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Serum Proteomic Analysis of Scrub Typhus Patients for Screening Antigenic Proteins Originating from *Orientia tsutsugamushi*

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Abstract : Scrub typhus is an acute febrile disease caused by the pathogenic bacterium *Orientia tsutsugamushi*, belonging to the Rickettsiaceae family. The shotgun proteomic analysis was performed using the sera of scrub typhus patients to identify the proteins having their origin in *O. tsutsugamushi*. Three different databases approaches were used for the identification of the proteomes. We identified the RsmD, an RNA methyltransferase as the commonly detected protein from all three approaches. This protein was not detected in the sera of healthy negative controls. We believe that this protein is a potential biomarker of *Orientia tsutsugamushi* present in the sera of scrub typhus patients.

Keywords : Scrub typhus, *Orientia tsutsugamushi*, proteomic analysis, serum

Introduction

Orientia tsutsugamushi is a pathogen that causes scrub typhus. Scrub typhus is an infectious disease (mortality rate: approximately <1%–50%) affecting the people residing in East Asia (the 'tsutsugamushi triangle' region; Korea, China, and Japan).[1] Because of high mortality, early POCT (point of care test) of Scrub typhus is important. However, a clinical investigation is a routine method for early diagnosis of suspected patients, which is relatively inaccurate and highly subjective depending on clinicians. In general, molecular diagnostics (PCR method) or serology tests (ELISA) are considered to be the 'gold standard' methods, but these can be performed only in well-organized laboratories.[2,3] Therefore, it is important to develop simple and accurate diagnostic methods. For this purpose, screening of novel biomarkers of *O. tsutsugamushi* had been performed in the proteomic research field.[4-6] These potential biomarkers can be used directly for the test of antiserum of Scrub typhus patients or used for the production of antibodies, which can be used for the detection of *O. tsutsugamushi* in the clinical samples of Scrub typhus patients.

We used the shotgun proteomics method for screening the novel *O. tsutsugamushi* biomarkers in the serum samples of scrub typhus patients. There are various challenges that need to be overcome during the direct detection of proteins that have their origin in pathogens. Numerous proteins (such as albumin, globulins, and fibrinogen) are present in high concentrations in the samples collected from patients. The high-concentration proteins can potentially mask the low copy number proteins (including proteins of *O. tsutsugamushi* present in clinical samples), making their detection difficult. Therefore, removing step of the abundant proteins can be helpful but more or less the loss of low copy proteins including pathogen-originated proteins is inevitable. It is also difficult to optimize the tandem mass spectrometry (MS/MS) spectral analysis method. It is important to efficiently utilize the proteomic databases for the accurate identification of the pathogen-originated proteins. We tried three different methods to accurately identify and verify the accuracy of the results to identify the proteins present in *O. tsutsugamushi*. We concluded that the RsmD family RNA methyltransferase, present in the clinical samples (sera) of scrub typhus patients, was a potential biomarker of scrub typhus.

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Experimental

Patient information and preparation of clinical serum samples for proteomic analysis

Information on the patients and the protocols followed for the preparation of clinical samples have been previously reported.[7] In brief, blood serum samples were isolated from three scrub typhus patients who were being treated in the Chonbuk national university hospital. The samples were collected before the patients were performed antibiotic treatment. A positive IgM titer against *O. tsutsugamushi* was confirmed. Three normal subjects were selected as the healthy negative controls.

Proteomic analysis using liquid chromatography with tandem mass spectrometry

Proteomic analysis was performed following previously reported protocols.[7] Clinical serum samples were applied into the ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit (Sigma-Aldrich, St. Louis, MO, USA) to remove albumin and IgG. Following this, the prepared protein samples were separated using 10% Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE). The samples were then subjected to the process of tryptic digestion. The tryptic peptide samples were loaded onto an Acclaim PepMap RSLC C18 reverse-phase column (10 cm × 75 μm ID, Thermo Scientific) at a flow rate of 300 nL/min. All MS and MS/MS spectra were generated using the Q Exactive Plus Orbitrap mass spectrometer. MS/MS spectra were searched in three approaches using MASCOT v2.6 (Matrix Science, Inc., Boston, MA, USA) for protein identification. The search parameters for identification of proteins were as followed: maximum missed cleavages:2; carboxymethyl (C) as a fixed modification; oxidation (M) as variable modifications; a precursor mass tolerance of 10 ppm; and a MS/MS mass tolerance of 0.8 Da. The second approach were identified proteins using two steps in MASCOT program. First, Human database was applied as reference database. Among them, the spectra with probability that match is random > 1 in 20 were used for identifying *O. tsutsugamushi* derived proteins.

