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Insight of diagnostic performance using B-cell epitope antigens derived from triple P44-related proteins of *Anaplasma phagocytophilum*

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ABSTRACT

Human granulocytic anaplasmosis (HGA) is caused by *Anaplasma phagocytophilum*. Indirect immunofluorescence assay (IFA) is generally used for HGA serodiagnosis. *A. phagocytophilum* immunodominant P44 major outer membrane proteins are encoded by *p44/msp2* multigene family, responsible for IFA reactivity. However, because multiple P44-related proteins may involve immunoreactivity in IFA, the available diagnostic antigens remain obscure. In this study, we identified 12 B-cell epitopes on triple P44-related proteins using peptide array that reacted with 4 HGA patients' sera. Then, peptide spot immunoassay using 14 synthetic peptides derived from those 12 epitopes as antigens was applied for the detection of antibody to *A. phagocytophilum* from patients with fever of unknown origin. The sensitivities and diagnostic efficiencies of this immunoassay were higher than those of Western blot analysis using 3 recombinant proteins previously developed. Thus, the immunoassay using our epitope-derived antigens, which has higher diagnostic performances, may have significant benefit for HGA serodiagnosis.

1. Introduction

Human granulocytic anaplasmosis (HGA) is a tick-borne infectious disease caused by *Anaplasma phagocytophilum*, an intracellular bacterium in the family *Anaplasmataceae*, the order *Rickettsiales* (Bakken and Dumler, 2008). HGA is clinically characterized by fever, headache, chills, myalgia, thrombocytopenia, and elevated liver transaminase levels, and will be more severe in the absence of rapid treatment (Rikihisa, 2011). For serodiagnosis of HGA, indirect immunofluorescent assay (IFA) using *A. phagocytophilum* cultured in HL-60 cells as an antigen is usually used. *A. phagocytophilum* produces immunodominant 44-kDa outer membrane proteins (P44s) which strongly react with patients' sera (Zhi et al., 1997) and are responsible for IFA reactivity. P44s were later found to be encoded by a *p44/msp2* multigene family and characterized as protein structures consisting of a central hypervariable region flanked by N-terminal and C-terminal conserved regions (Dunning Hotopp et al., 2006; Murphy et al., 1998; Zhi et al., 1998, 1999). Our previous study has reported that HGA patients' sera in Japan reacted with *A. phagocytophilum* cultured in THP-1 rather than HL-60 cells as antigens in IFA (Ohashi et al., 2013). Subsequently, we found that P44-47E and P44-60 transcripts were predominantly expressed in *A. phagocytophilum* cultured in THP-1, while P44-18ES expression was predominant in the infected HL-60 (Gaowa et al., 2014), suggesting the production of different P44-related proteins between *A. phagocytophilum* propagated in THP-1 and HL-60 cells. Most recently, the predominant shift of different P44-expressing *A. phagocytophilum* in the cultures with different cell lines has been reported (Shimada et al., 2019). Hence, the reactivities of IFA using the different host cell lines as antigens might sometime be variable. Our previous study also showed that sera form 4 HGA patients in Japan reacted with 3 recombinant P44-related proteins (rP44-18ES, rP44-47E, and/or rP44-60)

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prepared by *in vitro* insect translation system (Gaowa et al., 2014). In this study, we performed B-cell epitope mapping on those 3 P44-related proteins (P44-18ES, P44-47E, and P44-60) to identify their amino acid sequence regions that are available as epitope-derived antigens for HGA serodiagnosis by peptide array. Then, the diagnostic performance of those epitope-derived antigens was evaluated using sera from patients with fever of unknown origin.

