TABLE 4 Mutations in gyrases A and B and MICs of ciprofloxacin for *H. cinaedi* isolates

No. of isolates	Mutation(s) in:		Ciprofloxacin MIC (mg/liter)	
	GyrA	GyrB	Range	50%[a]
16	T84I		16–64	16
25	T84I	D423N	64–128	64
1	T84I	P442S	64	
2	T84I, D88G		64	
1	T84I, D88H			
1	T84I, D88N		64	

[a] 50%, MIC_{50}.

In conclusion, we analyzed 46 *H. cinaedi* and 3 *H. fennelliae* isolates from the same hospital in Japan during 2008 to 2012. Most *H. cinaedi* isolates belonged to the same clonal complex and were isolated from the same ward, which mainly hosts immunocompromised patients. *H. fennelliae* strains were isolated from the same ward and possessed the same PFGE patterns, indicating these isolates had undergone human-to-human transmission. Antimicrobial susceptibilities were similar between species; however, the mutations that conferred resistance to clarithromycin differed from those in *H. cinaedi*.

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Hemin-Binding Proteins as Potent Markers for Serological Diagnosis of Infections with Bartonella quintana

Mayumi Matsuoka, Toshinori Sasaki, Naomi Seki, Mutsuo Kobayashi, Kyoko Sawabe, Yuko Sasaki, Keigo Shibayama, Tsuguo Sasaki and Yoshichika Arakawa
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Hemin-Binding Proteins as Potent Markers for Serological Diagnosis of Infections with *Bartonella quintana*

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It is difficult to distinguish infections with different *Bartonella* species using commercially available immunofluorescence (indirect immunofluorescent antibody [IFA]) assay kits. To identify appropriate proteins for serodiagnosis of *Bartonella quintana* infections, we investigated the antigenicity of *B. quintana* proteins using sera from homeless people with high *B. quintana* IgG titers in IFA assay. These sera reacted strongly to an outer membrane protein, hemin-binding protein D (HbpD). Further, serum from an endocarditis patient infected with *B. quintana* reacted to HbpB and HbpD. To locate the antigenic sites within the proteins, we generated deletion mutants of HbpB and HbpD. Amino acid residues 89 to 220 of HbpB and 151 to 200 of HbpD were identified as the minimum regions required for recognition by these sera. Several oligopeptides comprising parts of the minimum regions of HbpB and HbpD were synthesized, and their immunoreactivity with the above-mentioned sera was tested by enzyme-linked immunosorbent assay (ELISA). Serum from the endocarditis patient reacted similarly to synthetic peptides HbpB2 (amino acid residues 144 to 173 of HbpB) and HbpD3 (151 to 200 residues of HbpD), while sera from the other subjects reacted to HbpD3. These results indicate that synthetic peptides HbpB2 and HbpD3 might be suitable for developing serological tools for differential diagnosis of *B. quintana* infections from other *Bartonella* infections.

Bartonella is a genus of ubiquitous, fastidious, slow-growing, and hemotropic Gram-negative bacteria, of which 24 species are known to date. Among them, *Bartonella henselae* and *B. quintana* are common microbes responsible for human infections (1). *B. henselae* is the causative pathogen of cat scratch disease (CSD) and is present in various animals, including wild and domestic cats, which act as a natural reservoir. Although *B. henselae*-infected cats show no typical disease symptoms, the bacterium may cause CSD, bacillary angiomatosis (BA), peliosis hepatis, chronic bacteremia, and endocarditis after transmission to humans (2). On the other hand, *B. quintana* was the causative agent of epidemic trench fever (also called 5-day fever) in World Wars I and II. It grows extracellularly in the midgut of human body lice, and the bacterium in crushed lice or in louse feces is transmitted to humans via broken skin (3, 4). In the 1990s, trench fever reemerged in refugee camps and prisons and also in developed countries among homeless people and drug addicts. These outbreaks are referred to as urban trench fever, to distinguish them from classical trench fever (5). *B. quintana* also causes BA, chronic bacteremia, and endocarditis in humans, as *B. henselae* does (6–11).

Since culture of *Bartonella* is time-consuming, complicated, and often unsuccessful, serological methods are considered preferable for diagnosis of *Bartonella* infections. Several serodiagnostic methods, including Western blotting, enzyme-linked immunosorbent assay (ELISA), and indirect immunofluorescent antibody (IFA) assay, have been proposed (12, 13). IFA assay is most commonly used for routine clinical diagnosis of *Bartonella* infections, since a quality-controlled commercial kit is available, using fixed bacterial cells cocultured with Vero cells on a slide as the antigen, for semiquantitative measurements of human serum IgG against *B. henselae* and *B. quintana*. The availability of independent IgG titers for both *B. henselae* and *B. quintana* at once is one of the advantages of this kit. However, it is difficult to distinguish *B. henselae* and *B. quintana* infections reliably by using the IFA test, since the kit utilizes whole cells and the genomes of the two species show a high degree of overall similarity.

We have conducted an epidemiological survey of trench fever in Japan in conjunction with a rescue outreach program for homeless persons in Tokyo. In the survey, blood samples from nonhospitalized homeless people were prepared and examined for IgG antibodies against *B. quintana* (7). Because the subjects may be infected with various bacteria, including *B. henselae*, it is important that the diagnostic tools used for serological differentiation should exhibit high species specificity without cross-reaction. Recently, several bacterial outer membrane proteins, including Pap31 and BH11510 from *B. henselae* (14–20) and VompA, VompB, PpI, and hemin-binding protein E (HbpE) from *B. quintana* (21), have been proposed as candidate proteins for development of serodiagnostic tools for *Bartonella* infections. In this study, we attempted to identify species-specific antigenic proteins from *B. quintana*, using sera from homeless people and a *B. quintana*-infected endocarditis patient. We also synthesized fragment peptides of two of the identified proteins as candidates for development of a novel serodiagnostic assay.

MATERIALS AND METHODS

Bacterial strains. *Bartonella* and *Escherichia coli* strains used in this study are listed in Table 1. *B. quintana* Oklahoma was kindly provided by P.

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference
Strains		
B. henselae	ATCC 49882	ATCC
B. quintana Oklahoma	Human isolate	P. Brouqui
E. coli TOP10	Host strain for cloning	Invitrogen
E. coli BL21Star(DE3)	Host strain for gene expression	Invitrogen
Plasmids		
pCR4-TOPO	TA-cloning vector	Invitrogen
pET100D/TOPO	Expression vector	Invitrogen
pHbpA	pET100D/TOPO containing *hbpA*	This study
pHbpB	pET100D/TOPO containing *hbpB*	This study
pHbpC	pET100D/TOPO containing *hbpC*	This study
pHbpD	pET100D/TOPO containing *hbpD*	This study
pHbpE	pET100D/TOPO containing *hbpE*	This study
pHbpB1	pET100D/TOPO containing *hbpB* (1–354)	This study
pHbpB2	pET100D/TOPO containing *hbpB* (265–660)	This study
pHbpB3	pET100D/TOPO containing *hbpB* (601–900)	This study
pHbpB4	pET100D/TOPO containing *hbpB* (901–1362)	This study
pHbpD1	pET100D/TOPO containing *hbpD* (1–300)	This study
pHbpD2	pET100D/TOPO containing *hbpD* (151–450)	This study
pHbpD3	pET100D/TOPO containing *hbpD* (301–600)	This study
pHbpD4	pET100D/TOPO containing *hbpD* (451–750)	This study
pHbpD5	pET100D/TOPO containing *hbpD* (601–885)	This study

[a] Numbers indicate the positions in the nucleotide sequence of the appropriate gene.

Brouqui (WHO Collaborative Center for Rickettsial Reference and Research, Marseilles, France) in 2003. *B. quintana* and *B. henselae* were cultured on Columbia agar with 5% sheep's blood (Sysmex-bioMérieux, Tokyo, Japan) for 14 days at 37°C in 5% CO_2.

TABLE 3 Sequences of peptides synthesized as antigens for ELISA[a]

Hbp	Position of ORF (no. of amino acids)	Amino acid sequence (from N terminus to C terminus)
HbpB1	144–173 (20)	LENQLLGKSPKRKVQRTASA
HbpB2	144–163 (30)	LENQLLGKSPKRKVQRTASAAPAAPAAPAP
HbpD1	151–180 (30)	TEFPGGVEFPDPVPGPERPPFEYDRIIQAT
HbpD2	171–200 (30)	FEYDRIIQATLKQKWSGATRVRVGFSGERL
HbpD3	151–200 (50)	TEFPGGVEFPDPVPGPERPPFEYDRIIQATL KQKWSGATRVRVGFSGERL

[a] Hemin-binding protein (Hbp) sequences used for peptide design were derived from the open reading frame (ORF) of the sequences with the accession numbers AY126673 for HbpB and AY126674 for HbpD.

Plasmids. Primers used in this study are listed in Table 2. Genomic DNA of *B. quintana* was prepared, and genes for Hbp were amplified by standard PCR methods. Purified fragments were then cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA), and DNA sequences were verified. To generate Xpress-tagged full-length versions and deletion mutants of Hbp, a Champion pET Directional TOPO Expression kit (Invitrogen) was used according to the manufacturer's instructions. His_6-tagged proteins were purified by using His-Bind kits (Novagen, Darmstadt, Germany).

Sera and antibodies. Serum from nonhospitalized homeless people had been obtained during an epidemiological investigation of trench fever in Japan. Informed consent had been obtained, and data were anonymized. Demographic, clinical, and other information about the subjects of the present study was described previously (7). Serum from an anonymized endocarditis patient infected with *B. quintana* had been obtained at three time points (22). The IFA titers of these sera were determined with a *Bartonella* IFA IgG kit (Focus Diagnostics, Cypress, CA). Pooled serum from 30 healthy donors (Dako, Glostrup, Denmark) was used as a control in ELISA and Western blotting. Anti-Xpress antibody was purchased from Invitrogen. Alkaline phosphatase (AP)- and peroxidase (PO)-conjugated secondary antibodies for human and mouse IgG were obtained from Dako.

