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Running head: rTSAs in the serological diagnosis of scrub typhus
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Summary

Scrub typhus is a mite-borne rickettsiosis caused by the intracellular bacterium *Orientia tsutsugamushi* (OTS), which is classified as a biosafety level-3 (BSL-3) pathogen. For serological tests of scrub typhus, mouse fibroblast cells infected with the five prevalent serotypes of OTS in Japan are generally used as antigens for indirect immunofluorescent assay (IFA). In this study, *sf*-9 insect cells infected with the recombinant type-specific antigens (rTSA)-expressing baculovirus were applied for IFA. The paired sera samples of 15 scrub typhus-patients, 10 rickettsiosis-patients, and 10 control individuals were used. Both IgM and IgG IFA titers determined by the rTSA-based IFA were correlated with those determined with the OTS-infected cell-based IFA ($R^2 = 0.7319$ to $0.7956$). Based on the criteria for serological diagnosis, such as the suitable cutoff for single serum (IgM ≥ 1:160) and/or significant increase in IgG titer between paired sera (≥ 4 times), all of the 15 scrub typhus patients diagnosed as positive with OTS-infected cell-based IFA were also diagnosed as positive by the rTSA-based IFA, whereas all 10 rickettsiosis patients and 10 control individuals were not. The rTSAs, which can be prepared in BSL-2 laboratories, are efficacious in the serological diagnosis of scrub typhus.
Introduction

Scrub typhus is a mite-borne rickettsiosis caused by an intracellular bacterium, *Orientia tsutsugamushi* (OTS) (1), which is classified as a biosafety level-3 (BSL-3) pathogen. Scrub typhus (ST) is widely found in several countries of the Asia-Pacific region, including Japan (2). In Japan, approximately 300 to 500 cases of ST, including a few fatal cases, are reported annually (3). Most cases are confirmed by serological diagnosis using immunofluorescent assay (IFA). For IFA, OTS-infected cells are generally used as antigens. The use of at least five serotypes of OTS is generally recommended in Japan, because the serotypes, including the Kato, Karp, Gilliam, Kuroki (4), and Kawasaki (5) types, are prevalent in Japan, and serological cross-reactivity between the serotypes can be limited in patient sera (6). In other words, the serum-antibody to humoral immune response of a patient sometimes elevates only against a certain serotype and not others. Preparation of the OTS-infected cells requires a BSL-3 facility, which possibly restricts the development of new commercially available tests and kits for the diagnosis of scrub typhus.

The type-specific antigen (TSA) of OTS is a 56 kDa major outer membrane protein, considered an immunodominant protein and major determinant of OTS serotypes (2). In previous reports, recombinant TSA was used as the antigen for serological assays,
such as enzyme-linked immune assays (7-9). All of the recombinant TSAs were expressed in *Escherichia coli* expression systems and have not been used as antigens for IFA thus far.

In this study, to develop alternative antigens to be used instead of OTS-infected cells, we developed recombinant TSA proteins of the five aforementioned OTS serotypes, including three unique isolates found in Japan, and evaluated them as antigens for IFA in the serological diagnosis of ST.

**Materials and Methods**

**Expression of TSAs with the baculovirus system**

A gene of the core TSA region, except the hydrophobic regions at both ends, was cloned by Gateway recombination cloning technology (Thermo Fisher Scientific, MA, USA). The genes of the major five OTS strains in Japan were amplified using a pair of specific primers with attB1 or attB2, attB1-TSA56core-F(N^His) (5’-G GGG ACA AGT TTG TAC AAA AAA GCA GGC TTC AGA GCA GAG MTA GGK GTT ATG TAC C-3’, 56-mer) and attB2-TSA56core-R(end) (5’-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTA ACC AGC ATA TAT TGA GAC TGA TTC AGT-3’, 60-mer), by polymerase chain reaction (PCR). The amplified core of the gene was cloned
into an entry vector, pDONR™221, and then subcloned into a destination vector, pDEST™10 (N-terminal 6×His tag). The inserted gene was confirmed by DNA sequencing, using M13 (5’-GTAAAACGACGGCCAGT-3’) and M13Rv primers (5’-CAGGAAACAGCTATGAC-3’).

