Microarray of surface-exposed proteins of *Rickettsia heilongjiangensis* for serodiagnosis of Far-eastern spotted fever

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**Abstract**

**Background:** Far-eastern spotted fever (FESF) is an important emerging infectious disease in Northeast Asia. The laboratory diagnosis of FESF in hospitals is mainly based on serological methods. However, these methods need to cultivate rickettsial cells as diagnostic antigens, which is both burdensome and dangerous.

**Methods:** Eleven surface-exposed proteins (SEPs) were identified in our previous study and their recombinant proteins (rSEPs) fabricated on a microarray were serologically analyzed with seventeen paired sera from patients suffered from FESF in this study.

**Results:** All the rSEPs showed sensitivities of between 53% and 82% to acute-phase sera and of between 65% and 82% to convalescent-phase sera, and all the rSEPs except rRplA showed specificities of between 80% and 95%. The combination assay of two, three, or four of the four rSEPs (rOmpA-2, rOmpB-3, rRpsB, and rSdhB) showed better sensitivities of between 76% and 94% to the acute-phase sera or between 82% and 100% to the convalescent-phase sera and acceptable specificities of between 75% and 90%.

**Conclusions:** Our results suggest that the four rSEPs are more likely candidate antigens for serological diagnosis of FESF.

**Keywords:** Far-eastern spotted fever, *Rickettsia heilongjiangensis*, Protein microarray, Serological diagnosis

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conorii [10], in which, many surface-exposed proteins (SEPs) like Adr2, Omp1, PLD, RickA, Sca1, Sca10, and Sca13 were used. In fact, many SEPs have been found to be suitable as diagnostic antigens, such as a 56 kDa outer membrane protein in detection of Orientia tsutsugamushi infection [11,12] and a surface protein Pap31 in detection of Bartonella bacilliformis infection [13]. These findings indicate that SEPs are more likely to be diagnostic candidates. In our previous study, 24 SEPs of R. heilongjiangensis were identified and their recombinant proteins (rSEPs) fabricated on a microarray were serologically analyzed and eleven of them were recognized as major seroreactive proteins and potential candidate antigens for serological diagnosis of FESF by sera from mice experimentally infected with R. heilongjiangensis [9]. Also, these rSEPs in microarray assay showed a higher specificity in recognizing R. heilongjiangensis-infected mouse sera compared with that in ELISA [9]. In the present study, these rSEPs fabricated on a protein microarray were assayed with paired sera from FESF patients during the acute and convalescent phase.

Methods
Patient sera
FESF was diagnosed in patients by PCR using whole blood [14] as well as clinical symptoms consistent with tick-bite fever, multiple inoculation eschars and cutaneous rash in hospital. IgG antibody titers of patient sera were determined by IFA with R. heilongjiangensis antigen as described previously [9]. Each case of FESF was confirmed by a single serum with the specific IgG titer of ≥1:128 or the paired sera with a fourfold or greater increase of the specific IgG titers. The paired sera were collected from 17 patients suffered from FESF during the acute and convalescent phase. The acute-phase sera were collected from the patients at the date of onset of illness, and the convalescent-phase sera were collected from the same patients approximately two weeks after the first sampling. Also, 20 sera, collected from acute febrile patients with uncertain diagnoses and their titers of IgG antibodies to R. heilongjiangensis being determined to be less than or equal to 1:8 in IFA, were used as negative control or as reference sera to assess diagnostic specificity of the microarray assay in this study.

All of the patient sera were obtained from a hospital in northeast China. The serum samples of patients were collected as part of the routine management of patients without any additional sampling. All patients gave their informed consent and all patient data were deidentified. The Institutional Review Board of the Beijing Institute of Microbiology and Epidemiology approved the research involving human materials.

Preparation of recombinant proteins
Eleven rSEPs of R. heilongjiangensis, including rGroEL, rOmpA-2, rOmpB-3, rPrsA, rRplA, rRplY, rRpsB, rSdhB, rSurA, rYbgF, and rRho54_02285, were used in the present study. The preparation and purification of these recombinant proteins were described in our previous study [9,15].

