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Original Article

Genetic Analyses of Beta-Lactamase Negative Ampicillin-Resistant Strains of *Haemophilus influenzae* Isolated in Okinawa, Japan

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SUMMARY: Recently, the instance of β-lactamase-negative ampicillin (AMP)-resistant (BLNAR) strains of *Haemophilus influenzae* has exhibited a marked increase in Japan. Our group determined the MICs of 160 clinical isolates of *H. influenzae* at a university hospital in Okinawa, the southernmost part of Japan, and found that 27 strains were BLNAR, while 24 strains were β-lactamase-producing. Among the latter, 2 strains were resistant to AMP/clavulanic acid. BLNAR strains were shown to be more resistant to cephems than non-BLNAR strains. The competitive affinity assay using biotinylated AMP for penicillin-binding protein (PBP) showed that the binding of cefotiam to PBP 3A/3B of BLNAR strain C2163 was lower than that of the AMP-susceptible strain, while bindings to other PBPs were not changed. The sequences of *ftsI*, the gene encoding transpeptidase domain of PBP 3A and/or PBP 3B, were determined, and it was found that sequences of the *ftsI* gene of BLNAR strains were heterogeneous mutations. Deduced amino acid sequence analyses of BLNAR strains showed that three residues (Asn-526, Val-547, and Asn-569) were replaced with Lys, Ile, and Ser, respectively. In addition, some BLNAR strains had an additional three residues (Met-377, Ser-385, and Leu-389) in *ftsI* replaced with Ile, Thr, and Phe, respectively. Furthermore, changes from Asp-350 to Asn-350 and from Ser-357 to Asn-357 were also found in most BLNAR strains. These substitutions were located around the penicillin binding sites of PBP3. Multiple substitutions in the amino acid sequence seemed to be closely related with extended resistance against β-lactams, including third-generation cephems. Randomly amplified polymorphism DNA fingerprinting of clinical isolates of BLNAR strains showed genetic heterogeneity of the strains, suggesting that the prevalence of BLNAR in this region was a result of the emergence of multiple clones of this phenotype.

INTRODUCTION

Haemophilus influenzae is one of the important pathogens that cause both acute and chronic respiratory tract infections in both adults and children. Furthermore, the type b serotype of this bacterium may cause severe meningitis in children. *H. influenzae* may acquire ampicillin (AMP) resistance by means of two different mechanisms. One is the production of β-lactamases, referred to as TEM-1 (1), and ROB-1 (2), which hydrolyse AMP enzymatically. Another is the conformational change of penicillin-binding proteins (PBPs), the enzyme for peptideglycan synthesis, resulting in reduced affinity with AMP (3-5). Strains with resistance due to the latter mechanism were isolated in 1978-1980 in New Zealand for the first time (3), and termed β-lactamase-negative AMP-resistant (BLNAR) *H. influenzae*.

Genetic analysis revealed that BLNAR strains have several amino acid substitutions in the *ftsI* gene product, PBP 3A and/or PBP 3B. The transformation of the AMP-susceptible strain with the *ftsI* gene from the BLNAR strain results in the elevation of MIC for AMP and other β-lactams (6). According to the genetic characterization of BLNAR isolates in France (7), amino acid substitutions in PBP3A/B of BLNAR strains are diverse, and BLNAR strains were classified into six groups: I, IIa, IIb, IIc, IId, and III. Recently, Hasegawa et al. (8) also found that there are at least six groups of deduced amino acid substitution in the *ftsI* gene and corresponding phenotypes.

In the United States, the proportion of BLNAR isolates remains very low, while during the last decade the proportion of BLNAR isolates has rapidly increased in Japan (8,9). Recently, Nakasone et al. found that the incidence of BLNAR was up to 37.9% during 1999-2000 in Okinawa, the southernmost part of Japan (10). These BLNAR strains show more resistance to second- and third-generation cephems than do non-BLNAR strains, which may cause serious clinical problems. Therefore, it is important to determine out how the organism acquired the resistance and how BLNAR spreads in this region. The present study includes: (i) a survey of the antimicrobial susceptibilities of clinical isolates of *H. influenzae* in Okinawa, Japan, (ii) an analysis of PBP affinity to β-lactam in a representative BLNAR isolate, and (iii) a determination of the *ftsI* gene sequence and randomly amplified polymorphic DNA (RAPD) fingerprinting of BLNAR isolates. This study suggests the emergence of multiple clones of BLNAR strains in this area. This study also provided additional information concerning point mutations within the *ftsI* gene in BLNAR strains.