2. Materials and methods

2.1. B-cell epitope mapping on 3 P44-related proteins of *A. phagocytophilum*

To prepare a peptide array sheet, 594 spots (approx. 3-mm diameter of each spot) consisting of 15 amino acid residues each, frame-shifted by 1 residue covering the amino acid sequences of 3 P44-related proteins (P44-18ES, P44-47E, P44-60) from *A. phagocytophilum*, were synthesized on a cellulose membrane (IntavisAG, Bioanalytical Instruments, Nattermannallee, Germany) with a ResPep SL Automatic Peptide Synthesizer (IntavisAG, Bioanalytical Instruments, Nattermannallee, Germany) based on Fmoc chemistry (Frank, 1992). Those spots consist of 194 peptide spots from 154 to 347 amino acid positions for P44-18ES, 203 spots from 154 to 356 amino acid positions for P44-47E, and 197 spots from 17 to 213 amino acid positions for P44-60. The side-chain protecting groups were removed by treatment with trifluoroacetic acid after completion of peptide synthesis. Then, the membrane was cut to make 3 sheets for respective P44-related protein sheets. Those sheets were blocked with 5% skim milk in 20 mM Tris-buffered saline including 0.1% Tween 20 (TBS), pH 7.4, for 90 min at room temperature. After washing with TBS, the sheets were incubated for overnight at 4 °C with diluted sera (1:250) from 4 HGA patients that were previously diagnosed (Gaowa et al., 2014). After washing again, the sheets were further incubated with alkaline-phosphatase conjugated goat anti-human IgM (μ-chain specific, 1:15,000 dilution, Invitrogen; Thermo Fisher Scientific, Waltham, MA) or anti-human IgG (γ-chain specific, 1:15,000 dilution, Invitrogen) for 60 min at room temperature. Antibody-binding peptide spots were stained by enzyme reactions with BCIP/NBT substrates for 15 min (Promega, Madison, WI). After reaction, the sheets were scanned, and the reactive intensity of each spot was analyzed using ImageJ software (https://imagej.nih.gov/ij/index.html). The peptide sequences of the reactive spots identified as B-cell epitopes of 3 P44-related proteins were confirmed that there are no similar sequences other than *A. phagocytophilum* by BLAST search.

2.2. Selection of febrile patients' sera for peptide spot immunoassay with B-cell epitope-derived antigens

The sera (including paired sera) from 76 patients with fever of unknown origin (2008–2017) were obtained from 22 prefectures in Japan. The study was approved by the Ethics Committee of the University of Shizuoka (approval no. 26-34) and National Institute of Infectious Diseases (approval no. 482). We first performed IFA using both *A. phagocytophilum* cultured in THP-1 and HL-60 cells as antigens, and the sera with an antibody titer of 20 or more were selected. Western blot analysis using an insect lysate including each of 3 recombinant P44-related proteins (rP44-18ES, rP44-47E, and rP44-60) prepared by *in vitro* translation system as described previously (Gaowa et al., 2014) was further conducted for the confirmation of IFA reactivities.

2.3. Diagnostic application and performance of peptide spot immunoassay with B-cell epitope-derived antigens

The cellulose strips with the synthetic peptide spots corresponding to B-cell epitopes were prepared and tested for the detection of antibody to *A. phagocytophilum* in the sera from the febrile patients. Twenty-five normal human sera were purchased from PromedDx (Norton, MA) to use as negative controls. The cutoff value between positive and negative reactions for each spot was determined using the 25 normal human sera (the mean plus 3 standard deviations of the reactive intensity with sera from the normal humans on each spot). Positive immunoreaction was defined by binding to at least 1 peptide spot. The diagnostic performances of the peptide spot immunoassay with B-cell epitope-derived antigens were compared with those of Western blot analysis based on the IFA reactivities in a total of 80 patients tested: 76 patients with fever of unknown origin and the 4 HGA patients (Gaowa et al., 2014).

2.4. Statistical analysis

All statistical analyses including sensitivity, specificity, and diagnostic efficiency, and heatmap illustration in this study were performed using Rstudio (version 1.1.442).

3. Results

3.1. B-cell epitope mapping on 3 P44-related proteins using peptide array with HGA patients' sera

Peptide array with spots consisting of the overlapping 15 amino acid residues derived from each of 3 P44-related proteins (P44-18ES, P44-47E, P44-60) as antigens was used for the examination of immunoreactivities with sera from 4 HGA patients that have been previously diagnosed (Gaowa et al., 2014). By this epitope mapping using the array, we successfully identified 12 epitope regions on 3 P44-related proteins that well reacted with either IgM or IgG, or both in the 4 patients' sera (Fig. 1). Epitope 1 and epitope 12 were included in N-terminal and C-terminal conserved regions of P44-related proteins, respectively. Epitope 2 to epitope 4 for P44-18ES, epitope 5 to epitope 9 for P44-47E, and epitope 10 and epitope 11 for P44-60 were contained in a central hypervariable region within the respective P44-related proteins. We next prepared spot strips consisting of truncated peptides with 1 amino acid sequential deletion from 15 amino acid residues to identify suitable peptide length as antigens and examined for the reactivities of shorter-length peptides with patients' sera. In epitope 1 to epitope 11, the synthetic peptides with 15 amino acid residues than those with shorter length well reacted with the sera, whereas in epitope 12, the peptides with shorter length showed higher reactivity than those with 15 amino acid residues (their representatives shown in Fig. 2). Therefore, in epitope 12, 3 peptides with different length were prepared (Fig. 1) and used in the following experiments for the detection of antibody to *A. phagocytophilum* in febrile patients.