Next, the gene encoding the core region of TSA was expressed through a Bac-to-Bac baculovirus expression system (Thermo Fisher Scientific, MA USA). DH10Bac E. coli cells were transformed with the pDEST™10, incorporating the gene of the core region of TSA, and then the recombinant Bacmid DNA was selectively collected. The sf-9 insect cells were transfected with the recombinant DNA (pDEST™10, including the gene of the core region of TSA) to produce recombinant baculovirus, which express the core region of TSA (rTSA). Finally, the recombinant baculovirus clones were obtained after plaque purifications.

**Analyses of expression and immunogenicity of rTSA**

The sf-9 insect cells were inoculated with the recombinant baculovirus and incubated in Sf-900™ II SFM medium (Thermo Fisher Scientific, MA, USA) at 23°C for 72 hours. Then, the infected cells were detached with a scraper and 100 µl of the culture medium containing the infected cells (approximately 1x10⁶ cells) was collected for western blot
analysis and IFA to confirm rTSA expression. Western blot analyses were performed as previously described (10, 11), using anti-His-tag monoclonal antibodies (014-23221; FUJIFILM Wako Pure Chemical Industries, Ltd., Osaka, Japan) and rabbit-anti-OTS serum at a 1:1000 dilution. The expected molecular weight of the expressed protein was calculated for the core region of TSA combined with the linker, including 6×His-tag at N-terminus (MSY YHH HHH HDY DIP TTE NLY FQG ITS LYK KAG S), by the Compute pI/Mw tool provided by bioinformatics resource portal, ExPASy (https://web.expasy.org/compute_pi/) (Table 1). Immunofluorescent assays were performed as previously described (12, 13), using guinea pig-anti-OTS serum at a 1:1000 dilution.

**Micro-IFA**

A previously reported micro-IFA method (14) was modified and applied to determine the antibody titer of each serum sample. In brief, the recombinant baculovirus was inoculated into the sf-9 insect cells and incubated in Sf-900™ II SFM medium at 23°C for 3 to 5 days, until the infected cells were observed in over 80% of the cells. The sf-9 insect cells infected with the recombinant baculovirus, which express the TSAs of each serotype, were spotted in each well of a multi-well slide. The serum underwent a two-
fold serial dilution, from an initial 1:40 dilution with PBS. After the diluted serum was added to one well, the slides were incubated at 37°C for 1.5 h in a humid chamber.

After washing with PBS-T, Alexa Fluor 488-conjugated AffiniPure goat-anti-human IgM, Fe5μ or IgG Fcγ fragment-specific antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) were diluted to 1:200 with PBS and added into the well. The slides were then incubated at 37°C for 1.5 h. After washing with PBS-T, the slides were embedded in a mounting fluid (Mount, PermaFluor, Thermo Fisher Scientific, Waltham, MA, USA), topped with a micro cover glass, and observed under a fluorescent microscope (Axioskop2 Plus; Carl Zeiss, Oberkochen, Germany).

The micro-IFA titer was calculated as the highest dilution of serum at which the fluorescence-stained cells were clearly observed compared to the control, i.e., non-infected sf-9 insect cells. The micro-IFA titer was determined in two or three independent observations.

**Human patient sera**

Fifteen pairs of sera samples from scrub typhus patients (N = 30), 10 pairs of sera samples from healthy subjects (N = 20), and 10 pairs of sera samples from patients with other types of rickettsiosis, including 7 pairs of patients with Japanese spotted fever (N
rTSAs through IFA. Serum samples were collected between 2000 and 2012, and sent to our laboratory for serological tests of scrub typhus and other types of rickettsiosis. All the serum samples were kept at -80°C. This study was approved by the Ethics Committee for Medical and Health Research involving Human Subjects of the National Institute of Infectious Diseases (474: January 16, 2014).