MATERIALS AND METHODS

Clinical isolates: The strains used in this study were col-

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lected in an affiliate hospital of the University of the Ryukyus Medical School in Okinawa during 1995-2000. BLNAR strains isolated in other hospitals in Okinawa were also included in the RAPD study. All strains were isolated from respiratory specimens and identified using conventional techniques, including the requirement of β-NAD^+ (V factor) and hemin (X factor) for growth. The production of β-lactamase was confirmed by a nitrocefin test (Showa Chemical Inc., Tokyo, Japan) with whole cells. *H. influenzae* ATCC49766 (AMP-susceptible) and *H. influenzae* ATCC49247 (BLNAR) strains were used as controls.

Media and antimicrobials: The medium used for determining the MIC was Mueller-Hinton broth (Difco Laboratories, Detroit, Mich., USA) supplemented with 2% defibrinated and heat-treated horse blood, 0.5% yeast extract, and β-NAD^+ (15 μg/mL). Chocolate agar plates (Kyokuto Co., Tokyo, Japan) were routinely used for bacterial growth. Plate and broth cultures were incubated at 37°C in 5% CO_2 air. The antimicrobial agents used in this study were as follows: AMP (Meiji Seika Kaisya, Ltd., Tokyo, Japan), AMP with clavulanic acid (AMP/CVA) (GlaxoSmithKline KK, Tokyo, Japan), piperacillin (PIPC) (Toyama Chemical Co. Ltd., Toyama, Japan), cefazolin (CEZ) (Nippon Roche KK, Tokyo, Japan), cefotiam (CTM) (Takeda Chemical Industries Ltd., Osaka, Japan), ceftizoxime (CZX) (Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan), cefotaxime (CTX) (Aventis Pharma, Tokyo, Japan), ceftazidime (CAZ) (GlaxoSmithKline KK), cefpirome (CPR) (Takeda Pharmaceuticals), imipenem (IPM) (Banyu Seiyaku Co., Ltd., Tokyo, Japan), meropaenem (MEPM) (Sumitomo Pharmaceutical Co., Ltd., Osaka, Japan), aztreonam (AZT) (Takeda Pharmaceuticals), and levofloxacin (LVFX) (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan).

Antimicrobial susceptibility testing: Antimicrobial susceptibility was determined by the twofold broth microdilution method according to guidelines set by the National Committee for Clinical Laboratory Standards (11). Bacterial suspensions were inoculated with an automatic MIC-2000 inoculator (Dynatech Laboratories, Inc., Alexandria, Va., USA). Final inocula contained approximately 5×10^5 CFU/mL. MIC was defined as the lowest concentration of antibiotic that inhibited visible growth. Microplates were examined after 20 h of incubation at 37°C in 5% CO_2.

PBP profiles and PBP affinities to β-lactams: Membrane preparations of *H. influenzae* and PBP binding reactions with [^{3}H]-penicillin (Amersham Bioscience Co., Piscataway, N. J., USA) or biotinylated AMP (BIO-AMP) were performed as described previously (12). BIO-AMP was always freshly prepared before use. A mixture of bacteria membrane preparations and the labeled drug was subjected to 10% SDS-PAGE, and the proteins were then transferred to a nitrocellulose membrane. After blotting, the membrane was incubated in a blocking agent and then quickly rinsed. A streptavidin-peroxidase conjugate (Boehringer Mannheim Canada, Dorval, Quebec, Canada) was then allowed to bind to BIO-AMP-PBP complexes on blots for 1 h at room temperature. The unbound conjugate was removed by several washes. The detection of peroxidase-streptavidin-BIO-AMP-PBP complexes on nitrocellulose membranes was performed by the chemiluminescence of oxidized luminol, which was catalyzed by peroxidase in the presence of hydrogen peroxide (ECL western blotting detection system). Oxidized luminol provokes a subsequent light emission that can be visualized on a film. In competition experiments, the membrane preparations were incubated with various concentrations of a competing β-lactam (CTM) for 10 min, prior to the addition of 10 μg of BIO-AMP per mL for an additional incubation period of 45 min at room temperature. The samples were then processed as stated above.