3.2. Diagnostic application and performance of peptide spot immunoassay with B-cell epitope-derived antigens

We first examined the sera from 76 patients with fever of unknown origin by IFA using both *A. phagocytophilum* cultured in HL-60 and THP-1 cells as antigens. The sera from 21 patients, which had IFA titers of 20 or more (highest titers of 320) selected as "potentially antibody-positive," and from the 4 HGA patients (Gaowa et al., 2014) (total 25 patients) were used for the peptide spot immunoassay using 14 epitope-derived peptides B-cell epitope-derived antigens. The reactive intensities of spots were plotted, and the cutoff value between potentially positive and negative reactions in each spot was determined in Fig. 3. Based on those cutoff values, the reactivities of the 14 peptides with the sera from case 1 to case 25 were visualized by heatmap (Fig. 4). Serological evidences of the peptide spot immunoassay show that the multiple peptides in Peptide_1 to Peptide_14 were immunoreactive in IgM, but the variety of reactive peptides was less in IgG and the certain spots had high intensities. Furthermore, Western blot analysis using an insect lysate including each of 3 recombinant P44-related proteins (rP44-18ES,

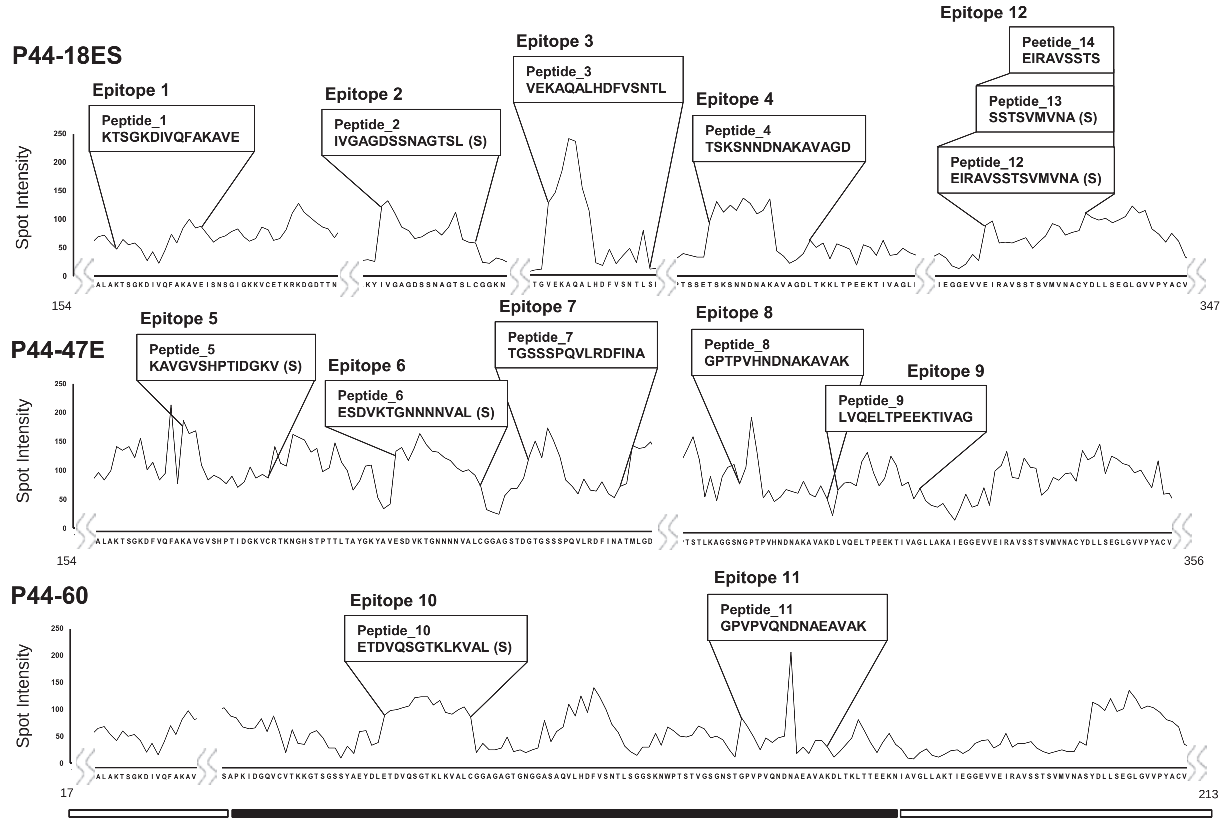

Fig. 1. Potential B-cell epitopes of 3 P44-related proteins of *A. phagocytophilum* (P44-18ES, P44-47E, and P44-60). The 12 B-cell epitopes that were identified by peptide array using the sera from 4 HGA patients previously serodiagnosed (Gaowa et al., 2014) are shown on respective P44-related protein sequences. The spot intensity of the immunoreactivities is indicated by line plots. Synthetic peptides (Peptide_1 to Peptide_14) corresponding to those epitopes that are shown by boxed amino