Western blotting. Bacterial cells of *B. quintana* and *B. henselae* were collected from plates, and whole cells were lysed by sonication. *B. quintana* or *B. henselae* proteins were separated by SDS-PAGE and immunoblotted with sera from homeless people. For detection of Xpress-tagged Hbp, *E. coli* lysates suspended in lysis buffer (50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton

TABLE 2 Primers for construction of hemin-binding proteins in *Bartonella quintana*

Construct	Sequence (5′→3′)	
	Forward primer[a]	Reverse primer
HbpA	CACCATGAATATAAAATCTTTAATAACG	TTAGAATTTATAAGCTACACCAAAACGG
HbpB	CACCATGAATACGAAACGTTTAATAAC	TTAGAATTTATAAGCTACACCAATACG
HbpC	CACCATGAATATGAAATGGTTAATAAC	TTAAAATTTGTAAGCCACACCAACGC
HbpD	CACCATGGCTAAAAAATATTTAATCAC	TTAAAATTTGTACGCTACACCAACACGG
HbpE	CACCATGAATATGAAATTTATAATAGG	TTAGAATTTGTAAGCTACACCCACACG
HbpB1	CACC ATG AATACGAAACGTTTAATA	TTATGTGTATTTTTGTCCAGACCA
HbpB2	CACCGGTCTTTACGCCGGTTCTAAT	TTATGTTAATGATGGTCGGGCTAC
HbpB3	CACCCCAGCAGCACCAGCGTCGGTA	TTACTCACCTTGTGCACTCCGTCC
HbpB4	CACCCAAACGGTGAATGAAAAAAGT	TTAGAATTTATAAGCTACACCAAT
HbpD1	CACCATGGCTAAAAAATATTTAATC	TTAGAAATCAAAATTGAAACCGCC
HbpD2	CACCATAGGTAATTTTTCAAGTAAG	TTACCCTGGAATCTCTATCCCTGA
HbpD3	CACCGGCAAGGGTTTTGTTATAGGT	TTACAAGCGCTCCCCAGAAAAACC
HbpD4	CACCACAGAGTTTCCAGGAGGGGTA	TTAAAAATCAACACCGCCTCCAAG
HbpD5	CACCATGCCTTATATTGCTGGAGGT	TTAAAATTTGTACGCTACACCAAC

[a] CACC at the 5′ end is a sequence added to allow pET directional cloning.

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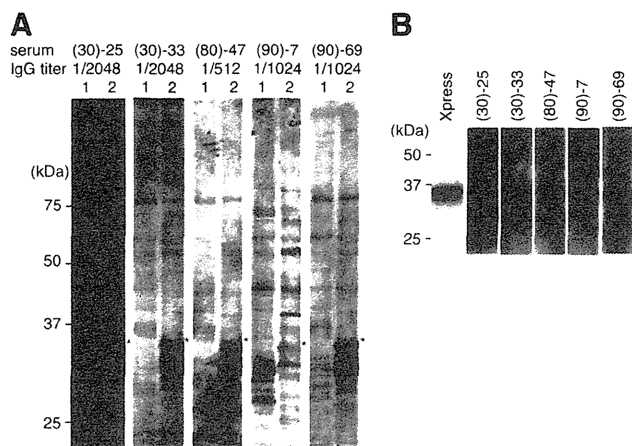

FIG 1 Western blots of sera from homeless people with HbpD of *B. quintana*. (A) Western blots of *B. henselae* and *B. quintana* with sera from homeless people. Whole-cell lysates of *B. henselae* (lanes 1) and *B. quintana* (lanes 2) were separated on a 12.5% SDS-PAGE gel and subjected to Western blotting with five sera (top). IgG titers for *Bartonella*, measured with a *Bartonella* IFA IgG kit, are indicated under the serum numbers. Asterisks indicate a protein of approximately 35 kDa in *B. quintana* lysate that reacts strongly to these sera. (B) *E. coli* lysates expressing Xpress-tagged HbpD were subjected to Western blotting with anti-Xpress or sera from homeless people as indicated at the top.

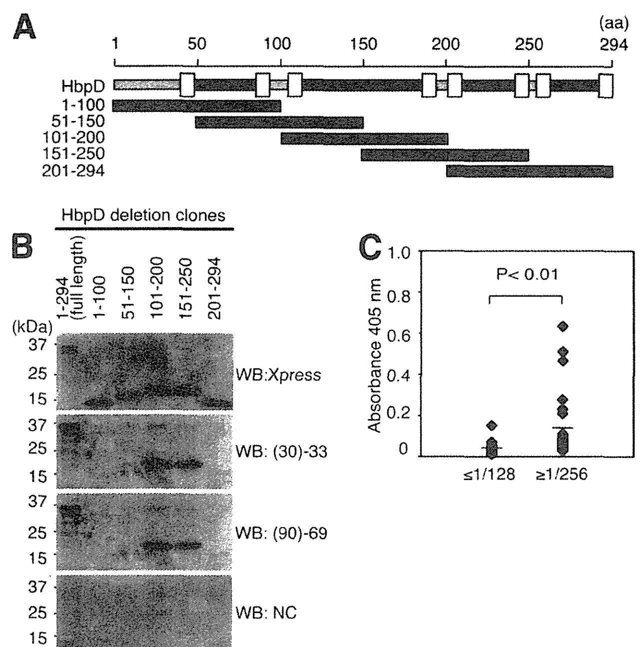

FIG 2 Antibody against the second extracellular domain of HbpD is increased in sera from homeless people. (A) Schematic structure of HbpD of *B. quintana*. Amino acid (aa) numbering is indicated at the top. The regions corresponding to the five HbpD deletion mutants are also indicated. The intracellular domain (gray bar), the extracellular domain (black bar), and the transmembrane domain (TM; open box) are shown. The transmembrane domains are located at residues 41 to 49, 85 to 93, 105 to 113, 187 to 195, 200 to 208, 242 to 250, 256 to 264, and 286 to 294 in HbpD. (B) Full-length forms and deletion mutants of HbpD were separated on a 15 to 25% SDS-PAGE gel and subjected to Western blotting with anti-Xpress antibody (top panel), sera from two homeless people (middle panels), and control serum (NC; bottom panel). Positions of molecular mass markers are indicated on the left. (C) Purified HbpD/101–200 was applied as a coating to ELISA plates, and antibody titers of sera from 65 homeless people were measured. The sera were separated into two groups based on the IFA IgG titer, as indicated at the bottom. Geometric means of the ≤1/128 and ≥1/256 groups were 0.042 and 0.127, respectively, as indicated by lines ($P < 0.01$).

X-100, 10 mM imidazole) were separated by SDS-PAGE and immunoblotted with anti-Xpress antibody or human serum as indicated.

Amino acid sequencing. Whole-cell lysate of *B. quintana* was separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was stained with Coomassie brilliant blue (CBB) staining solution, and the 35-kDa band was cut out. Protein was extracted, and the N-terminal amino acid sequence was determined.

ELISA. Peptides used as ELISA antigens are listed in Table 3. One-microgram samples of peptides synthesized by TaKaRa Bio Inc. (Shiga, Japan) were applied as a coating on immunoplates (Maxisorp; Nunc, Roskilde, Denmark) with coating buffer (15 mM Na_2CO_3, 35 nM $NaHCO_3$, 3 mM NaN_3, pH 9.6). Serum diluted to 1:100 was added, and the plates were incubated for 1 h at room temperature and then further incubated with AP-conjugated anti-human IgG (1:1,000) and *p*-nitrophenyl phosphate disodium (Sigma, St. Louis, MO). The absorbance at 405 nm was measured.

Statistical analysis. Mean values of *Bartonella* IFA titer were calculated, and the numbers of sera with titers of ≤1/128 and ≥1/256 were compared using Student's *t* test. Statistical analysis was performed using Stata software, version 11 (StataCorp. LP, College Station, TX).

RESULTS

Identification of HbpD from *B. quintana* as an antigen strongly reactive to sera from homeless people. To identify *B. quintana* proteins that react to sera from *B. quintana*-infected subjects, whole-cell lysates of *B. quintana* and *B. henselae* were separated by means of SDS-PAGE and subjected to Western blotting with 50 selected sera from homeless people, in which the IgG titer to *B. quintana* measured with the *Bartonella* IFA IgG kit was ≥1/512. As shown in Fig. 1A, several proteins with molecular masses below 37 kDa were detected in both *B. quintana* and *B. henselae* lysates. It is noteworthy that a strong signal at approximately 35 kDa was detected only in *B. quintana* lysate (asterisks in Fig. 1A), with almost all sera (five representative sera that showed a strong signal are shown). These results indicated that antibody to this 35-kDa protein is strongly induced by *B. quintana* infection, and therefore, this is a potential candidate for developing a diagnostic tool. To identify the 35-kDa protein, its N-terminal amino acid sequence was determined by the Edman degradation method. We obtained two N-terminal amino acid sequences corresponding to malate dehydrogenase and hemin-binding protein D (HbpD). Since HbpD was reported to be an outer membrane protein located at the bacterial surface (23), we considered that the strong signal observed at 35 kDa in *B. quintana* lysate represented HbpD. To investigate whether antibodies against HbpD were actually increased in these sera, Xpress-tagged HbpD was subjected to Western blotting with individual sera. As shown in Fig. 1B, Xpress-tagged HbpD was detected at approximately 37 kDa with anti-Xpress antibody (Fig. 1B, leftmost lane). A signal at the same position was detected with all the sera. These results suggest that antibody against HbpD of *B. quintana* is typically increased in sera from homeless people.