Results and Discussion

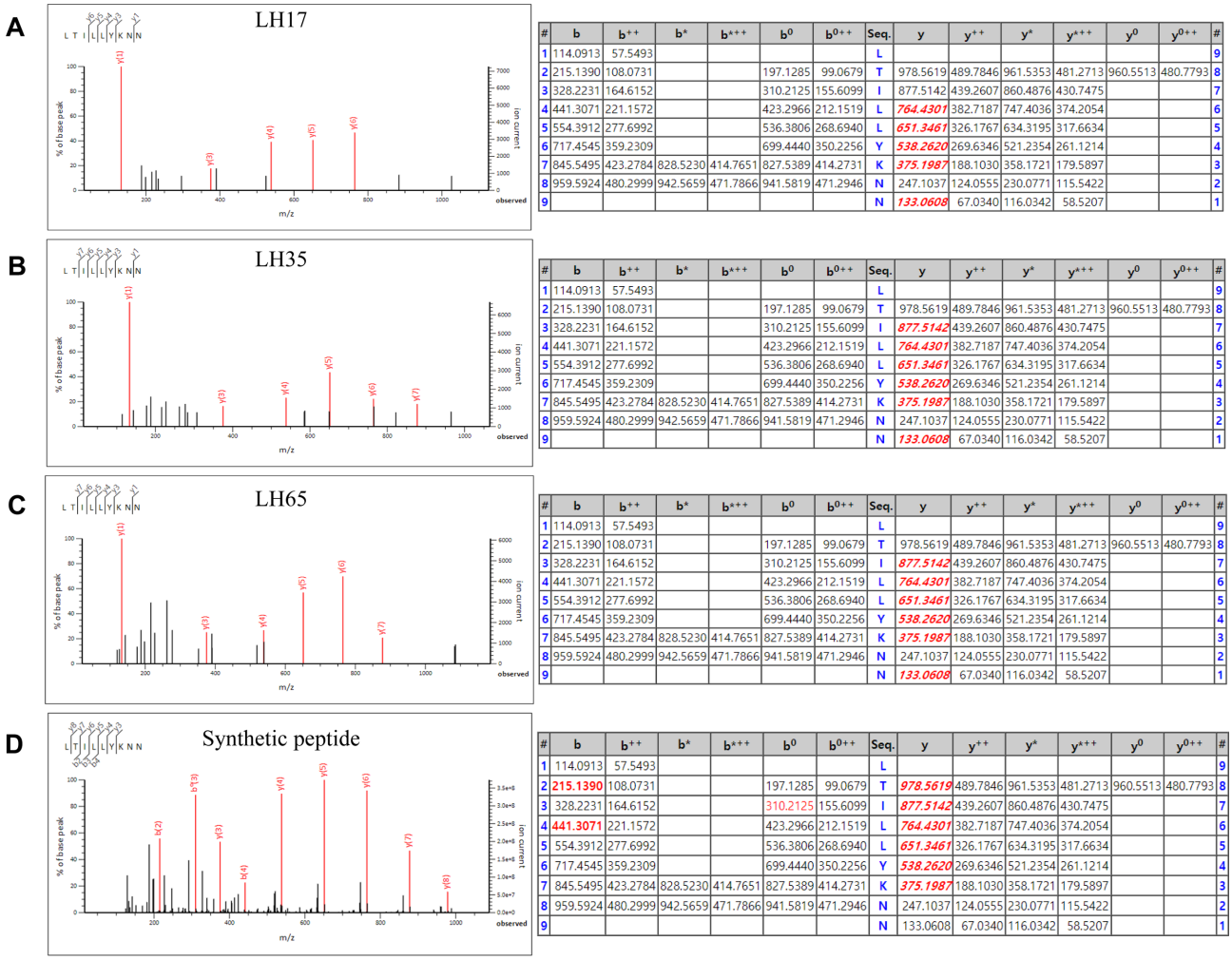
The serum proteomics using the sera of three patients of scrub typhus was performed. As first approach for MS/MS spectrum analysis for the protein identification of pathogen, the genome database of *O. tsutsugamushi* strain Boryong (Accession number: NC_009488.1) was applied as the reference database.[8] From each sample, 109~199 tryptic peptides and 24~27 proteins of *O. tsutsugamushi* were identified (Table 1). However, as the major serum proteins originate from human proteins, the use of the *O. tsutsugamushi* strain Boryong as the sole reference genome for protein identification can potentially result in the generation of inaccurate data during spectral analysis. Therefore, we applied the Uniprot human reference proteome (Proteome ID: UP000005640) as well as genome data of *O. tsutsugamushi* as second approach for MS/MS spectrum analysis. All MS/MS spectra that matched with

Table 1. Summary of serum proteomic results of scrub typhus patients.