acid sequences were prepared for the detection of antibody to *A. phagocytophilum* from patients with fever of unknown origin. The replacement of cysteine with serine residues to avoid nonspecific reaction due to disulfide bond that is usually used is shown by "S" in parenthesis. The numbers of right and left sides on the bottoms of 3 panels show amino acid sequence positions of respective P44-related proteins. Black and white bars on the bottom of each panel indicate the central hypervariable region and parts of N- and C-terminal conserved regions among P44-related proteins, respectively.

rP44-47E, and rP44-60) (Gaowa et al., 2014) was done for comparison of diagnostic performances with the peptide immunoassay using B-cell epitope-derived antigens (Supplementary Fig. 1). The sensitivities and diagnostic efficiencies of the peptide spot immunoassay were higher than those of Western blot analysis, although its specificity was a little lower (Table 1).

4. Discussion

For HGA serodiagnosis, IFA is usually used in several countries. However, as far as we know, there is no available *A. phagocytophilum* human isolate that has been used as antigens for HGA serodiagnosis in own countries other than United States to date, although the isolation of

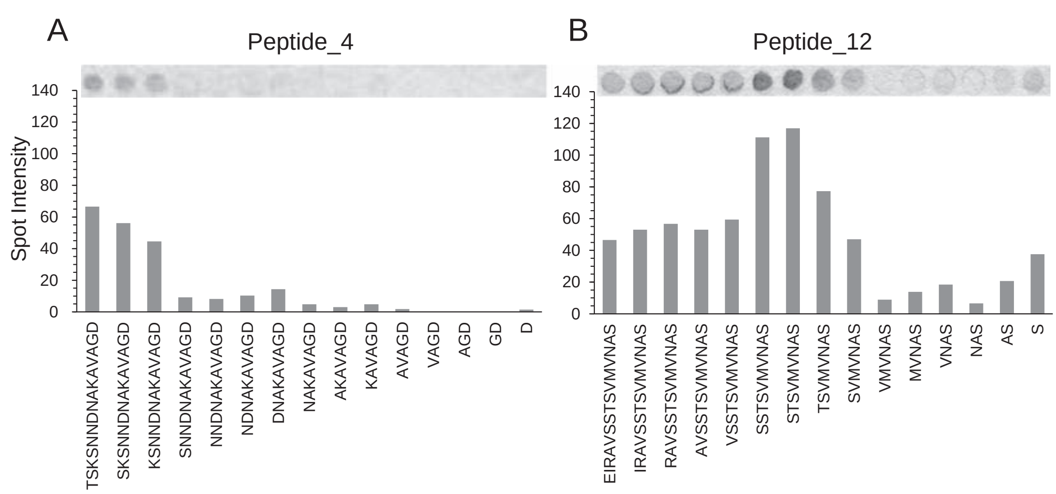

Fig. 2. Analysis of immunoreactive peptide length. For the confirmation of peptide length with the reactivity of antibody to *A. phagocytophilum*, truncated peptides that were generated by reducing the peptide length were synthesized on the cellulose membrane and tested for the antibody-positive patients' sera. The reactivities of Peptide_4 (A) and Peptide_12 (B) with the antibody-positive sera (IgM) from case 4 and case 6, respectively, are shown as representatives.