Antibodies reacting to the second extracellular domain of HbpD are increased by *B. quintana* infection. To identify the antigenic site in HbpD, we prepared a series of deletion mutants, which contain approximately 100 amino acids each and cover the entire region of HbpD (Fig. 2A). As shown in Fig. 2B, Xpress-

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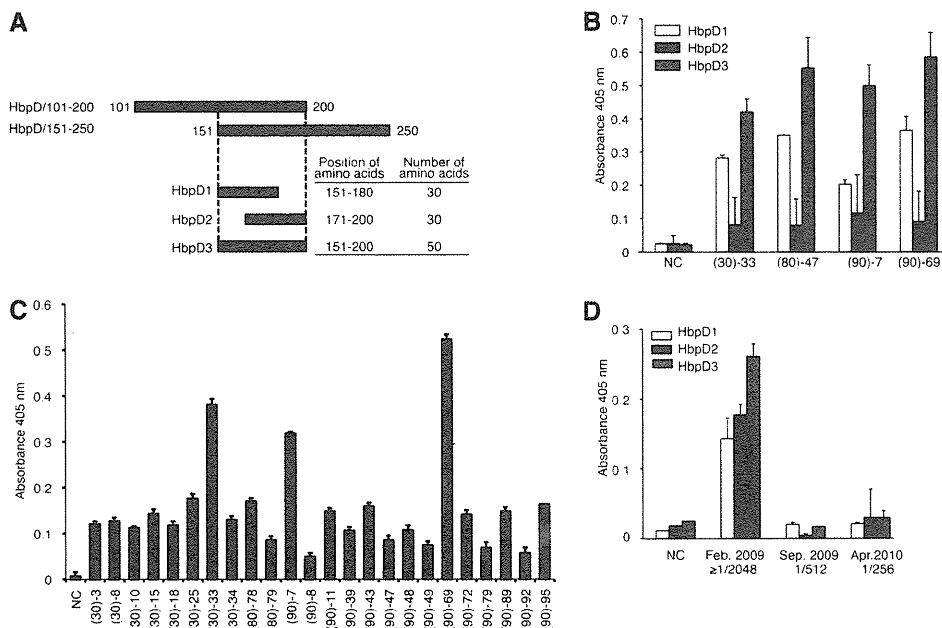

FIG 3 Reaction of sera from homeless people and the *B. quintana*-infected patient with HbpD synthetic peptides. (A) Schematic diagram of the HbpD synthetic peptides. HbpD1, HbpD2, and HbpD3 were designed to cover overlapping regions of HbpD/101–200 and HbpD/151–250. The positions and numbers of amino acids of the three peptides are indicated on the right. (B) HbpD1 (white bars), HbpD2 (black bars), and HbpD3 (gray bars) were applied as a coating to plates, and the avidities of four sera from homeless people and control serum (NC) were measured by ELISA. Data are the means of three independent experiments. (C) Sera from 24 homeless people and control serum were examined by ELISA with an HbpD3-coated plate. Data are the means of three independent experiments. (D) Serum from an endocarditis patient infected with *B. quintana* was measured by ELISA with HbpD1 (white bars), HbpD2 (black bars), and HbpD3 (gray bars). Sera were prepared at indicated time points during the course of the infection. IgG titers for *B. quintana* measured by IFA are shown. Data are the means of three independent experiments.

tagged full-length proteins and deletion mutants were detected by anti-Xpress antibody at the expected position (Fig. 2B, top panel). Full-length HbpD was also detected with two sera, (30)-33 and (90)-69, whereas this signal was not detected with control serum. Among five deletion mutants, only two, HbpD/101–200 and HbpD/151–250, were detected with both (30)-33 and (90)-69 (Fig. 2B, middle panels). These results indicated that antibodies against the 101–200 and 151–250 sequences of HbpD are present in these two sera, which exhibited a high IFA titer for *B. quintana*. We next tested whether or not antibody against HbpD/101–200 was increased in sera from 65 homeless people by using ELISA with the purified HbpD/101–200 fragment. These sera covered various ranges of IFA titers and were divided into two groups (30 sera with IFA titers of ≤1/128 and 35 sera with IFA titers of ≥1/256). As shown in Fig. 2C, the geometric mean of the ≥1/256 group was 3-fold higher than that of the ≤1/128 group (mean absorbance values at 405 nm were 0.127 and 0.042, respectively).

HbpD is a membrane protein with eight β-stranded transmembrane domains and four extracellular domains (23). The reactive HbpD-derived fragments found here include the second or the third extracellular domain. Amino acids 151 to 200, which are shared by these two fragments, correspond to the latter half of the second extracellular domain (Fig. 2A). We then investigated whether synthetic peptides corresponding to this region could be potential epitopes for antibodies in sera with high IFA titers for *B. quintana*. To this end, three peptides, HbpD1, HbpD2, and HbpD3 (corresponding to amino acid residues 151 to 180, 171 to 200, and 151 to 200 of HbpD, respectively), were synthesized (Fig. 3A; Table 3), and the avidities of four sera from homeless people, (30)-33, (80)-47, (90)-7, and (90)-69, for these targets were measured by ELISA (Fig. 3B). In terms of absorbance at 405 nm, the titers of these sera were 8- to 15-fold for HbpD1, 3- to 5-fold for HbpD2, and 20- to 28-fold for HbpD3, relative to the control serum. All four sera showed the highest avidity for HbpD3, though the avidity for HbpD1 was only slightly lower. Avidity for HbpD2 was weak compared to that for HbpD1 or HbpD3. The lower avidity for HbpD2 was not unexpected, since the C-terminal half of HbpD2 corresponds to transmembrane and intracellular domains, which may be unlikely to act as epitopes. HbpD3 peptide was further tested with another 24 sera belonging to the ≥1/256 IFA titer group. As shown in Fig. 3C, all sera tested showed high avidity for HbpD3 compared to the control serum. Among them, four sera, (30)-25, (30)-33, (90)-7, and (90)-69, which gave a strong signal in Western blot assays with the 35-kDa protein of *B. quintana* lysate (Fig. 1), showed relatively high avidity for HbpD3 in ELISA. These results also indicated that sera showing high IgG titers for *B. quintana* in the standard IFA test contain antibodies against HbpD, and the epitope of these antibodies is probably located at the C-terminal region of the second extracellular domain, corresponding to HbpD3 peptide.

Given that the avidity in HbpD ELISA and the IFA titer of these sera were well correlated, we next investigated serum from a patient with prosthetic valve endocarditis caused by *B. quintana* infection, by means of ELISA with the HbpD synthetic peptides. Serum samples had been obtained at three different time points during the course of infection, i.e., on admission (February 2009), on discharge (September 2009), and at an outpatient clinic (April 2010), and showed IFA titers for *B. quintana* of ≥1/2,048, 1/512,

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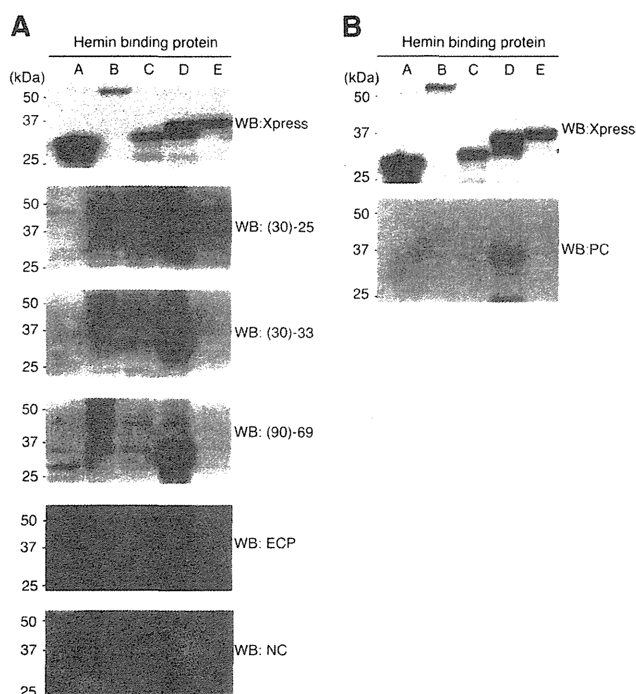

FIG 4 Antibody against HbpB was increased in serum from the *B. quintana*-infected patient. (A) Five Xpress-tagged Hbps (as indicated at the top) were separated on a 7.5 to 15% SDS-PAGE gel and subjected to Western blot analysis with anti-Xpress antibody (top panel), three sera from homeless people [(30)-25, (30)-33, and (90)-69], serum from the *B. quintana*-infected endocarditis patient (ECP), and control serum (NC). Positions of molecular mass markers are indicated on the left. (B) The same membrane as that in panel A was subjected to Western blotting with the positive control (PC) from the *Bartonella* IFA kit.

and 1/256, respectively. As shown in Fig. 3D, increased avidity for all three peptides was observed with the serum obtained in February 2009. The avidity of sera obtained at the other time points was similar to that of the control serum. These results suggested that HbpD3 is the most suitable peptide for an ELISA antigen among the three peptides.

Antibodies against HbpB, but not HbpA, HbpC, or HbpE, are increased in sera of *B. quintana*-infected subjects. We next tested whether or not antibodies against other Hbp family proteins are also increased in sera from homeless people and the *B. quintana*-infected patient by using five Xpress-tagged Hbps. As shown in Fig. 4A, expression of all Hbps was confirmed by Western blotting with anti-Xpress antibody, although the expression level of HbpB was slightly lower than those of the others (Fig. 4A, top panel). When these extracts were subjected to Western blotting with sera from homeless people [Fig. 4A, panels (30)-25, (30)-33, and (90)-69], strong signals corresponding to HbpD were observed as expected, while no signals corresponding to HbpA, HbpC, and HbpE were detected. In the cases of (30)-33 and (90)-69, very weak signals for HbpB were detected. Serum from the endocarditis patient showed signals with HbpB and HbpD but not HbpA, HbpC, or HbpE. No Hbp was detected with control serum. These results indicated that, in addition to antibody against HbpD, antibody against HbpB is also increased by *B. quintana* infection. The five Hbps were also subjected to Western blotting with the polyvalent murine antibody supplied with the *Bartonella* IFA IgG kit as a positive control. Only a signal corresponding to HbpD was detected (Fig. 4B). This result suggested that the positive-control antibody in the IFA kit predominantly recognizes HbpD among the Hbp family members, and thus, the standard diagnosis of *B. quintana* infection by IFA might depend predominantly on the presence of HbpD.