Immunoblotting assay
The purified rSEPs were immunoblotted with the paired sera from one patient with FESF. Briefly, rSEPs separated by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked with 1% [w/v] bovine serum albumin (BSA) in phosphate buffer saline (PBS, containing 8.1 mM Na2HPO4, 1.9 mM NaH2PO4, and 154 mM NaCl) at pH 7.4 overnight. Then, rSEPs on the PVDF membrane were incubated with the acute- or convalescent-phase serum (1:250 dilution) that was previously neutralized with E. coli lysate (5 mg/ml) for 1 h. After three washes in PBST (pH 7.4 PBS containing 0.05% [v/v] Tween 20), the PVDF membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (1:5 000 dilution; Beijing CoWin Biotech, Beijing, China) for 1 h. Following an additional three washes in PBST, the PVDF membrane was developed using a diaminobenzidine (DAB) kit (Boster, Wuhan, China).

Fabrication of protein microarray
Each of the purified rSEPs diluted in PBS to a concentration of 0.3 mg/ml was printed on epoxy slides (CapitalBio, Beijing, China) in 5 replicate spots as described previously [16]. Human IgG with serial dilutions (2.5, 5, 10 and 20 μg/ml) was used to fit the internal calibration curves or as positive controls. BSA in PBS or lysate of E. coli cells transformed with pET-32a plasmids at a concentration of 0.3 mg/ml was used as negative controls [16]. For quality control, the microarray slides were incubated with mouse anti-His tag IgG-Cy5 (SBA, Birmingham, AL) and the fluorescence intensity (FI) of each protein on the slides was scanned by GenePix Personal 4100A (Molecular Devices, Sunnyvale, CA) for 1 h. Following an additional five washes in PBST, the PVDF membrane was developed using a diaminobenzidine (DAB) kit (Boster, Wuhan, China).

Analysis of proteins on microarrays by patient sera
The rSEPs on the microarray slide were probed using patient sera according to previous descriptions [9]. Briefly, the microarray slide was blocked with 1% [w/v] BSA in PBS overnight. Then each well on the slide was incubated with mouse anti-His tag IgG-Cy5 (SBA, Birmingham, AL) for 1 h. Following an additional five washes in PBST and a final wash in deionized water, the air dried microarray slide was scanned with a GenePix

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Personal 4100A scanner and the scanned images were analyzed by GenePix Pro 6.0. The FI value of each protein was calculated by averaging the FI values of five replicate spots, which had been background-subtracted [9].

Microarray data analysis
Human IgG dose-FI value curves were fitted with linear regression analysis using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). The relative amounts of specific IgG (RASIgG) to individual rSEPs in each serum were determined by interpolating the calculated FI value with the IgG internal calibration curve [17].

The cutoff value for individual rSEPs in the microarray assay was generated as described previously using Youden’s index [13]. The reaction was considered positive if the RASIgG to one rSEP in any of the patient sera was higher than the cutoff value.

Statistical analysis
The log-transformed IgG titers to R. heilongjiangensis in IFA and the numbers of rSEPs recognized by FESF patient sera in microarray assay were analyzed for potential correlations by linear regression using GraphPad Prism 5 software.

The IgG titer of the convalescent-phase serum divided by the IgG titer of the acute-phase serum was calculated as the increased titer for each paired sera. The increase in RASIgG to each rSEP in each paired sera was calculated as follows: The increased RASIgG = RASIgG to one rSEP in the convalescent-phase serum/RASIgG to the same rSEP in the acute-phase serum.

In paired sera, the correlation between the increase in IgG titers (log transformed) to R. heilongjiangensis and the increase in RASIgG to individual rSEPs was analyzed by linear regression using GraphPad Prism 5 software.

Results
IgG titers of sera determined by IFA
The IgG titers of 17 paired sera from FESF patients were determined by IFA with R. heilongjiangensis antigen. Fourteen of these paired sera showed a fourfold or greater rise in specific IgG titers and the other 3 paired sera showed high specific IgG titers greater than or equal to 1:128 (Table 1).

