GATTAGAATG AAAAAAATTA TGTTAATTGC
Karp    AAATTTAATT CTTTTAAGGA GATTAGAATG AAAAAAATTA TGTTAATTGC
Kato    AAATTTAATT CTTTTAAGGA GATTAGAATG AAAAAAATTA TGTTAATTGC
          701                                                       750
Gilliam TGGGTGAGGA AGGAGGATTA GAGTGTGGTC CTTACGGTAA AGTTGGAATC
Karp    TGGGG...GA AGAAGGATTA GAGTGTGGTC CTTATGCTAA AGTTGGAGTT
Kato    TGGGGGATGA AGGAGGATTA GAGTGTGGTC CTTATGCTAA AGTTGGAGTC
          801                                                       850
Gilliam TTCTGAGGGA AAAAAACATT TGTCATTAAC AACTGGACTG CCATTTGGTG
Karp    TGCTGAAGGC AAAAAACACT TGTCATTAAC AAATGGGCTG CCATTTGGTG
Kato    TGCTGGTGGC AAAAAACAAT TGCCATTAAC AACCTCGATG CCATTTGGTG
          901                                                       950
Gilliam GGTGTTATGT ACCTTAGAAA TATAAGCGCT GAGGTTGAAG TAGGTGAAGT
Karp    GGTGTTATGT ACCTTACAAA TATAACTGCT CAGGTTGAAG AAGGTAAAGT
Kato    GGGGTTATGT ACCTTGCGAA TGTAAAAGCA GAGGTGGAAT .......CAGG
          1001                                                      1050
Gilliam GGTGGGACAG ATACTCGCGG TTTAAACTTA CACCACCTCA CCTACTGATA
Karp    ATGCTCCTAT ACGTAAGCGG TTTAAACTTA CACCTCCTCA GCCTACTATA
Kato    ATTCTCCTAT GCCTCAGCGG TATAAACTTA CACCACCTCA GCCTACTATA
          1101                                                      1150
Gilliam TGCTCAAGCT GCTG..... ... CTGGGC... . AACCACAG  CTTACTGTTG
Karp    GACCTCAGCA G.... CAAGC  ACAAGC... CG CAGCCTCAGG CTTAATGATG
Kato    ACCTCAAGGA GGAGCTAATC ACCTGGGTGA TAACCTTGGT GCTAATGATA
          1201                                                      1250
Gilliam GACTATATGG TCCCAGATCC TCAGAATCCT ..........  GTATTGTTAA
Karp    GACTATAGGG TAAAAAACCC TAATGATCCT AATGGGCCTA TGGTTATAAA
Kato    GACTATATGG TTCCAGATCC TAATAATCCT CAGG... CTA GAATTGTAAA

          551                                                       600
. AAGTTAGTG TGGCTAAATA ATTAGTTTAG AATGGTTACC ACTAAAAAAT
  AAAGTTAGTG TGGCTAAATA ATTAGTTTAG AATGGTTACC ACTAAAAAAT
. AAGTTAGTG TGGCTAAATA ATTAGTTTAG AATGGTTACC ACTAAAAAAT
          651                                                       700
  TAGTGCAATG TCTGCATTGT CATTGCCGTT TTCAGCTAGT GCAATAGAAT
  TAGTGCAATG TCTGCGTTGT CGTTGCCATT TTCAGCTAGT GCAATAGAAT
  TAGTGCAATG TCTGCATTGT CATTGCCGTT TTCAGCTAGT GCGATAGAAT
          751                                                       800
  GTTGGAGGAA TGATTACTGG TGCAGAATCT ACTCGCTTGG ATTCAACTGA
  GTTGGAGGAA TGATTACTGG CGTAGAATCT GCTCGCTTGG ATCCAGCTGA
  GTTGGAGGAA TGATTACTGG CGTAGAATCT ACTCGCTTGG ATCCAGCTGA
          851                                                       900
  GTACATTAGC TGCGGGTATG ACAATTGCAC CAGGATTTAG AGCAGAGCTA
  GAACGTTGGC TGCAGGTATG ACAATCGCTC CAGGATTTAG AGCAGAGATA
  GTACATTAGC TGCAGGTATG ACAATCGCGC CAGGATTTAG AGCAGAGCTA
          951                                                       1000
  AGGTAAAGGC AAGGTAGATT CTAAAGGTGA GATAAAGGCA GATTCTGGAG
  TAAGGCAGAT TCTGTAGGTG AGACAAAGGC AGATTCTGTA GGTGGGAAAG
  TAAAACTGGC TCTGATGCTG  ATA.... ....  ..TTAGATC. . . TGGTGCAG
          1051                                                      1100
  ATGCCTATAA GTATAGCTGA TCGTGATGTG GGGGTTGATA CTGATATTCT
  ATGCCTATAA GTATAGCTGT ACGTGACTTT GGGATTGATA TTCCTAACCA
  ATGCCTATAA GTATTGCGGA TCGTGACCTT GGGGTTGATA TTCCTAACGT
          1151                                                      1200
  AGCGGGCTGC TGCAGATAGG ATTGCTTGGT TGAAGAATTA TGCTGGTATT
  AGCAACGTGC TGCAGCTAGG ATCGCTTGGT TAAAGAATTG TGCTGGTATT
  TTCGGCGTGC TGACGATAGG ATCACTTGGT TGAAGAATTA TGCTGGTGTT
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**Figure 1** Nucleotide sequences of the 56-kDa protein gene of the *O. tsutsugamushi* reference serotypes, obtained from the GenBank database.