Given that anti-HbpB antibody was detected in serum from the *B. quintana*-infected patient, we next investigated the antigenic region of HbpB in order to design a suitable synthetic peptide for use as an ELISA antigen. Four Xpress-tagged HbpB fragments covering the entire region of HbpB were generated (Fig. 5A), and bacterial extracts including these HbpB fragments were subjected to Western blotting. As shown in Fig. 5B, signals corresponding to the four fragments were detected by anti-Xpress antibody. Among these four fragments, HbpB/89–220 reacted to serum from the *B. quintana*-infected patient. HbpB contains four extracellular domains, and HbpB/89–220 corresponds to the second extracellular domain. These results indicated that, in addition to anti-HbpD antibody, anti-HbpB antibody is also induced by *B. quintana* infection, and the epitope may be located at the N-terminal region of the second extracellular domain of HbpB. Given that antibody against HbpB/89–220 was predominant in serum of the *B. quintana*-infected patient, we synthesized two peptides corresponding to HbpB/89–220 for use in ELISA (Fig. 5C). HbpB1 and HbpB2 correspond to amino acid residues 144 to 163 and 144 to 173 of HbpB, respectively. As shown in Fig. 5D, the avidity to HbpB1 and HbpB2 of serum obtained on admission from the *B. quintana* patient was increased 11- and 14-fold, respectively, compared to the control serum. In the case of sera from homeless people, (90)-7 showed the highest avidity, and the avidity for HbpB1 and HbpB2 was increased 5.5- and 5.7-fold versus the control, respectively. These results suggest that detection of antibodies against HbpB1 and HbpB2 may be an effective basis for diagnosis of *B. quintana* infections.

DISCUSSION

We previously used a commercial IFA assay to examine the incidence of *B. quintana* infections in homeless people in urban areas of Japan (7), and our results suggested an unexpectedly high rate of exposure to *B. quintana*. Such a high rate of putative *B. quintana* infection might have several possible explanations. In particular, the IFA system used might generate a significant number of false positives, since it employs bacterial whole cells as the antigen for detecting anti-*B. quintana* antibodies. For example, antibodies to other infectious agents may cross-react to *B. quintana*, or nonspecific antibodies reacting to this microbe might be present in the population. The possibility of asymptomatic infection with *B. quintana* should also be taken into account.

We considered that the use of a single, potent peptide antigen instead of whole bacterial cells might virtually eliminate false positives caused by cross-reaction of other antibodies. In the present study, therefore, we first examined the antigenicity of *B. quintana* proteins. As a result, we identified hemin-binding protein B (HbpB) and HbpD of *B. quintana* as potential candidates for developing a novel serological tool for diagnosis of *B. quintana* infections. Importantly, HbpB and HbpD of *B. quintana* reacted to *B. quintana*-infected sera but not sera from *B. henselae*-infected CSD patients in either Western blotting or ELISA (data not shown). Consequently, it should be possible to distinguish *B. quintana* and *B. henselae* infections by using these antigens. To

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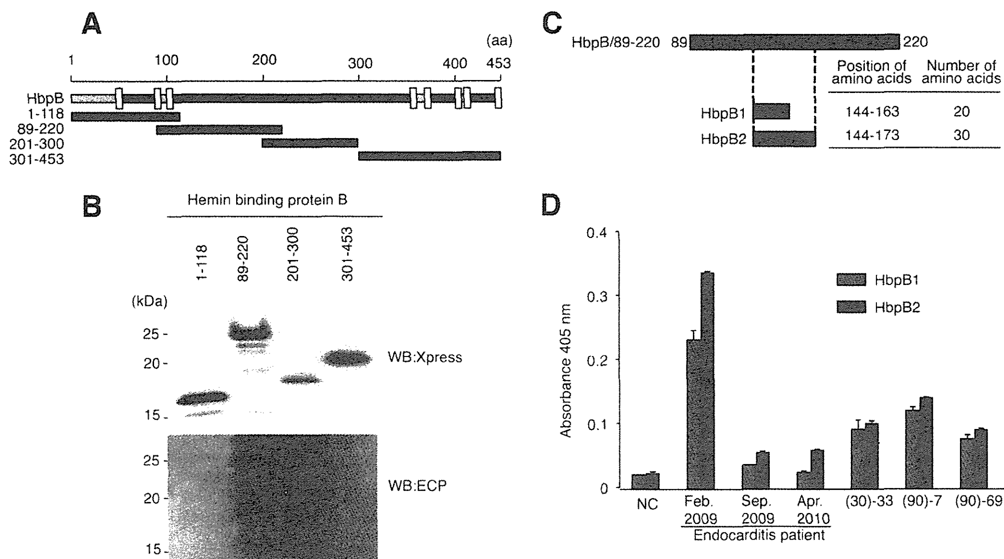

FIG 5 The sequence of amino acid residues 89 to 220 of HbpB is recognized by serum from the *B. quintana*-infected patient. (A) Schematic structure of HbpB of *B. quintana*. Regions corresponding to four HbpB deletion mutants are also shown. The intracellular domain (gray bar), the extracellular domain (black bar), and the transmembrane domain (TM; open box) are indicated. The number of amino acids is shown at the top. Transmembrane domains are located at residues 43 to 52, 83 to 91, 103 to 111, 364 to 372, 377 to 385, 401 to 409, 415 to 423, and 445 to 453. (B) Xpress-tagged HbpB mutants (indicated at the top) were separated on a 15% SDS-PAGE gel and subjected to Western blotting by using anti-Xpress antibody (top panel) and serum from the *B. quintana*-infected endocarditis patient (ECP; bottom panel). Positions of molecular mass markers are indicated on the left. (C) Schematic diagram of two HbpB synthetic peptides. HbpB1 and HbpB2 were designed based on the sequence of the extracellular domain of HbpB (residues 89 to 220). Positions and numbers of amino acid residues of the two synthetic peptides are indicated on the right. (D) HbpB1 and HbpB2 were applied as a coating to ELISA plates, and sera from the endocarditis patient and homeless people were examined. Data are the means of three independent experiments.

further characterize the specificity of the *B. quintana* ELISA, it would be necessary to examine patients infected with *Bartonella* species other than *B. quintana*. Also, it would be desirable to confirm that the 35-kDa protein, which we identified as HbpD by N-terminal sequence analysis, is truly specific for *B. quintana* by testing sera from homeless people against antigens from other *Bartonella* species. We hope to address these issues in association with the next epidemiological survey of trench fever in Japan.

Hbp proteins are located at the bacterial surface and bind to environmental heme (23–27). The *hbp* gene family in *B. quintana* consists of five members (*hbpA* to *hbpE*), and all are expressed under usual bacterial culture conditions (14). For *in vitro* culture of *Bartonella* species, erythrocytes, hemoglobin, or hemin is usually supplied as a source of iron, because *Bartonella* lacks genes for heme biosynthesis. Genes *hbpB* and *hbpC* appear to be expressed predominantly under high-environmental-hemin conditions, based on the environment in lice, while *hbpA*, *hbpD*, and *hbpE* appear to be expressed predominantly under low-environmental-hemin conditions, based on the environment in humans (24). In this study, we found that sera from homeless people reacted to HbpD, while serum from the endocarditis patient reacted to HbpB. This finding may be attributable to differences in the expression levels of HbpB and HbpD in the pathogens in response to interindividual differences in patients' background factors or differences in the sites of infections or other factors. To examine this possibility, sera from a larger number of *B. quintana*-infected patients should be examined. The five Hbps in *B. quintana* show a high degree of identity at the amino acid sequence level, and the positions and the amino acid sequences at transmembrane domains are particularly well conserved. They are also similar in size except for HbpB, which has a 170-amino-acid extension in the second extracellular domain. Although we found anti-HbpB and anti-HbpD antibodies in individuals infected with *B. quintana*, no antibodies against HbpA, HbpC, or HbpE were detected, despite the high degree of identity among these proteins. Therefore, we considered that the target epitopes of antibodies directed to HbpB and HbpD may be located at unique regions in these molecules, probably at regions exposed on the bacterial outer membrane. It is possible that only HbpB and HbpD form immunogenic complexes in the bacterial outer membrane, though the interacting partners of Hbp proteins have not been elucidated yet.

As a next step, we established the locations of the antigenic sites within these proteins by generating a series of deletion mutants of HbpB and HbpD and identified residues 89 to 220 of HbpB and 151 to 200 of HbpD as the minimum regions required for recognition by the sera. Several oligopeptides comprising parts of the minimum regions of HbpB and HbpD were synthesized, and their immunoreactivity with the above sera was tested by ELISA. In this way, we identified synthetic peptides HbpB2 (amino acid residues 144 to 173 of HbpB) and HbpD3 (residues 151 to 200 of HbpD) as candidates for the development of novel serological tools for differential diagnosis of *B. quintana* infections from other *Bartonella* infections. Further work is under way to develop and validate these assays.

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Detection of Tripoli metallo-β-lactamase 2 (TMB-2), a variant of bla_{TMB-1}, in clinical isolates of *Acinetobacter* spp. in Japan

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Sir,

Metallo-β-lactamase is an important resistance determinant among Gram-negative bacteria, and some metallo-β-lactamase genes are encoded on mobile gene elements that can spread among various clinically important bacterial species.[1] TMB-1 (Tripoli metallo-β-lactamase 1) was first identified in 2012 in an *Achromobacter xylosoxidans* strain isolated from a hospital environment sample in Tripoli, Libya.[2] Here, we report two cases in which bla_{TMB}-positive non-*baumannii Acinetobacter* spp. were isolated from patients with no history of international travel.

The first case, a carbapenem-resistant *Acinetobacter* sp. (MRY12-142) was isolated from a urine sample in December 2011. This case had no recent history of international travel. For the second case, *Acinetobacter* sp. (MRY12-226) was isolated from necrotic tissue in July 2012. This patient also had no international travel history. These two cases were identified in two hospitals that are separated by more than 1000 km, and there was no epidemiological link between the two cases.