Immunoblotting assay
Eleven rSEPs of R. heilongjiangensis were immunoblotted by the acute- or convalescent-phase serum from one of FESF patients (patient 8 in Table 1). As shown in Figure 1, seven rSEPs (rGroEL, rOmpA-2, rRh054_02285, rRplA, rRpsB, rSurA, and rYbgF) were recognized strongly by both acute- and convalescent-phase serum and the rest were recognized weakly. Seven rSEPs (rGroEL, rOmpA-2, rRpsA, rRplA, rRpsB, rSdhB, and rYbgF) showed a stronger staining reaction with the convalescent-phase serum than with the acute-phase serum.

Quality control of protein microarray
For quality control, the microarray slides printed with rSEPs were incubated with mouse anti-His tag IgG-Cy5 (SBA, Birmingham, AL) and scanned for their FI values. The coefficient of variations (CV) was calculated as the SD of the FI value for each SEP divided by the average FI value. As a result, the within-slide CV (n = 6) and between-slide CV (n = 6) of individual rSEPs on the microarray ranged from 8% to 18%.

Sensitivity and specificity of rSEPs in microarray assay
Internal calibration curve of the microarray was generated by probing a serial dilution of human IgG solution with goat-anti human IgG-Cy5 and the FI value of BSA probed with goat-anti human IgG-Cy5 was set as the first point of the internal calibration curve. Linear regression analysis revealed that all the calibration curves gave the good correlation coefficients (r²) ranging from 0.967 to 0.997 (Figure 2).

Eleven rSEPs on the microarray were probed with the patient sera. As a result (Table 2), all the rSEPs showed sensitivities of between 53% and 82% in recognizing acute-phase sera and of between 65% and 82% in recognizing convalescent-phase sera, and all the rSEPs except rRplA showed specificities of between 80% and 95%. Four rSEPs (rOmpA-2, rRplA, rRpsB, and rSdhB) showed

| Patients no. | Acute-phase serum sample | Convalescent-phase serum sample | Diagnostic criteria |
|--------------|--------------------------|-------------------------------|--------------------|
| 1            | 128                      | 512                           | Fourfold increase  |
| 2            | 64                       | 1024                          | Fourfold increase  |
| 3            | 256                      | 512                           | IgG titer ≥128     |
| 4            | 64                       | 256                           | Fourfold increase  |
| 5            | 32                       | 512                           | Fourfold increase  |
| 6            | 128                      | 1024                          | Fourfold increase  |
| 7            | 128                      | 1024                          | Fourfold increase  |
| 8            | 256                      | 512                           | IgG titer ≥128     |
| 9            | 32                       | 512                           | Fourfold increase  |
| 10           | 256                      | 256                           | IgG titer ≥128     |
| 11           | 64                       | 1024                          | Fourfold increase  |
| 12           | 64                       | 1024                          | Fourfold increase  |
| 13           | 64                       | 512                           | Fourfold increase  |
| 14           | 128                      | 1024                          | Fourfold increase  |
| 15           | 128                      | 1024                          | Fourfold increase  |
| 16           | 64                       | 512                           | Fourfold increase  |
| 17           | 64                       | 1024                          | Fourfold increase  |
The sensitivities of ≥71% in recognizing both acute- and convalescent-phase sera. Also the summary of sensitivities of each protein to both acute- and convalescent-phase sera and specificity of each protein was calculated to generally evaluate its ability as a candidate antigen for diagnosis of FESF. As a result (Table 2), four rSEPs (rOmpA-2, rOmpB-3, rRpsB, and rSdhB) scored higher than the rest.

Relationship between specific IgG titers of sera and seroreactivity of rSEPs
Linear regression analysis revealed a significant positive correlation between the log-transformed IgG titers to \( R. \) heilongjiangensis in all the patient sera and the numbers of proteins recognized by these sera (Figure 3, \( r^2 = 0.5381, P < 0.0001, n = 54 \)).

In addition, linear regression analysis revealed that significant correlations between the increase in the log-transformed IgG titers to \( R. \) heilongjiangensis for paired sera and the rising in RAS IgG to rOmpA-2 (\( r^2 = 0.2621, P = 0.0356 \)) or rRpsB (\( r^2 = 0.2838, P = 0.0277 \)) in these sera (Table 3).