Table 1. Primers for *ftsI* gene sequencing

Set A	PBP3-1-U872	5′-AAG TGT TAG CTA TGG CGA CTG-3′
	PBP3-1-L1267	5′-CCA GCG TTT ACG ATT TGC -3′
Set B	PBP3-2-U1008	5′-CGC ACT TCA ACG AGG TG-3′
	PBP3-2-L1402	5′-AAC TGG CGG ATC AAC TTT AGT-3′
Set C	PBP3-3-U1404	5′-TAA AGT TGA TCC GCC AGT TAT-3′
	PBP3-3-L1800	5′-GAT TCT TGT GTT CGC CAA TA-3′

Sequencing: The 1 kb fragment encoding the PBP 3 transpeptidase domain was amplified from the genomic DNA of *H. influenzae* by PCR. Genomic DNA was prepared from several colonies of *H. influenzae* grown on a chocolate agar plate by boiling with Chelex-100 (Bio-Rad, Hercules, Calif., USA). Subsequently, 5 μl of the extract was added to 50 μl of a PCR solution (1 × PCR buffer, 200 μM of each dNTP, 2.5 U of *Taq* polymerase [Takara Biomedical, Kyoto, Japan], and 0.5 μM of sense and reverse primers). Three sets of primers were used for sequencing the *ftsI* gene (Table 1). PCR conditions were as follows: 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. The PCR products were electrophoresed on an agarose gel to confirm the presence of the product, and were then purified with a PCR purification kit (QIAGEN Sciences, Germantown, Md., USA) to prepare a sequencing template. The sequencing reaction was conducted with a Rhodamine Terminator Cycle Sequencing FS Ready Reaction kit (PE Biosystems, Foster, Calif., USA). The reaction mixtures were placed in a thermal cycler and denatured at 94°C for 2 min. They were then subjected to 25 PCR cycles, each of which were at 94°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. The nucleotide sequences were determined with an ABI PRISM377 DNA sequencer.

RAPD fingerprinting: RAPD fingerprints were generated by using primer AP2 (5′-ATG TAA GCT CCT GGG GAT TCT C-3′) (Amersham Biosciences, Uppsala, Sweden). This primer was arbitrarily chosen from enterobacterial repetitive intergenic consensus sequences, and successfully used for *H. influenzae* genotyping (13). The PCR amplification included 45 cycles of 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min. Amplification products were separated through 2% agarose gels at 100 V, with bromophenol blue as the tracking dye. One hundred base pair DNA ladders as external reference standards were run in each gel. DNA fragments were visualized by staining with ethidium bromide for 30 min. Patterns of DNA fragments were recorded and stored as TIFF formatted image files with image capture equipment for UV trans-illuminator (FAS-III MINI; Toyobo, Osaka, Japan). The relatedness of RAPD patterns was analyzed using the Diversity Database (PDI, Inc., New York, N.Y., USA).

RESULTS

Antimicrobial susceptibility of *H. influenzae* and characterization of BLNAR strains: A total of 160 clinical isolates of *H. influenzae* were collected. MICs of 11 β-lactams and LVFX were determined for these isolates (Table 2). Among them, 27 isolates showed the BLNAR phenotype (MIC of AMP ≥2 μg/mL and β-lactamase negative). Twenty-four strains were β-lactamase positive and AMP-resistant

Table 2. MIC distribution of *H. influenzae* for various antimicrobials

Phenotype (strains)	MIC_{50}/MIC_{90}		
	BLNAS (109)	BLNAR (27)	BLPAR (24)
AMP	0.25/1	2/4	16/≥32
AMP/CVA*	0.25/1	2/4	1/4
PIPC	≤0.125/≤0.125	≤0.125/≤0.125	4/≥32
CEZ	4/≥32	≥32/≥32	4/≥32
CTM	1/8	≥32/≥32	2/≥32
CZX	≤0.125/0.5	1/4	≤0.125/0.5
CPR	≤0.125/0.25	0.5/0.5	≤0.125/0.25
CAZ	≤0.125/1	1/2	≤0.125/1
AZT	≤0.125/1	2/2	≤0.125/1
IPM	0.5/1	1/4	1/4
MEPM	≤0.125/0.25	0.25/0.25	≤0.125/0.25
LVFX	≤0.125/≤0.125	≤0.125/≤0.125	≤0.125/≤0.125