Acinetobacter spp. were identified by sequencing the partial *rpoB* gene and the 16S–23S rRNA gene spacer region,[3,4] which revealed *Acinetobacter pittii* in the first case and *Acinetobacter* genomospecies 14BJ in the second case. Both isolates were resistant to penicillins, cephalosporins, imipenem, meropenem and trimethoprim/sulfamethoxazole, but susceptible to fluoroquinolones, amikacin, and minocycline according to MICs determined by the VITEK2 system (bioMérieux, Lyon, France) and the recommended breakpoints of CLSI 2012.[5] Metallo-β-lactamase production was screened using a disc containing sodium mercaptoacetic acid (SMA) (Eiken, Tokyo, Japan).[6] For both isolates, the growth inhibitory zone around the imipenem and ceftazidime discs expanded upon the addition of the SMA disc, which is strongly indicative of metallo-β-lactamase production.

Based on PCR analyses, both isolates were negative for bla_{NDM-1}, bla_{KPC}, bla_{IMP} bla_{VIM-1}, bla_{VIM-2}, $bla_{OXA-23-like}$, $bla_{OXA-24-like}$, $bla_{OXA-51-like}$ and $bla_{OXA-58-like}$. However, PCR analyses for class 1 integron gene cassettes, in which primers targeted the 5′-conserved region (CS) and 3′-CS, revealed two bands of ~1.2 kbp and 1.8 kbp in both isolates. Sequence analysis of the 1.2 kbp PCR products of both isolates showed that the class 1 integron gene cassette contained only one gene that had 99% amino acid identity with TMB-1, and was thus designated TMB-2. The 738 bp sequence of bla_{TMB-2} was identical to that of bla_{TMB-1}, except for one substitution at nucleotide position 544, which caused an amino acid change from serine to proline at position 228 according to the class B standard numbering[7] (GenBank accession numbers AB758277 and AB758278). Sequence analysis for the 1.8 kbp PCR product of MRY12-142 was conducted and showed that the class 1 integron gene cassette contained *aac(6′)-Ib* and a hypothetical open reading frame.

The PCR product of the class 1 integron gene cassette containing bla_{TMB-2} was ligated into pGEM-T (Promega, WI, USA) and transformed into *Escherichia coli* strain DH5α. In addition, pGEM-T harbouring bla_{TMB-1} was obtained by site-directed mutagenesis and transformed into *E. coli* DH5α to evaluate the role of this single amino acid substitution on antimicrobial susceptibility, the MICs being determined by Etest (bioMérieux). As shown in Table 1, the TMB-2-producing transformant was resistant to ceftazidime and susceptible to aztreonam, similar to the TMB-1-producing transformant. However, the TMB-2-producing transformant showed >256-fold and 16-fold lower MICs for doripenem and meropenem, respectively, compared with the TMB-1-producing transformant. The MICs of imipenem were not

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Table 1. Antimicrobial susceptibility of isolates and strains determined by Etest

Antimicrobial agents	MICs (mg/L) for isolates and strains						
	A. pittii MRY12-142	*A.* genomospecies 14BJ MRY12-226	*E. coli* DH5α (pGEM-T-TMB-2)	*E. coli* DH5α (pGEM-T-TMB-1)	*E. coli* DH5α (pGEM-T)	*E. coli* DH5α	*A. pittii* ATCC 19004
Aztreonam	32	32	0.064	0.094	0.047	0.047	16
Ceftazidime	>256	>256	>256	>256	0.25	0.38	6
Imipenem	16	>32	2	1	0.38	0.38	0.25
Meropenem	>32	>32	2	32	0.064	0.064	0.75
Doripenem	>32	>32	0.064	32	0.032	0.032	0.19

different for the two strains. Both transformants also showed an apparent expansion of the growth inhibitory zone around the ceftazidime disc upon addition of the SMA disc.

It has been reported that carbapenem resistance among non-*baumannii Acinetobacter* spp. is usually due to the production of metallo-β-lactamase.[8-10] To our knowledge, this study is the first to report an *Acinetobacter* spp. positive for bla_{TMB} and to identify a new variant, bla_{TMB-2}. It is also the first report to identify bla_{TMB} in clinical isolates. The low MICs of carbapenems for transformant cells suggests that additional resistance mechanisms, such as the production of other classes of β-lactamase, a reduction in outer membrane protein expressions and an acceleration of efflux pump activities, may be involved in the carbapenem resistance of parental clinical isolates of *Acinetobacter* spp. Although the same phenomenon was reported in the IMP-type,[11] it is notable that one amino acid substitution from serine to proline in TMB-2 has drastically decreased the MICs of meropenem and doripenem. As neither patient had a history of international travel nor any epidemiological link, it is possible that bla_{TMB-2} had been endemic in Japan but unrecognized because of its reduced ability to hydrolyse carbapenems. The unrecognized spread of bla_{TMB-2} could be a concern as it can turn to bla_{TMB-1} by only one nucleotide substitution. Although this report discusses only two cases, it may be important to evaluate the spread of this emerging metallo-β-lactamase gene among non-*baumannii Acinetobacter* spp.

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Transparency declarations

None to declare.

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Ability of the VITEK® 2 system to detect group B streptococci with reduced penicillin susceptibility (PRGBS)

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Keywords: group B *Streptococcus*, GBS, penicillin G

Sir,

Group B *Streptococcus* (*Streptococcus agalactiae*, GBS) is the leading cause of neonatal sepsis and meningitis and an important pathogen among elderly people and those suffering from underlying medical disorders.[1,2] The highest GBS mortality and morbidity result from invasive infections in neonates.[1,2]

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High cephalosporin resistance due to amino acid substitutions in PBP1A and PBP2X in a clinical isolate of group B *Streptococcus*

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Objectives: Group B *Streptococcus* (GBS; *Streptococcus agalactiae*) has been regarded as uniformly susceptible to penicillins. However, we recently reported the existence of GBS with reduced penicillin susceptibility (PRGBS), with amino acid substitutions in penicillin-binding protein (PBP) 2X. Although most PRGBS show high MICs of ceftizoxime (4–64 mg/L) and cefotaxime (0.12–1 mg/L), those for strain B1 are exceptionally high (ceftizoxime MIC ≥256 mg/L and cefotaxime MIC 2 mg/L). We previously found an amino acid substitution (G539S) neighbouring the conserved K_{540}TG motif in PBP1A in addition to the PRGBS-specific amino acid substitution Q557E in PBP2X of B1. The aim of this study was to reveal the effect of the amino acid substitutions in PBP1A and PBP2X of B1 on the high cephalosporin resistance.

Methods: A ceftizoxime competition assay was performed to reveal the PBPs that are the main targets of ceftizoxime. We generated two allelic exchange mutants from β-lactam-susceptible GBS BAA-611. BAA-611 (B1PBP2X) contained the PBP2X gene derived from B1 and BAA-611 (B1PBP2X, B1PBP1A) contained both the PBP2X and the PBP1A gene derived from B1. These allelic exchange mutants and strain B1 were subjected to susceptibility testing.

Results: The ceftizoxime competition assay revealed that PBP1A and PBP2X were the main targets of ceftizoxime. Although the MICs of ceftizoxime and cefotaxime for BAA-611 (B1PBP2X) were 64 and 0.5 mg/L, respectively, BAA-611 (B1PBP2X, B1PBP1A) showed high cephalosporin resistance (ceftizoxime MIC ≥256 mg/L and cefotaxime MIC 2 mg/L) comparable to B1.

Conclusions: The high cephalosporin resistance of GBS was caused by amino acid substitutions in PBP1A and PBP2X.

Keywords: GBS, penicillin-binding proteins, PBPs, antibiotic resistance, group B streptococci with reduced penicillin susceptibility, PRGBS

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Introduction

Group B *Streptococcus* (GBS; *Streptococcus agalactiae*) is the leading cause of neonatal sepsis and meningitis. The resulting meningitis is associated with high mortality and morbidity, and no licensed vaccines are currently available. The use of prophylactic intrapartum antibiotics has been recommended by the CDC[1–4] and the rate of early-onset (during the first post-natal week), though not late-onset, GBS infections has reduced to some extent.

On the other hand, GBS is a medically important pathogen in both pregnant women and non-pregnant adults. It is especially prevalent among the elderly and those suffering from underlying medical disorders.[1,2,4] Elderly adults account for >40% of persons with invasive GBS disease and for >50% of GBS-associated deaths.[5] Moreover, GBS disease in adults is frequently nosocomial and may be related to the placement of an intravenous catheter.[6]

Penicillins, including penicillin G, are first-line antimicrobial agents in intrapartum antibiotic prophylaxis and also treatments for GBS infections. All clinical GBS isolates have been found to be uniformly susceptible to β-lactams, including penicillins.[1,2] No criteria for penicillin resistance have yet been established by the CLSI.[7] However, we recently reported the existence and

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molecular mechanisms of clinical GBS isolates with reduced penicillin susceptibility (PRGBS)[8–14] and subsequently other research groups also identified similar PRGBS isolates in Japan, the USA and Canada.[15–18]

In our earlier study,[8] PRGBS showed penicillin MICs of 0.25–1 mg/L (non-susceptible range) and ceftizoxime MICs of 4–128 mg/L in 12 geographically separate hospitals in Japan during 1995–98 and 2005. We determined that the non-susceptibility to penicillin and ceftizoxime was caused by the acquisition of amino acid substitutions in penicillin-binding protein (PBP) 2X.[8] Among the PRGBS strains tested, markedly high MICs of ceftizoxime and cefotaxime were observed for strain B1, compared with other PRGBS strains. Moreover, PBP1A of strain B1 contained an amino acid substitution (G539S) neighbouring the conserved K_{540}TG motif, which is thought to form a part of the active site (Figure S1, available as Supplementary data at *JAC* Online).

The purpose of the present study was to reveal the effect of the amino acid substitutions in PBP1A and PBP2X of B1 on the high cephalosporin resistance. We approached this task by generating allelic exchange mutants with PBP2X and PBP1A genes derived from B1.

Materials and methods

Strains

S. agalactiae ATCC BAA-611 and ATCC 12403 were purchased from ATCC. *S. agalactiae* B1 was clinically isolated from sputum in Japan in 1995 and stored in 20% glycerol until analysed. The MICs of ceftizoxime (≥256 mg/L) and cefotaxime (2 mg/L) for strain B1 were the highest among the PRGBS we tested, while the MICs of penicillin G (0.5 mg/L) and ampicillin (0.06 mg/L) for B1 were comparable to those for the other PRGBS.[8] *S. agalactiae* B1 contained an amino acid substitution (G539S) neighbouring the conserved K_{540}TG motif of PBP1A (Figure S1; GenBank accession number AB279794) in addition to the PRGBS-specific amino acid substitution Q557E in PBP2X (GenBank accession number AB279798).[8]

MIC determination

To determine MICs, the agar dilution method was performed as recommended by the CLSI.[7] *Streptococcus pneumoniae* ATCC 49619 was used as a quality control.