### Discussion
In our previous study [9], eleven SEPs of \( R. \) heilongjiangensis were recognized as major seroreactive antigens and potential candidate antigens for serological diagnosis of FESF in microarray assay with \( R. \) heilongjiaensis-infected mouse sera. In the present study, their recombinant proteins fabricated on a microarray were

| Proteins | Sensitivity (%) | Specificity (%) | Summary (%) |
|----------|----------------|----------------|-------------|
| rGroEL   | 65             | 80             | 209         |
| rOmpA-2  | 71             | 85             | 238         |
| rOmpB-3  | 65             | 90             | 237         |
| rPrsA    | 53             | 95             | 224         |
| rRho54_02285 | 71           | 90             | 225         |
| rRplA    | 71             | 65             | 218         |
| rRplY    | 65             | 85             | 232         |
| rRpsB    | 71             | 95             | 236         |
| rSdhB    | 82             | 90             | 249         |
| rSurA    | 59             | 90             | 231         |
| rYbgF    | 65             | 90             | 219         |

*The summary of sensitivities of each protein to both acute- and convalescent-phase sera and specificity of each protein was calculated to evaluate its ability as a candidate antigen for diagnosis of FESF.
assayed with paired sera from FESF patients so as to identify potential candidate antigens for serological diagnosis of FESF, as well as to explore the kinetic change of the specific antibodies to individual SEPs in FESF patients.

Firstly, these rSEPs were immunoblotted by paired sera from one FESF patient, and most of them showed a stronger reaction with the convalescent-phase serum than with the acute-phase serum, which suggested that more specific antibodies to these SEPs appeared in the convalescent-phase serum. This could not be quantitatively determined in the immunoblotting assay. However, the reactivity of each rSEP with individual sera was quantitatively determined by the microarray assay. In addition, the FI value of each protein probed with individual sera was interpolated with the calibration curve, which minimized variability in this quantitative determination so as to improve the within-slide and between-slide analytical precision.

The individual rSEPs were analyzed by both immunoblot and microarray assay using the paired sera from patient 8. All of the rSEPs except OmpB-3 and YbgF, to which the RASIgG were higher in microarray, showed a stronger staining reaction in immunoblot assay (Figure 1B,C). The exception may be due to the different states of OmpB-3 and YbgF existing in different assays. OmpB-3 was stained very lightly in immunoblot while the RASIgG in the serum detected with microarray was big, which might be due to the tertiary structure of OmpB-3 on the microarray slide that might have exerted a steric effect to promote non-specific absorption of IgG from the sera, one effect that would not apply to the denatured OmpB-3 in the immunoblot assay. YbgF was stained strongly in immunoblot assay while the RASIgG in microarray assay was small. YbgF was denatured and might provide more epitopes to bind the specific antibodies in immuno blot assay, while it maintained its native structure and might provide less epitopes to bind the specific antibodies in the microarray assay.

In this microarray assay, only 5% to 20% of reference sera from the acute febrile patients without antibodies to *R. heilongjiangensis* reacted positively to individual rSEPs except rRplA, suggesting these rSEPs had a good specificity. The cross-reaction might be caused by the conservative SEPs such as the ribosomal protein RplA and patients from whom the reference sera were collected have suffered from other infection caused by bacteria which shared the conservative SEPs with *R. heilongjiangensis*. All of these rSEPs gave a sensitivity of over 65% to the convalescent-phase sera from FESF patients while five rSEPs (rOmpA-2, rOmpB-3, rRplA, rRplY, and rSurA) had a higher sensitivity of 82% to them. However, only rSdhB had a higher sensitivity of 82% to the acute-phase sera from FESF patients and the other rSEPs gave sensitivities of only between 53% and 71% to them.