Patient name	LH17	LH35	LH65
First approach	**Database of *O. tsutsugamushi* str. Boryong**		
p-value	0.005234	0.005623	0.00524
FDR	1	0.997	0.999
matched peptide	133	109	199
Proteins	24	27	27
Sum of emPAI	2.81	2.87	2.88
Second approach	**Step1, Human genome database; Step 2, Database of *O. tsutsugamushi* str. Boryong**		
p-value	0.0005199	0.0004325	0.0001327
FDR	1.408	1.176	1.136
matched peptide	71	85	88
Proteins	1	2	2
Sum of emPAI	0.15	0.17	0.48
Third approach	**Human genome database and Database of *O. tsutsugamushi* str. Boryong**		
p-value	0.005099	0.006606	0.005899
FDR	0.999	0.998	1
matched peptide	38339	39071	38117
Proteins	3	2	3
Sum of emPAI	0.5	0.48	0.5

Table 2. Representative *Orientia tsutsugamushi* proteins identified from three different approaches.

	Accessions	Description	Control #1	Control #2	Control #3	LH17	LH35	LH65
First approach	WP_011944465.1	**RsmD family RNA methyltransferase**	-	-	-	0.15	0.15	0.15
	WP_011945013.1	acyl-[ACP]--phospholipid O-acyltransferase	-	-	-	0.02	0.02	0.02
	WP_011945103.1	50S ribosomal protein L27	-	-	-	0.33	0.33	0.33
Second approach	WP_011944465.1	**RsmD family RNA methyltransferase**	-	-	-	0.15	0.15	0.15
	WP_011945013.1	acyl-[ACP]--phospholipid O-acyltransferase	-	-	-	-	0.02	-
	WP_011945103.1	50S ribosomal protein L27	-	-	-	-	-	0.33
Third approach	WP_011944465.1	**RsmD family RNA methyltransferase**	-	-	-	0.15	0.15	0.15
	WP_011945013.1	acyl-[ACP]--phospholipid O-acyltransferase	-	-	-	0.02	-	0.02
	WP_011945103.1	50S ribosomal protein L27	-	-	-	0.33	0.33	0.33



#	b	b++	b*	b*++	b0	b0++	Seq.	y	y++	y*	y*++	y0	y0++	#
1	114.0913	57.5493					L							9
2	215.1390	108.0731			197.1285	99.0679	T	978.5619	489.7846	961.5353	481.2713	960.5513	480.7793	8
3	328.2231	164.6152			310.2125	155.6099	I	877.5142	439.2607	860.4876	430.7475			7
4	441.3071	221.1572			423.2966	212.1519	L	764.4301	382.7187	747.4036	374.2054			6
5	554.3912	277.6992			536.3806	268.6940	L	651.3461	326.1767	634.3195	317.6634			5
6	717.4545	359.2309			699.4440	350.2256	Y	538.2620	269.6346	521.2354	261.1214			4
7	845.5495	423.2784	828.5230	414.7651	827.5389	414.2731	K	375.1987	188.1030	358.1721	179.5897			3
8	959.5924	480.2999	942.5659	471.7866	941.5819	471.2946	N	247.1037	124.0555	230.0771	115.5422			2
9							N	133.0608	67.0340	116.0342	58.5207			1

Figure 1. The spectra of tryptic peptide 'LTILLYKNN' of RsmD family RNA methyltransferase of *Orientia tsutsugamushi*. These spectra were collected from three scrub typhus patients and synthetic peptide. A; LH17, B; LH35, C; LH65, D; synthetic peptide.

the human database were filtered out and the remaining MS/MS spectra were applied to the *O. tsutsugamushi* database. In this approach, 2~3 proteins were identified from each sample (Table 1). Finally, two databases (the *O. tsutsugamushi* strain Boryong database and human database) were simultaneously applied for the MS/MS spectrum analysis and similar results were obtained (Table 1). The proteins of *O. tsutsugamushi*, identified in scrub