Ceftizoxime competition assay

The membrane fractions were prepared from *S. agalactiae* ATCC 12403 as described previously.[8] The membrane fractions (400 μg) were incubated for 10 min at 37°C in various concentrations of ceftizoxime (0–64 mg/L), followed by incubation for 30 min at 37°C in 12.5 μM Bocillin FL. A 3× SDS sample buffer was then added, followed by SDS–PAGE and fluorescent image analysis with an LAS-3000 multicolour analyser (FUJIFILM, Tokyo, Japan).

Allelic exchange experiment

To generate *S. agalactiae* ATCC BAA-611 allelic exchange mutants containing the chromosomally encoded PBP2X and PBP1A genes derived from PRGBS B1 (designated B1PBP2X and B1PBP1A, respectively), we performed allelic exchange experiments as described previously, using a thermosensitive *Escherichia coli–Streptococcus* shuttle vector, pG+host6Δamp.[8] To generate targeting vectors, fragments containing the nucleotide region from 309417 to 311834 of the GBS ATCC BAA-611 genome were amplified from chromosomal DNA of the B1 clinical isolate, using Pyrobest DNA polymerase (Takara Bio Inc., Ohtsu, Japan), and then ligated into pG+host6Δamp. Several inserts were sequenced and no additional mutations were found.

Electrocompetent cells were made from *S. agalactiae* ATCC BAA-611 (B1PBP2X) as described previously, but with a minor modification.[8] We introduced 1 μg of targeting vector into electrocompetent cells derived from BAA-611 (B1PBP2X) and transformants were selected on agar plates containing 0.5 mg/L erythromycin at 30°C. To obtain cells with target vector insertion into the chromosome of recipient GBS cells, transformants were incubated in liquid medium containing 5 mg/L erythromycin at 37°C. These integrant strains were successively cultivated for 3 days in liquid medium at 30°C without erythromycin selection to facilitate the excision of vector pG+host6Δamp. Allelic exchange mutants were selected on agar plates containing 64 mg/L ceftizoxime and susceptibility to erythromycin was confirmed using agar plates containing 5 mg/L erythromycin. The PBP1A genes of each allelic exchange mutant were confirmed by sequencing and then the MICs for the strains were determined.

Results

Ceftizoxime competition assay

The results of the ceftizoxime competition assay are shown in Figure 1. In the absence of ceftizoxime, six bands were found in a lane. The PBP1A and PBP2X bands (arrows) were found as reported previously.[8,19] As the concentration of ceftizoxime increased, the densities of the PBP1A band, the PBP2X band and another band all diminished. These data suggest that ceftizoxime mainly binds three PBPs, including PBP1A and PBP2X. Because there were no amino acid substitutions neighbouring the conserved motifs of the other PBPs in strain B1, we focused on PBP1A and PBP2X.

MICs for allelic exchange mutants

Elevation of the cephalosporin MICs for the allelic exchange mutants is shown in Table 1. The MICs of ceftizoxime (64 mg/L) and cefotaxime (0.5 mg/L) for BAA-611 (B1PBP2X) were comparable to those for the previously analysed PRGBS strains, except B1. The MICs of ceftizoxime (≥256 mg/L) and cefotaxime (2 mg/L) for BAA-611 (B1PBP2X, B1PBP1A) were comparable only to those for strain B1. The MICs of penicillin G (0.25–0.5 mg/L) for the two allelic exchange mutants were comparable to those for the previously analysed PRGBS.

Discussion

In the present study, we generated two allelic exchange mutants, BAA-611 (B1PBP2X) and BAA-611 (B1PBP2X, B1PBP1A), and revealed that the MICs of ceftizoxime and cefotaxime for BAA-611 (B1PBP2X, B1PBP1A) were comparable to those for strain B1. These results indicate that the amino acid substitutions in both PBP1A and PBP2X of B1 are responsible for the elevation of the ceftizoxime and cefotaxime MICs for *S. agalactiae*. Moreover, the results of the ceftizoxime competition assay show that PBP1A and PBP2X are involved as the main ceftizoxime targets in *S. agalactiae* (Figure 1). These findings suggest the

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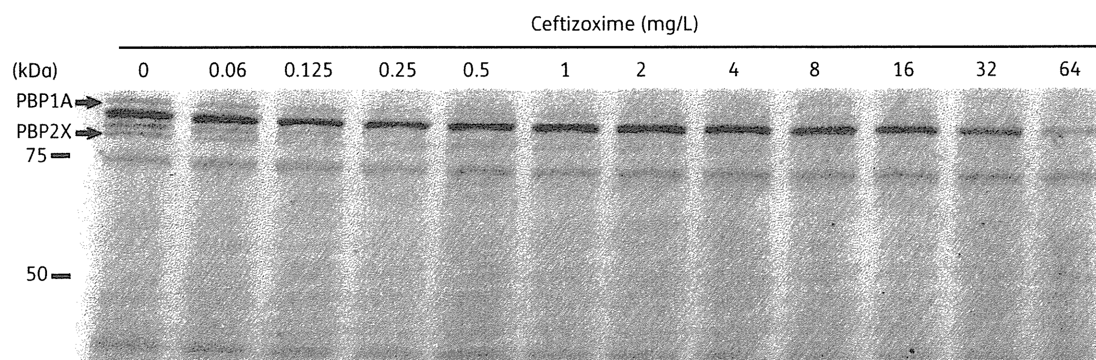

Figure 1. Ceftizoxime competition assay. The membrane fractions of β-lactam-susceptible *S. agalactiae* ATCC 12403 were first incubated with various concentrations of ceftizoxime and then incubated with fluorescent-conjugated penicillin V. This was followed by SDS-PAGE and fluorescence detection.

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Table 1. Elevation of MICs of cephalosporins for allelic exchange mutants

	MIC (mg/L)							
Strain	PEN	AMP	OXA	CFZ	FEP	CTX	ZOX	MEM
S. pneumoniae ATCC 49619	0.25	0.06	1	1	0.12	0.06	0.5	0.06
S. agalactiae ATCC 12403	0.03	0.06	0.25	0.12	0.06	0.06	0.25	0.03
S. agalactiae ATCC BAA-611	0.03	0.06	0.25	0.12	0.06	≤0.03	0.25	0.03
S. agalactiae ATCC BAA-611 (B1PBP2X)	0.25	0.06	2	0.5	0.25	0.5	64	0.03
S. agalactiae ATCC BAA-611 (B1PBP2X, B1PBP1A)	0.5	0.06	4	2	1	2	≥256	0.03
S. agalactiae B1	0.5	0.06	4	2	1	2	≥256	0.03

PEN, penicillin G; AMP, ampicillin; OXA, oxacillin; CFZ, cefazolin; FEP, cefepime; CTX, cefotaxime; ZOX, ceftizoxime; MEM, meropenem.

amino acid substitutions in PBP1A and PBP2X are responsible for the elevation of the MICs of cephalosporins for *S. agalactiae*.

We also observed that the amino acid substitutions in PBP1A and PBP2X found in strain B1 did not markedly elevate the MICs of penicillins, such as penicillin G and ampicillin. However, since the penicillin MICs for penicillin-resistant *S. pneumoniae* (PRSP) are evidently increased by acquiring various amino acid substitutions in PBPs, including PBP1A and PBP2X (amino acid substitutions in PBP2B are known to contribute to penicillin resistance in PRSP), we cannot deny the possibility that other amino acid substitutions in PBP1A and PBP2X in GBS would result in the elevation of these MICs. In the present study, we successfully revealed the importance of these amino acid substitutions in high cephalosporin resistance in GBS. If we consider the amino acid substitutions in PBP2X as the first step in the β-lactam resistance of GBS, these mechanisms, the amino acid substitutions in PBP1A and PBP2X, might be second steps in that resistance, i.e. the evolution from PRGBS to highly cephalosporin-resistant GBS.

Recently, we reported that PRGBS tend to show multidrug resistance to fluoroquinolones and macrolides, as well as reduced penicillin susceptibility.[14] Therefore, drug choice for the prevention and treatment of infections caused by these multidrug-resistant PRGBS may be limited. Moreover, if the cephalosporin MIC increases due to amino acid substitutions in PBPs, including PBP1A and PBP2X (as found in B1 in the present study), it may become more difficult to achieve positive outcomes with antimicrobial treatment for such multidrug-resistant PRGBS infections. In addition, although the MICs of penicillins for PRGBS are not so high (0.25–1 mg/L) at present, as the penicillin MICs for PRSP are increasing due to the accumulation of various amino acid substitutions in PBPs, the MICs of penicillins in addition to cephalosporins for GBS may increase in the near future by similar mechanisms. If the MICs of penicillins and cephalosporins for GBS increase in addition to fluoroquinolone and macrolide resistance, drug choice for the prevention and treatment of infections caused by such multidrug-resistant PRGBS may become quite limited. Because of the above-described reasons, PRGBS could become a serious medical concern in the near future and further research on this issue is recommended.

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Transparency declarations

The authors have no conflicts of interest to declare.
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Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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different for the two strains. Both transformants also showed an apparent expansion of the growth inhibitory zone around the ceftazidime disc upon addition of the SMA disc.