**Data analysis**

Antibody titers of the human sera against rTSAs in this study were compared to those against OTS-infected cells in the conventional IFA reported previously (15). Correlations between the antibody titers against rTSAs and OTS-infected cells were analyzed by linear regression analysis, and the coefficient of determination ($R^2$) was calculated using Excel software (Microsoft Corporation, WA, USA). The correlation was considered strong when $R^2 > 0.7$. A suitable cutoff value of IgM titer against rTSA was evaluated by the receiver operating characteristic (ROC) analysis (16) using Bell curve software based on Excel software (SSRI Co., Ltd. Tokyo, Japan). The ROC curve was created by plotting the true positive fraction (TPF) against the false positive fraction (FPF) at various cutoff value settings. The point showing the highest TPF and
the lowest FPF was decided as the suitable cutoff value. Serological diagnosis was conducted according to the new cutoff value of IgM for a single serum sample and/or ≥4-fold elevation of micro-IFA titers for paired sera.

**Results**

**Expression and Immunogenicity of rTSA**

Western blot analysis, using the anti-His-tag monoclonal antibody, resolved a 44 kDa protein corresponding to the anticipated size of rTSA, across all strains, with slight size variations (Fig 1Aa). Extra bands, suggested to be the degraded rTSA protein, were also observed. Additionally, in the western blot assay using the rabbit-anti-OTS serum, the major protein bands corresponded to the bands in the assay using the anti-His-tag monoclonal antibody, although a common band was observed in both the infected cells and the non-infected control cells (Fig. 1Ab). In IFA, the guinea pig-anti-OTS serum strongly reacted to the infected cells, but not to the non-infected control cells (Fig 1B).

**Evaluation of rTSA-expressed cells for antigens in serological diagnosis by IFA**

Both IgM and IgG IFA titers determined by the rTSA-based IFA were correlated well with those determined by the OTS-infected cell-based IFA ($R^2 = 0.7319$ to $0.7956$) (Fig
Furthermore, the correlation coefficients of IgM and IgG titers between the rTSA-based and OTS-infected cell-based IFAs were strong across the five individual strains of OTS ($R^2 = 0.7476$ to $0.8722$) (Fig 2B).

**Evaluation of IgM-cutoff values by ROC analysis**

ROC analysis showed that the most suitable cutoff value for the IgM titer was 1:160 in the rTSA-based IFA (Fig. 3), whereas the most suitable cutoff value was 1:80 in the conventional OTS-infected cell-based IFA.

**Comparison of rTSA-expressed cells and OTS-infected cells in serological diagnosis**

Based on the criteria for serological diagnosis, such as the suitable cutoff for single serum (IgM ≥ 1:160) and/or significant increase in IgG titer between acute and convalescent phases ≥ 4 times for paired sera, all 15 of the scrub typhus-patients diagnosed as positive by OTS-infected cell-based IFA were also diagnosed as positive by the rTSA-based IFA, whereas all 10 rickettsiosis-patients and 10 control individuals were not (Table 2).
Discussion

In the present study, we developed the recombinant proteins of the major
immunodominant protein of OTS and evaluated them as the antigen for serological
diagnosis as an alternative to the conventional antigen, i.e., OTS-infected cells.
The core regions of the TSAs of the five major OTS strains presently found in Japan
were successfully expressed using a baculovirus system. In the system, expressed
proteins are sometimes modified in the insect cells (17), which may influence the
antigenicity of a protein (18, 19). However, in the present study, rTSA was likely
unmodified, because the size of the rTSA was very similar to the computed size
according to the amino acid sequence of the corresponding region of the TSA. In
addition, the immunoreactivity of the rTSA was highly conserved, as demonstrated by
the strong reaction of anti-OTS sera to rTSA, seen in the western blot analysis and IFA.
These results suggested that rTSA was a highly competent antigen for serological
testing of scrub typhus.