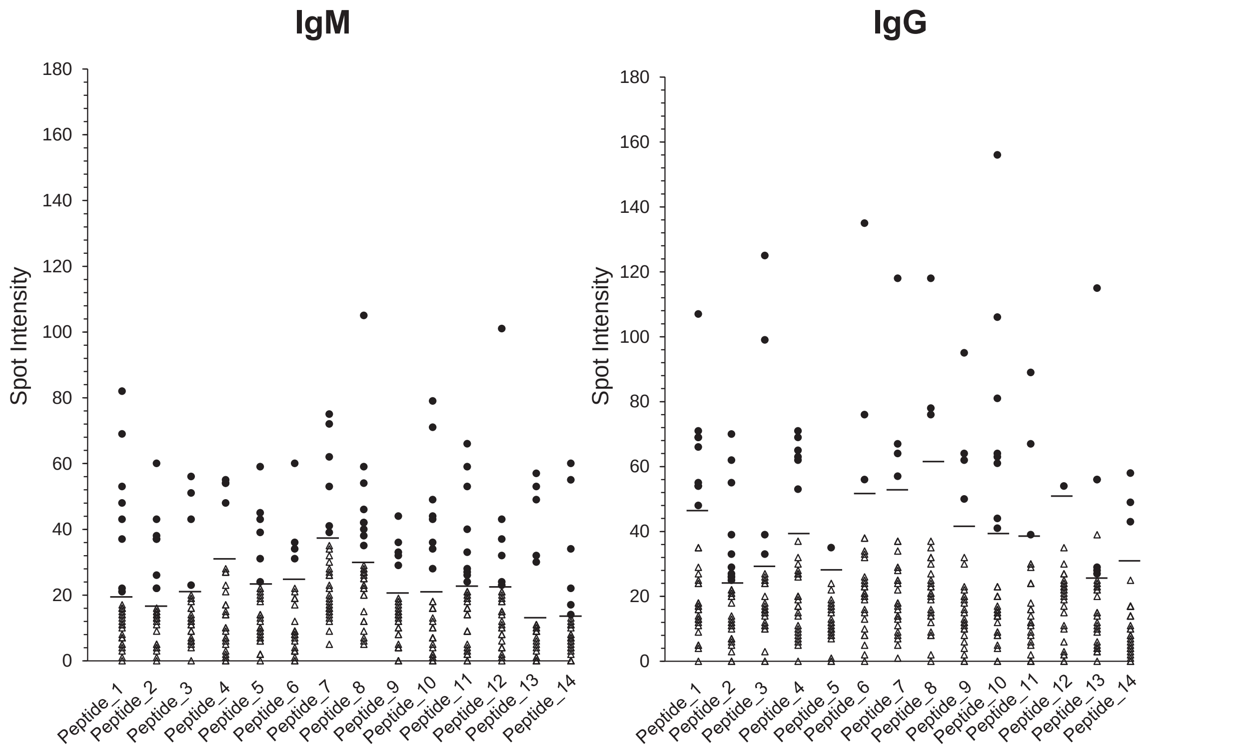

Fig. 3. Spot intensity plots of 14 peptides for specific detection of antibody to *A. phagocytophilum*. The cutoff values for the positive reaction of each peptide were obtained based on the mean plus 3 standard deviations of the intensity for the same spot in the 25 normal human sera as negative control. The peptide spots reactive to IgM and IgG from 25 febrile patients with specific antibody to *A. phagocytophilum* are plotted by closed circles, and the spots in 25 IFA-negative febrile patients as representatives are shown by open triangles. Cutoff in each spot is shown by a horizontal short bar between the positive and negative reaction.