It has been reported that carbapenem resistance among non-*baumannii Acinetobacter* spp. is usually due to the production of metallo-β-lactamase.[8–10] To our knowledge, this study is the first to report an *Acinetobacter* spp. positive for bla_{TMB} and to identify a new variant, bla_{TMB-2}. It is also the first report to identify bla_{TMB} in clinical isolates. The low MICs of carbapenems for transformant cells suggests that additional resistance mechanisms, such as the production of other classes of β-lactamase, a reduction in outer membrane protein expressions and an acceleration of efflux pump activities, may be involved in the carbapenem resistance of parental clinical isolates of *Acinetobacter* spp. Although the same phenomenon was reported in the IMP-type,[11] it is notable that one amino acid substitution from serine to proline in TMB-2 has drastically decreased the MICs of meropenem and doripenem. As neither patient had a history of international travel nor any epidemiological link, it is possible that bla_{TMB-2} had been endemic in Japan but unrecognized because of its reduced ability to hydrolyse carbapenems. The unrecognized spread of bla_{TMB-2} could be a concern as it can turn to bla_{TMB-1} by only one nucleotide substitution. Although this report discusses only two cases, it may be important to evaluate the spread of this emerging metallo-β-lactamase gene among non-*baumannii Acinetobacter* spp.

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Transparency declarations

None to declare.

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Ability of the VITEK® 2 system to detect group B streptococci with reduced penicillin susceptibility (PRGBS)

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Keywords: group B *Streptococcus*, GBS, penicillin G

Sir,

Group B *Streptococcus* (*Streptococcus agalactiae*, GBS) is the leading cause of neonatal sepsis and meningitis and an important pathogen among elderly people and those suffering from underlying medical disorders.[1,2] The highest GBS mortality and morbidity result from invasive infections in neonates.[1,2]

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Approximately 5% of GBS-infected infants die and survivors often suffer from severe neurological sequelae.[2] Intrapartum antibiotic prophylaxis has been recommended by the CDC[2] and is prescribed for pregnant women who have GBS isolated from vaginal specimens. Since the introduction of prophylaxis, the rate of GBS infection during the first post-natal week has decreased. Penicillins are the first-line agents in the prophylaxis and treatment of GBS infections because all clinical GBS isolates have been considered to be uniformly susceptible to β-lactams, including penicillins.[2,3] However, we identified and characterized several GBS isolates demonstrating reduced penicillin susceptibility (PRGBS) through acquisition of multiple mutations in the penicillin-binding protein 2X (*pbp2x*) gene,[4] and similar isolates were reported in the USA,[5] Canada[6,7] and Japan.[8] After our research was published, EUCAST (http://www.eucast.org/clinical_breakpoints/) defined a clinical penicillin MIC breakpoint for *Streptococcus* groups A, B, C and G, together with the penicillin MIC resistance breakpoint (>0.25 mg/L). The EUCAST breakpoint is higher than the breakpoint for penicillin susceptibility set by the CLSI (≤0.12 mg/L). Until recently, PRGBS were isolated from respiratory specimens, blood, decubitus ulcers and adult hip-joint fluid,[4–8] with no report of PRGBS isolated from neonates or vaginal specimens of pregnant women. The isolation rate of PRGBS from various sources is approximately 2.3% in Japan.[8] The MICs of penicillin G for PRGBS (0.25–1 mg/L) are near the breakpoint set by the CLSI (≤0.12 mg/L). Therefore it is unclear whether automated susceptibility testing machines such as VITEK® 2 can detect PRGBS accurately. Because the VITEK® 2 system is widely used in clinical laboratories in Japan, we used this system as an example in order to evaluate the ability of automated susceptibility testing machines to detect PRGBS.

The MICs of penicillin G were determined for 28 PRGBS using the agar dilution method as per CLSI recommendations.[3] *Streptococcus pneumoniae* ATCC 49619 was used as a quality control for MIC measurements. It was confirmed that these PRGBS harboured the amino acid substitutions in *pbp2x* genes, as described previously.[4,8] We performed the determination of the MICs of penicillin G for 28 PRGBS three times using the VITEK® 2 compact system with AST-P546 cards (bioMérieux Clinical Diagnostics, Marcy l'Étoile, France) in accordance with the manufacturer's instructions.

The results of the comparison between the MICs of penicillin G for 28 PRGBS, as determined by agar dilution and the VITEK® 2 system, are shown in Table 1. Although the MICs determined by the agar dilution method were 0.25–1 mg/L [above the breakpoint (≤0.12 mg/L) set by the CLSI], the MICs determined by the VITEK® 2 system were ≤0.12–1 mg/L. The MICs determined by the VITEK® 2 system were ≤0.12 mg/L in 38 instances (38/84, 45.2%; 84 instances = 28 strains × 3 times). The number of strains for which the MICs determined by the VITEK® 2 system were ≤0.12 mg/L at least two of three times was 13 (13/28, 46.4%).

Table 1. Comparison between MICs of penicillin G for 28 PRGBS determined by agar dilution and by VITEK® 2

Strain	MIC (mg/L) of penicillin G by agar dilution	MIC (mg/L) of penicillin G by VITEK® 2		
		1st	2nd	3rd
B1	0.5	0.25	0.5	0.5
B6	0.25	0.25	0.25	0.5
B7	0.25	≤0.12	≤0.12	≤0.12
B8	0.25	≤0.12	ND	≤0.12
B10	0.5	0.25	0.25	0.25
B12	0.25	ND	0.5	0.25
B40	0.5	0.5	0.5	0.5
B60	0.25	0.25	0.25	0.25
B68	0.5	0.5	0.25	0.5
B502	0.5	≤0.12	≤0.12	≤0.12
B503	0.25	≤0.12	≤0.12	≤0.12
B513	1	0.25	0.25	0.25
B514	0.25	≤0.12	≤0.12	≤0.12
B516	0.25	≤0.12	≤0.12	≤0.12
MRY06-238	0.5	≤0.12	0.25	0.25
MRY06-241	0.25	≤0.12	≤0.12	≤0.12
MRY08-517	0.25	0.25	0.25	0.25
MRY08-527	0.5	0.25	0.25	0.25
MRY08-528	0.25	≤0.12	≤0.12	≤0.12
MRY08-1422	0.25	≤0.12	≤0.12	≤0.12
R1	0.25	≤0.12	ND	≤0.12
R2	0.25	≤0.12	≤0.12	≤0.12
R3	0.25	0.25	0.25	0.25
R4	0.25	≤0.12	0.25	0.25
R5	0.25	≤0.12	≤0.12	≤0.12
R6	0.25	0.25	≤0.12	≤0.12
R7	0.25	0.5	1	0.25
R8	0.5	ND	ND	ND

ND, not determined.

In this study, we investigated the ability of the VITEK® 2 system to detect PRGBS. It detected only half of the PRGBS in this study. Automated susceptibility testing machines such as VITEK® 2 are used in clinical settings worldwide, and these results suggest that many PRGBS may be misclassified as 'susceptible' to penicillin G. We recently revealed that PRGBS tends to be resistant to fluoroquinolones and macrolides, in addition to having reduced penicillin susceptibility,[9] indicating that the classification of susceptibility to penicillin G is very important. The worldwide misclassification of PRGBS as 'susceptible' to penicillin G is undesirable and hinders attempts to clarify the clinical significance of reduced susceptibility to penicillin G.

The MICs of penicillin G for PRGBS (0.25–1 mg/L) are near the 'susceptible' breakpoint (≤0.12 mg/L) set by the CLSI, while the MICs of oxacillin (2–8 mg/L) and ceftizoxime (4–128 mg/L) for PRGBS are higher than those of penicillin-susceptible GBS.[4] However, the VITEK® 2 system AST-P546 cards for *S. agalactiae* do not include MIC determinations of oxacillin or ceftizoxime. We believe that inclusion of these MICs would enable more accurate detection of PRGBS by automated susceptibility testing machines. Moreover, it would be better for these machines to contain systems to alert operators to PRGBS-suspicious isolates when the MICs of penicillin G indicate a range near the susceptibility breakpoint, e.g. at 0.12 mg/L.

Previously we reported that disc diffusion methods using oxacillin, ceftizoxime and ceftibuten were useful for detecting PRGBS.[10] The disc diffusion method for detecting PRGBS does

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not require expensive or specialized equipment. Therefore, prior to any improvements in automated susceptibility testing machines, the disc diffusion method for detecting PRGBS will be useful for clinical microbiological laboratories worldwide.

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Transparency declarations

The authors have no conflicts of interest to declare.
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Ceftaroline in the treatment of concomitant methicillin-resistant and daptomycin-non-susceptible *Staphylococcus aureus* infective endocarditis and osteomyelitis: case report

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Sir,

A middle-aged male presented to the emergency department with fever and 1 week of sharp right hip pain worsening over the past 2 days. His past medical history included uncontrolled diabetes and chronic active hepatitis C. The patient also had a history of multiple methicillin-resistant *Staphylococcus aureus* (MRSA) infections, including bacteraemia 1 month prior, for which he was treated with vancomycin (MIC ≤0.5–1 mg/L).

Three sets of blood cultures were collected before the patient received a single dose of vancomycin and piperacillin/tazobactam. The blood Gram stain showed Gram-positive cocci. The infectious diseases team was consulted, and given recent vancomycin treatment and recurrent bacteraemia, we recommended high-dose daptomycin (8 mg/kg intravenously every 24 h). Dual therapy with rifampicin was considered, but due to limited *in vivo* data as well as concomitant hepatic dysfunction, we decided against it.[1] Initial blood cultures grew MRSA (3/3 bottles) on hospital day 3, with susceptibility tests showing a daptomycin MIC of 0.38 mg/L (Etest; AB Biodisk, Solna, Sweden) and a vancomycin MIC of 1 mg/L by broth microdilution. Repeat blood cultures on day 4 (2/2 bottles) showed no growth.

On hospital day 2, a transoesophageal echocardiogram (TEE) demonstrated no evidence of vegetations. On day 3, a contrast CT of the hip showed bone and retroperitoneal abscesses along with evidence highly suggestive of osteomyelitis. An MRI was less conclusive about the presence of early osteomyelitis. The surgical team was consulted and recommended that the patient to undergo CT-guided drainage of the retroperitoneal abscess as opposed to any surgical treatment, citing difficult surgical access, a high-risk patient with comorbidities and likelihood of the abnormal synovium in radiographic studies being reactive synovitis as opposed to osteomyelitis and septic arthritis. Drainage proceeded on day 7, though there was continued debate about the diagnosis and need for surgical intervention.