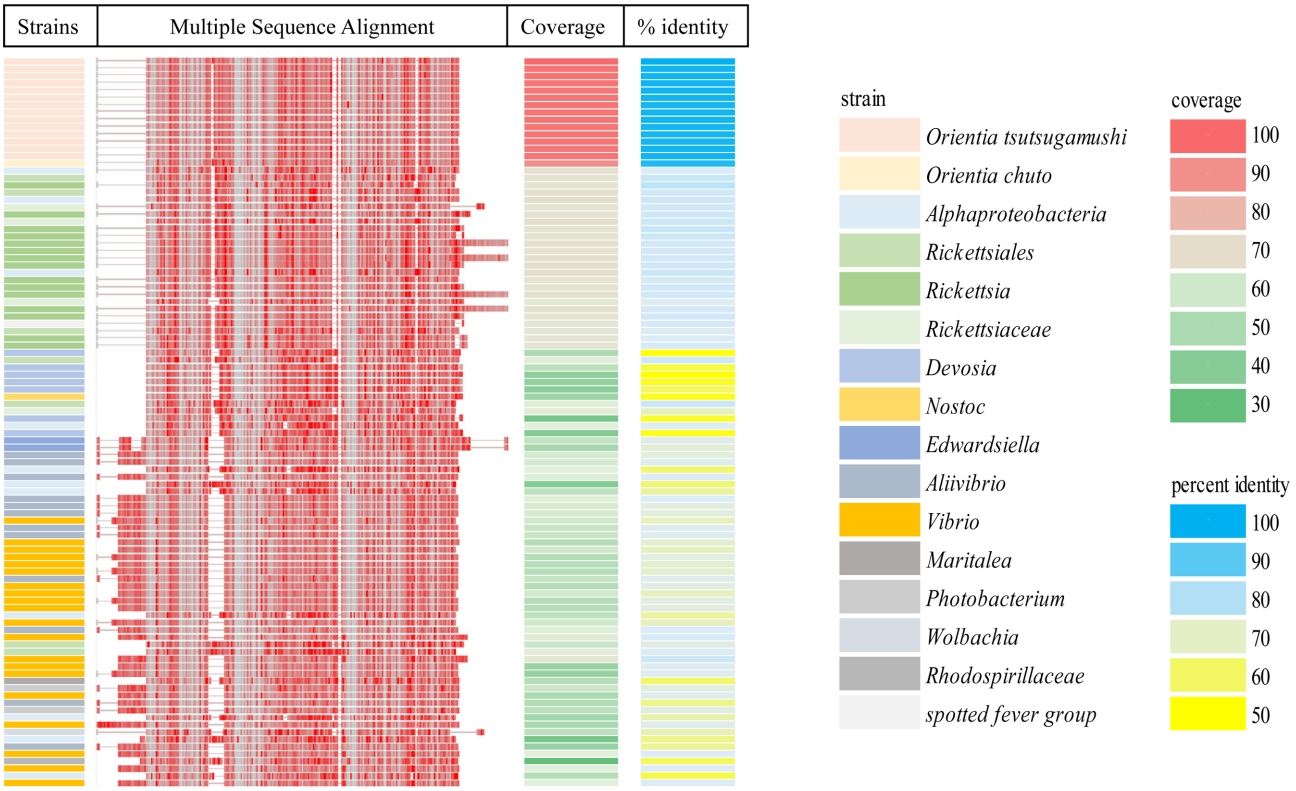


Figure 2. Sequence homology analysis of RsmD family RNA methyltransferase of *Orientia tsutsugamushi* with other pathogenic bacteria. Sixteen different bacterial categories and 53 bacterial strains were compared.

typhus patients, were not detected in the sera of the negative control samples (i.e., in samples collected from healthy people). Among the identified proteins, RsmD family RNA methyltransferase was found in the samples of all the patients. The presence of the RsmD family RNA methyltransferase was also confirmed from the results obtained using the three MS/MS analysis methods (Table 2). The C-terminal peptide 'LTILLYKNN' of RsmD family RNA methyltransferase was detected in all the patient samples (Figure 1). The spectral peak of 'LTILLYKNN' was confirmed using a synthetic peptide. Results obtained from the sequence homology analysis revealed that the RsmD family RNA methyltransferase had been conserved in *O. tsutsugamushi*. Except for *O. tsutsugamushi* and *Rickettsia*, the similarity to other species was less than 70% on coverage and percent identity. (Figure 2). A similar sequence was not found when the human reference data were analyzed. Based on these results, we concluded that the RsmD family RNA methyltransferase could be a potential biomarker of scrub typhus in the clinical samples (sera) of patients.

Conclusions

The prevalent diagnosis method for the diagnosis of scrub typhus is detection of seroconversion in the sera of scrub typhus patients using serological immunoassays such as immunofluorescence assay or ELISA.[2] The antibody of the 56-kDa protein, one of the outer membrane proteins, is the major target.[1] However, because one or two weeks are needed for the production of the antibodies, the serological test has the limit for the early detection of scrub typhus patients. To overcome this limit, various antigen detection methods have been applied. However, it is difficult to directly detect the antigenic proteins of *O. tsutsugamushi* as they are present in low amounts in scrub typhus patients. We used the highly sensitive proteomic methods for screening the antigenic proteins in the sera of Scrub typhus patients. The results revealed that RsmD family RNA methyltransferase is a potential biomarker protein. Further studies with more samples from scrub typhus patients should be conducted to understand the clinical significance of this protein and screen more candidate proteins.

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