In previous reports, E. coli-systems were used for the expression of TSAs (7-9, 20). In
this system, expressed protein must be generally purified before using it as an antigen in
some serological diagnostic tests, such as enzyme-linked immunosorbent assays
(ELISA). In the present study, the recombinant baculovirus-infected cells that expressed
rTSA were directly used as antigens for IFA. Antibody titers of IgM and IgG in the rTSA-based IFA had a good correlation to those of the conventional OTS-infected cell-based IFA. Furthermore, antibody titers against the five individual strains used in the present study showed a good correlation between the rTSA-based and the conventional OTS-infected cell-based IFA. In addition, serological diagnosis based on the antibody titers with the rTSA-based IFA using the paired sera corresponded entirely to those based on the conventional OTS-infected cell-based IFA. These results clearly suggested that the rTSAs are efficacious in the serological diagnosis of scrub typhus. The suitable IgM-cutoff value of the rTSA-based IFA was slightly higher than that of the conventional OTS-infected cell-based IFA. This fact probably suggested that we should optimize the cutoff value by more numbers of patient serum to avoid false positive cases in future study. Generally, for antigens used in serological diagnosis, it is recommended that a current strain prevalent in a certain area should be used, as the serum-antibodies of a patient sometimes only increase against a certain serotype and not others. In the future, the rTSA can potentially be applied for detecting multiple strains, both in Japan and globally where infection is endemic.

*Orientia tsutsugamushi* is classified as a BSL-3 pathogen. Biosafety level-3 laboratories are not available in some institutes and companies, potentially limiting the development
of diagnostic kits for scrub typhus. However, while OTS-infected cells require BSL-3 laboratories, the rTSA can be prepared in BSL-2 laboratories. Additionally, the recombinant baculovirus is cultured at room temperature, negating the use of special apparatus such as incubators. The rTSA developed in the present study can be readily purified because of the inclusion of a His-tag. While it is somewhat difficult to purify OTS through ultracentrifugation in BSL-3 laboratories, the rTSA developed in this study can be easily used in various serological diagnostic tests, such as ELISA and immunochromatography. To conclude, our results suggest high diagnostic potential of the rTSA in the serological diagnosis of scrub typhus.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee for Medical and Health Research involving Human Subjects of the National Institute of Infectious Diseases (474: January 16, 2014). The Ethics Committee waived the need for informed consent from the patients who provided the biological samples used in this study.

Consent for publication

Not applicable.
Conflict of interest

None to declare.

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Legends of figures

Figure 1 Expression of recombinant type specific antigens (rTSA) of the five endemic strains in recombinant baculovirus-infected sf-9 cells

A) Western blot analyses were performed as previously described (10, 11), using a) anti-
His-tag monoclonal antibody (014-23221; FUJIFILM Wako Pure Chemical Industries, Ltd., Osaka, Japan) and b) rabbit-anti-Orinetia tsutsugamushi (OTS) serum. B) Immunofluorescent assays (IFA) were performed as previously described (12, 13), using guinea pig-anti-OTS serum.

**Figure 2 Comparison of rTSA-expressed cells and OTS-infected cells in IFA**

Correlation of IgM and IgG antibody titers in paired sera (A), and in the individual strains of OTS (B). Correlation between the antibody titers against rTSAs and OTS-infected cells were analyzed by linear regression analysis, and the coefficient of determination ($R^2$) was calculated using Excel software (Microsoft Corporation, WA, USA). The coefficient of correlation was considered strong when $R^2 > 0.7$.