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Short Communication

Active Screening of Group B Streptococci with Reduced Penicillin Susceptibility and Altered Serotype Distribution Isolated from Pregnant Women in Kobe, Japan

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SUMMARY: Group B streptococcus (GBS; *Streptococcus agalactiae*) is a leading cause of neonatal invasive infections and was believed to be fully susceptible to penicillin. However, we recently identified several clinical GBS isolates with reduced penicillin susceptibility (PRGBS), which were mainly isolated from respiratory specimens of elderly people. An investigation of both the isolation rate of PRGBS and the serotype distribution among pregnant women is crucial to decisions regarding optimal prevention and strategies for GBS treatment in neonates. We collected 141 GBS isolates from vaginal specimens of 122 pregnant women in a hospital in Kobe, Japan, from 2007 to 2008. Of the 141 GBS isolates, 139 were subjected to antimicrobial susceptibility testing based on the results of screening for PRGBS by the disk diffusion method. All 139 isolates were susceptible to penicillin G, ampicillin, cefotaxime, cefepime, and meropenem; no PRGBS isolates were detected. However, the rates of erythromycin and clindamycin resistance in the isolates were 10.1% and 5.0%, respectively, which are much higher than the values previously reported in Japan. Serotypes VI and VIII accounted for 26% of GBS; a markedly decreased percentage from the rates observed around the year 2000. These findings suggested that penicillin remains an effective means of intrapartum antibiotic prophylaxis in Japan.

Group B streptococcus (GBS; *Streptococcus agalactiae*) is a leading cause of neonatal sepsis and meningitis (1-3). Some neonatal GBS infections occur due to vertical GBS transmission from the vaginal tracts of pregnant women to neonates during delivery; moreover, no GBS vaccines are available (4). Penicillins are used as first-line agents for intrapartum antibiotic prophylaxis and treatment because clinical GBS isolates are uniformly susceptible to β-lactams (3,5). However, in 2008, we identified and molecularly characterized GBS isolates that demonstrated reduced penicillin susceptibility (PRGBS); the isolates contained multiple amino acid substitutions in the penicillin-binding protein 2X (6,7). Similar PRGBS isolates were later reported in the United States (8), Canada (9,10), and Japan (11-13). PRGBS isolates primarily originate from respiratory specimens, blood, decubitus ulcers, and adult hip joint fluid (6-14). The isolation rate of PRGBS in pregnant women in Japan remains unknown.

Multivalent polysaccharide conjugate vaccines against 5 major serotypes (Ia, Ib, II, III, and V) have been proposed (4). A total of 10 GBS serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX) have been identified; serotypes Ia and III represent typical serotypes of neonatal invasive infections isolates (12). In the 1990s and early in the 2000s, GBS serotypes VI and VIII accounted for 60% of all GBS serotypes isolated from pregnant women's vaginal specimens in Japan (15-17). GBS serotypes VI and VIII have been reported to have a pathogenicity comparable to GBS serotypes Ia-III and V (18,19).

All of the clinical isolates in this study were recovered from vaginal specimens obtained from 3 groups of pregnant women who attended the Nishi-Kobe Medical Center between 2007 and 2008. The first group included 34 pregnant women; 34 GBS isolates were obtained between the 23rd and 25th gestational weeks. The second group included 61 pregnant women; 61 GBS isolates were obtained between the 34th and 36th gestational weeks. The third group included 27 pregnant women; a total of 48 GBS isolates were obtained during both of the periods stated above. We selected no more than 1 isolate per woman per time fram. All clinical isolates were confirmed as *S. agalactiae* by β-hemolysis on sheep blood agar plates and agglutination with Lancefield grouping anti-serum (Denka-Seiken, Tokyo, Japan).

The disk diffusion test using oxacillin, ceftizoxime, and ceftibuten disks for detecting PRGBS (20), was performed in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations (5).

The minimum inhibitory concentrations (MICs) of penicillin G, ampicillin, oxacillin, cefazolin, cefotaxime, cefepime, ceftizoxime, meropenem, erythromy-

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cin, and clindamycin were determined by CLSI agar dilution methods (5). *Streptococcus pneumoniae* ATCC49619 was used as the quality control strain.

The serotypes of 141 clinical isolates were determined by the agglutination method with anti-sera (Denka-Seiken) against the Ia, Ib, II, III, IV, V, VI, VII, and VIII capsular types. The serotypes of the 31 clinical isolates that were not typable by the agglutination method were determined genetically by polymerase chain reaction (PCR), as reported elsewhere (21), with minor modifications.

To detect PRGBS, we performed a disk diffusion test developed previously by our group (20); we used 3 Kirby-Bauer disks of oxacillin, ceftizoxime, and ceftibuten. The diameters of the growth-inhibitory zones around each disk for PRGBS were smaller than those for penicillin-susceptible GBS. Although an apparent growth-inhibitory zone appeared around the ceftibuten disk for penicillin-susceptible GBS, similar zones did not appear around the ceftibuten disk for most PRGBS isolates (20). We therefore used this disk diffusion test for the initial PRGBS screening of the 139 clinical isolates. In all 139 isolates, the growth-inhibitory zones around the oxacillin, ceftizoxime, and ceftibuten disks were 17-23, 29-35, and 19-24 mm, respectively; we did not observe any particularly small growth-inhibitory zones around any of the 3 disks. These results imply the absence of PRGBS in these 139 isolates.

We determined the MICs of the 3 antibiotics for each of the 139 isolates by the agar dilution method (Table 1). Previously, we demonstrated considerably high MICs of oxacillin and ceftizoxime for PRGBS (6). We therefore determined the MICs of oxacillin and ceftizoxime as a method of detecting PRGBS. The distributions of the MICs of 8 antibiotics are listed in Table 1. No established criteria exist for the evaluation of oxacillin, cefazolin, and ceftizoxime susceptibility in the CLSI protocol, but all 139 isolates were classified as "susceptible" to penicillin G, ampicillin, cefotaxime, cefepime, and meropenem. This result confirmed the absence of PRGBS in the 139 GBS isolates.

According to the CLSI criteria, 14 clinical isolates (14/139, 10.1%) were resistant to erythromycin and 7 (7/139, 5.0%) to clindamycin. Two isolates were intermediate to erythromycin and 2 to clindamycin. Among the remaining isolates, 123 were susceptible to erythromycin and 130 to clindamycin.

We determined the serotypes of 141 clinical isolates by agglutination methods using anti-serum. The number of GBS isolates for each of the serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII, and non-typable serotype were 10 (7%), 17 (12%), 15 (11%), 14 (10%), 0 (0%), 21 (15%), 19 (13%), 1 (1%), 13 (9%), and 31 (22%), respectively (Fig. 1A). The 31 non-typable isolates were subjected to PCR in order to confirm their genetic serotype. The number of the 31 non-typable GBS isolates for each of the serotypes listed above were 0 (0%), 9 (29%), 3 (10%), 3 (10%), 2 (6%), 8 (26%), 3 (10%), 0 (0%), 2 (6%), and 1 (3%), respectively (Fig. 1B). We combined the results from the agglutination and PCR methods and determined that the number of the 139 isolates for each of the serotypes listed above were 10 (7%), 26 (18%), 18 (13%), 17 (12%), 2 (1%), 29 (21%), 22 (15%), 1 (1%), 15 (11%), and 1 (1%), respectively (Fig. 1C). The 2 GBS isolates that were recovered during the middle and late stages of pregnancy from each of the 18 women who had tested positive for GBS exhibited identical serotypes.

Our study did not detect PRGBS (penicillin MIC, $>0.12\,\mu$g/ml) in any of the 139 GBS isolates, which suggests that the isolation rate of vaginal PRGBS was relatively low ($\leq 0.7\%$) in Japan between 2007 and 2008. However, continuous screening for PRGBS in GBS isolates obtained from pregnant women's vaginal specimens may become increasingly important if neonatal invasive PRGBS emerges.

Erythromycin was the alternative antibiotic to prevent from GBS infections in pregnant women who were

Table 1. Distribution of minimum inhibitory concentrations (MICs), MIC_{50}, and MIC_{90} for 8 β-lactams on 139 clinical isolates

Antibiotic	Distribution of		
	MIC (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
Penicillin G	0.015-0.06	0.03	0.06
Ampicillin	0.03-0.12	0.06	0.12
Oxacillin	0.12-0.5	0.25	0.5
Cefazolin	0.03-0.25	0.12	0.12
Cefotaxime	≤0.03-0.25	0.06	0.06
Cefepime	0.03-0.5	0.12	0.12
Ceftizoxime	0.06-1	0.25	0.25
Meropenem	0.015-0.06	0.03	0.06

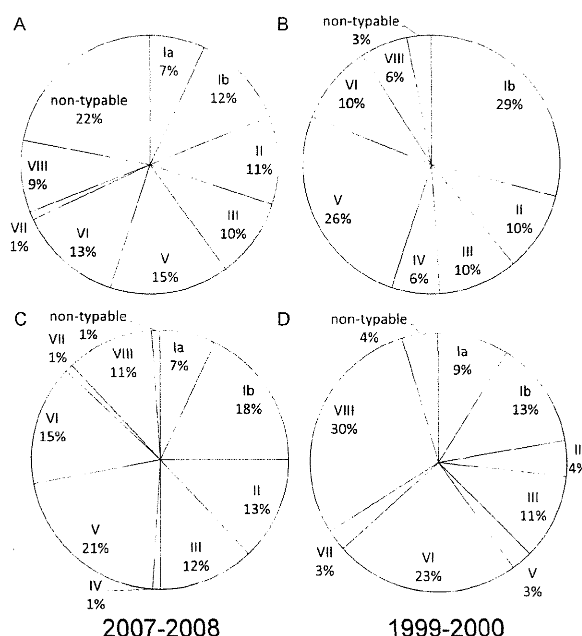

Fig. 1. Serotype distribution. (A) Serotype distribution determined by agglutination methods is represented in a circle graph. (B) Serotype distribution of clinical isolates determined non-typable by agglutination methods was determined by PCR methods. (C) The combined results of serotype distributions determined by agglutination and PCR methods are shown. (D) Serotype distribution of clinical isolates obtained from pregnant women between 1999 and 2000 as reported in ref. 16 is shown.