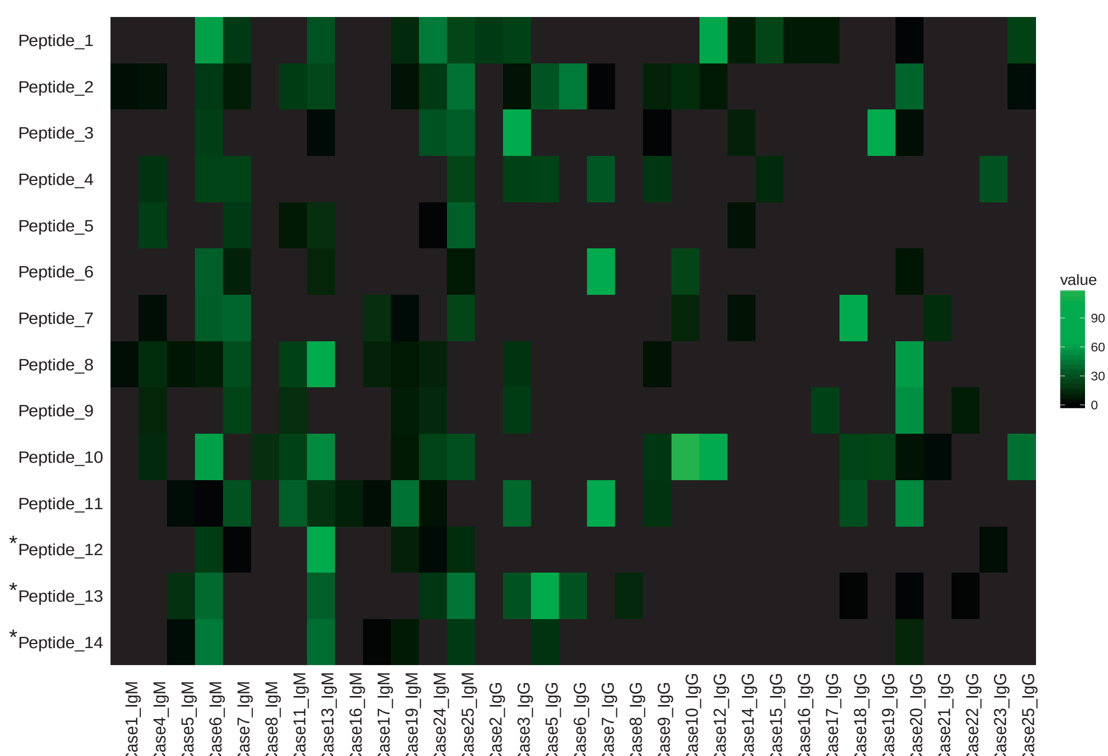

Fig. 4. Heatmap of the reactivities of peptide spots with antibody to *A. phagocytophilum* in febrile patients' sera. Heatmap is constructed based on the reactivities of 14 peptide spots (Peptide_1 to Peptide_14) with the antibody-positive sera using cutoff values in Fig. 3 from 25 febrile patients (case 1 to case 25). The immunoreaction of each spot intensity is represented by the graduation with green (the maximum value of 120) and black (the minimum value corresponding to cutoff value of each spot). Asterisks show 3 peptides derived from Epitope_12.

Table 1
Diagnostic performances of peptide spot immunoassay and Western blot analysis for the detection of antibody to *A. phagocytophilum* based on the IFA reactivities in 80 febrile patients tested.

Immunoassay	% Sensitivity[a]		% Specificity[a]		% Diagnostic efficiency[a]	
	IgM	IgG	IgM	IgG	IgM	IgG
Peptide spot immunoassay[b]	89.5	100.0	97.6	92.9	96.2	94.5
Western blot analysis[c]	51.7	40.0	98.9	100.0	87.9	85.5

[a] Formulae for the calculation of diagnostic performances are "true positive / (true positive + false negative) × 100" for sensitivity, "true negative / (true negative + false positive) × 100" for specificity, and "(true positive + true negative) / (true positive + true negative + false positive + false negative) × 100" for diagnostic efficiency.
[b] Fourteen peptide spots on a strip were used as antigens. Positive immunoreaction was defined by binding to at least 1 peptide spot.
[c] The results of Western blot analysis are indicated as positive reactions when bound by 1 of 3 recombinant P44 proteins (rP44-18ES, rP44-47E, rP44-60) prepared by *in vitro* insect translation system as described previously (Gaowa et al., 2014).