**Figure 3 Evaluation of IgM-cutoff value by receiver operating characteristic analysis (ROC)**

A suitable cutoff value of IgM titer against rTSAs was evaluated by ROC (16) using Bell curve software based on Excel software (SSRI Co., Ltd. Tokyo, Japan). TPF (Y axis) and FPF (X axis) mean true and false positive fraction respectively. The point
showing the highest TPF and the lowest FPF was decided as the optimal cutoff value and indicated by an arrow.
Table 1 Size of core region of TSA and expected molecular weight of the expressed protein

| Strain   | Core region of TSA (AA) | Expected molecular weight of expressed protein* (kDa) |
|----------|-------------------------|-----------------------------------------------------|
| Kato     | 1107 (369)              | 43.8                                                |
| Karp     | 1119 (373)              | 44.6                                                |
| Gilliam  | 1092 (364)              | 43.7                                                |
| Kuroki   | 1116 (372)              | 44.3                                                |
| Kawasaki | 1089 (363)              | 43.7                                                |

* Expected molecular weight of expressed protein was calculated for the core region of TSA combined with the linker including 6 x His tag at N-terminus (MSY YHH HHH HDY DIP TTE NLY FQG ITS LYK KAG S) by Compute pI/Mw tool, which was provided by bioinformatics resource portal, ExPASy.

TSA: type specific antigen, NA: nucleic acid, AA: amino acid
Table 2 Serological diagnosis using rTSA antigens compared to OTS infected cells

|                | rTSA-expressed cells | OTS-infected cells |
|----------------|----------------------|--------------------|
|                | Single serum<sup>a</sup> | Paired sera<sup>b</sup> | Judgement<sup>b</sup> | Single serum | Paired sera | Judgement |
| OTS patients   | 12/15                | 14/15              | 15/15              | 14/15        | 13/15       | 15/15     |
| Other rickettsiosis patients | 0/10               | 0/10               | 0/10               | 0/10         | 0/10        | 0/10      |
| Control group  | 1/10                 | 0/10               | 0/10               | 1/10         | 0/10        | 0/10      |

<sup>a</sup> Serological diagnosis using 1st serum by the cutoff value 1:80 for OTS-infected cells and 1:160 for rTSA antigens

<sup>b</sup> Serological diagnosis using paired sera by 4 times or more elevation of IgM or/and IgG antibody titer

<sup>c</sup> Judgement is the overall serological diagnosis both by the results using single serum and paired sera.

rTSA: recombinant type specific antigen, OTS: Orinetia tsutsugamushi
A

\[ a ) \]

\[ \begin{array}{cccccc}
Kt & Kp & G & Kr & Kw & C \\
\end{array} \]

\[ \text{Image 1} \]

\[ \begin{array}{cccccc}
Kt & Kp & G & Kr & Kw & C \\
\end{array} \]

\[ \text{Image 2} \]

B

\[ \text{B) Kato Karp Gilliam Kuroki Kawasaki Uninfected cells (C)} \]

\[ \text{Image 3} \]

\[ \text{Image 4} \]

\[ \text{Image 5} \]

\[ \text{Image 6} \]
IFA antibody titer using OTS-infected cells (2^n x 10)

**IgM**

- 1st sera
- 2nd sera
- Linear approximation of 1st sera
- Linear approximation of 2nd sera

- $R^2 = 0.7319$
- $R^2 = 0.7879$

**IgG**

- 1st sera
- 2nd sera
- Linear approximation of 1st sera
- Linear approximation of 2nd sera

- $R^2 = 0.7504$
- $R^2 = 0.7956$
rTSA-Kato

rTSA-Karp

rTSA-Gilliam

rTSA-Kuroki

rTSA-Kawasaki

All

IFA antibody titer using OTS-infected cells (2^n x 10)

IFA antibody titer using rTSA-expressed cells (2^n x 10)

IFA antibody titer using rTSA-expressed cells (2^n x 10)

IFA antibody titer using rTSA-expressed cells (2^n x 10)

IFA antibody titer using rTSA-expressed cells (2^n x 10)

IFA antibody titer using rTSA-expressed cells (2^n x 10)