Table 3 Linear regression analysis to examine potential relationships between the increased IgG titers to *R. heilongjiangensis* and the increased IgG level to individual rSEPs in paired sera

| Protein name | Coefficients of correlations (r²) | P value |
|--------------|----------------------------------|---------|
| rGroEL       | 0.09251                          | 0.2353  |
| rOmpA-2*     | 0.2621                           | 0.0356  |
| rOmpB-3      | 0.01372                          | 0.6544  |
| rPrsA        | 0.1329                           | 0.1502  |
| rRh054_02285 | 0.002353                         | 0.8533  |
| rRplA        | 0.06482                          | 0.3241  |
| rRplY        | 0.003742                         | 0.8156  |
| rRpsB*       | 0.2838                           | 0.0277  |
| rSdhB        | 0.005894                         | 0.7696  |
| rSurA        | 0.05963                          | 0.3449  |
| rYbgF        | 0.04017                          | 0.4405  |

*Statistically significant (P < 0.05) associations are marked.
better sensitivities of between 76% and 94% to the acute-phase sera or between 82% and 100% to the convalescent-phase sera and acceptable specificities of between 75% and 90% were obtained. Our results suggest that the remarkable variation in immune recognition patterns for FESF require multi-antigen combination to cover the different antibody responses and thus achieve the highest possible test sensitivity. Serological tests are the easiest methods for the diagnosis of rickettsiosis but seroconversion is usually detected 7–15 days after disease onset [18]. Our combination assay could recognize as many as 94% of the acute-phase sera and hopefully diagnose FESF rapidly at the early stage of infection. Therefore, the four rSEPs may be considered as more likely candidate antigens for the serological diagnosis of FESF, especially rSdhB, with its sensitivity of 82% to the acute-phase sera and 76% to the convalescent-phase sera with specificity of 90%. Furthermore, refinement of the production of fusion molecules comprised of these SEPs and the reaction conditions of microarray assay described herein may lead to improve the sensitivity and specificity for the serological diagnosis of FESF during both the acute and convalescent phase.

In conclusion, the eleven SEPs were serologically characterized with paired sera from FESF patients, and four rSEPs (rOmpA-2, rOmpB-3, rRpsB, and rSdhB) are more likely candidate antigens for the serological diagnosis of FESF. In addition, an optimized microarray composed with the four rSEPs may give an acceptable sensitivity for serological diagnosis of FESF during both the acute and convalescent phase.

### Table 4 The sensitivity and specificity of the combination assays composed of different rSEPs in recognizing the acute-phase sera (n = 17) or convalescent-phase sera (n = 17)

| Proteins                  | Sensitivity (%) | Specificity (%) |
|---------------------------|-----------------|-----------------|
|                           | Acute-phase     | Convalescent-phase |
| rOmpA-2&rOmpB-3           | 82              | 94              | 80              |
| rOmpA-2&rRpsB             | 76              | 94              | 80              |
| rOmpA-2&rSdhB             | 88              | 94              | 80              |
| rOmpB-3&rRpsB             | 82              | 94              | 90              |
| rOmpB-3&rSdhB             | 88              | 94              | 80              |
| rRpsB&rSdhB               | 82              | 82              | 85              |
| rOmpA-2, rOmpB-3&rRpsB    | 88              | 100             | 80              |
| rOmpA-2, rOmpB-3&rSdhB    | 94              | 100             | 75              |
| rOmpA-2, rRpsB&rSdhB      | 88              | 94              | 75              |
| rOmpB-3, rRpsB&rSdhB      | 88              | 94              | 80              |
| rOmpA-2, rOmpB-3, rRpsB&rSdhB | 94         | 100             | 75              |

Conclusions

In conclusion, the number of paired patient sera tested was small, which may influence the sensitivities and specificities of these rSEPs. Detection of specific IgM antibody to individual rSEPs might improve the sensitivity to acute-phase sera of FESF patients and unfortunately some paired patient sera were not enough to do this test. Therefore, it is necessary to get more serum samples of FESF patients for this microarray assay in the future.

### Competing interests

The authors declare that they have no conflict of interest.

### Authors’ contributions

YQ and WG carried out the experiments, data analyses and drafted the manuscript. XX assisted the analysis of data; JJ and YW provided the patient sera and helped to draft the manuscript; JJ and CD participated in its design and helped to draft the manuscript; BW designed the experiments and revised the manuscript. All authors read and approved the final manuscript.

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