A. phagocytophilum from a human patient has been reported in South Korea (Lee et al., 2017). Presently, we use *A. phagocytophilum* US-human isolate HZ strain for IFA as an antigen. It is unknown whether the antigenicity of human pathogenic *A. phagocytophilum* in Japan is similar to that of the pathogen in United States. Indeed, most IFA titers from patients' sera in Japan seem to be lower. Such lower titers may be due to weak cross-reactivities between the pathogenic *A. phagocytophilum* in Japan and United States. Another possible reason for the low titers is due to rapid treatment of rickettsiosis-suspected patients. In Japan, Japanese spotted fever caused by *Rickettsia japonica* and scrub typhus caused by *Orientia tsutsugamushi* frequently occur (NIID, 2017). One of potential vectors is known to be *Haemaphysalis hystricis* and *H. longicornis* for *R. japonica* and *A. phagocytophilum*, respectively, and both tick species are inhabited in the rickettsioses-endemic areas, especially central and western Japan (Gaowa et al., 2013). In those endemic areas, when the persons had fever, they immediately visit clinical hospitals. If the physicians suspect rickettsioses, they treat the febrile patients with tetracycline-related medicine promptly. Hence, the rise of antibody titers to *A. phagocytophilum* in the convalescent-phase sera from HGA patients after the rapid treatment is likely unexpected. Accordingly, the immunoassay with high sensitivity for HGA serodiagnosis other than IFA has been desired in Japan.

In this study, we identified B-cell epitopes on 3 P44-related proteins (P44-18ES, P44-47E, P44-60) by peptide array and the application of their epitope-derived antigens for HGA serodiagnosis attributed to be useful for detection of antibody to *A. phagocytophilum* in sera from patients with fever of unknown origin. Indeed, the peptide spot immunoassay using epitope-derived antigens in this study had higher sensitivity and diagnostic efficiency than Western blot analysis (Gaowa et al., 2014) in the comparison based on the results of IFA as a reference standard. The reason of higher sensitivities and diagnostic efficiencies of the peptide spot immunoassay than Western blot analysis may be the larger amounts of peptide antigens in the peptide spot immunoassay than that of protein antigens prepared by *in vitro* translation system in Western blot analysis. However, the diagnostic performances showed lower specificity of peptide spot immunoassay than Western blot analysis. The numbers of false-positive sera in peptide spot immunoassay may be more than those in Western blot analysis because of the larger amounts of antigens in peptide spot immunoassay than Western blot analysis. Tokarz et al. have reported a B-cell epitope of TSGKDIVQFAKAVEIS in the conserved region of P44-related proteins (Tokarz et al., 2018). We also found a similar epitope of KTSGKDIVQFAKAVE (Peptide_1) in this study. Although the sera from 13 of 25 patients with antibody to *A. phagocytophilum* in Japan reacted with Peptide_1, the sera from the rest of patients did not. Accordingly, our 14 synthetic peptides corresponding to 12 B-cell epitope-derived antigens identified in this study are much more useful for HGA serodiagnosis with the convenient preparation of antigens.

The 3 recombinant P44-related proteins previously prepared (Gaowa et al., 2013) consisted of a central hypervariable region and parts of N-terminal and C-terminal conserved regions but not full length (truncated proteins). The central hypervariable region is suggested to be hydrophilic and highly antigenic by *in silico* analysis, probably surface-exposed on *A. phagocytophilum*, whereas the conserved regions are hydrophobic and less antigenic, probably embedded into the outer membrane. Our peptide spot immunoassay covering protein length of 3 recombinant P44-related proteins showed that IgM rather than IgG in the sera had a larger variety of reactivities with Peptide_1 to Peptide_14 (Fig. 4). The result suggests that there are multiple epitopes within the hypervariable regions for IgM antibody.

In summary, the study showed the presence of 12 B-cell epitopes on the triple P44-related proteins by peptide array and the epitope-derived antigens could detect antibody to *A. phagocytophilum* in the sera from patients with fever of unknown origin. The immunoassay with these epitope-derived antigens, which has higher diagnostic performances, may have significant benefit for the detection of antibody to *A. phagocytophilum* in patients' sera and contribute to HGA serodiagnosis in foreign countries as well as Japan in near future.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2019.05.008.

Conflict of interest statement

The authors declare no conflict of interest.

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