AMP, ampicillin; CVA, clavulanic acid; PIPC, piperacillin; CEZ, cefazolin; CTM, cefotiam; CZX, ceftizoxime; CPR, cefpirome; CAZ, ceftazidime; AZT, aztreonam; IPM, imipenem; MEPM, meropaenem; LVFX, levofloxacin; BLNAS, β-lactamase negative AMP susceptible; BLNAR, β-lactamase negative AMP resistant; BLPAR, β-lactamase positive AMP resistant.
*: MIC of AMP/CVA was shown as concentration of AMP.

(BLPAR). In nine of them, the MIC of AMP/CVA was relatively high (MIC of AMP/CVA ≥2/1 μg/mL). BLNAR strains were more resistant to all cephems and carbapenems than non-BLNAR and BLPAR strains, while all strains were susceptible to LVFX (Table 2). Four isolates were CZX-resistant (MIC ≥4 μg/mL), and these were all BLNAR- or AMP/CVA-resistant.

PBP profiles and binding of CTM to PBPs: Figure 1 shows the PBP profiles of BLNAR strain C2163 and of AMP-susceptible strain ATCC49766. PBPs were named according to a previous report (14). In BLNAR strain C2163, for which the AMP MIC was 2 μg/mL, the binding of [^{3}H]-PEN to PBP 3A/3B was not observed (Figure 1A), but that of BIO-AMP was observed (Figure 1B) when compared with ATCC49766. The competitive assay for PBPs was carried out using BIO-AMP. Figure 2 shows fluorograms of the PBPs of *H. influenzae* ATCC49766 and BLNAR strain C2163, which were pretreated with various concentrations of non-labeled CTM. The competition assay using BIO-AMP showed that the band of PBP 3A/3B in C2163 was not diminished by the addition of 10 μg/mL of CTM, while the band of PBP 3A/3B in ATCC49766 was diminished by the addition of 1 μg/mL of CTM (Figure 2). This finding suggested that the PBP3A/3B affinity to CTM was apparently attenuated in BLNAR strain C2163. The competition assay using [^{3}H]-PEN showed a faint band of PBP 3A/3B in BLNAR strain C2163, and it was very difficult to interpret the inhibition by CTM pretreatment in the BLNAR strain (data not shown).

Nucleotide and deduced amino acid sequences of the *ftsI* gene: The nucleotide sequences of the *ftsI* gene were determined by direct sequencing for 20 BLNAR strains, 5 AMP-susceptible strains, and 2 control strains. The deduced amino acid sequences of AMP-susceptible strain ATCC49766, BLNAR strain ATCC49247, and representative BLNAS and BLNAR clinical isolates were compared (Table 3). Sequences of the active site (position 327-330) and the penicillin binding sites (positions 379-381 and 512-514) were preserved in all strains tested. In BLNAR strains, three residues (Asn-526, Val-547, and Asn-569) were replaced with Lys, Ile, and Ser, respectively. These substitutions also seemed to be

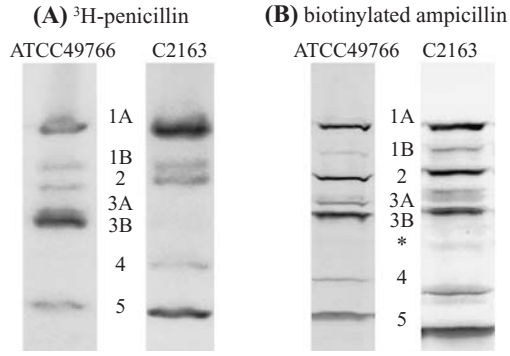

Fig. 1. PBP profiles of *H. influenzae* ATCC49766 (AMP susceptible) and C2163 (BLNAR phenotype) detected by autoradiography and chemiluminescence. Membrane fractions were labeled with 4 μg/mL of ^{3}H-penicillin (A) or biotinylated AMP (B). Samples were separated by SDS-PAGE, and the gels were dried for the detection of radio-labeled PBPs, or the proteins were transferred to a nitrocellulose membrane for the detection of biotinylated PBPs. The PBPs of *H. influenzae* are shown in the center of each panel. Note that a PBP* was visualized only by biotinylated AMP.

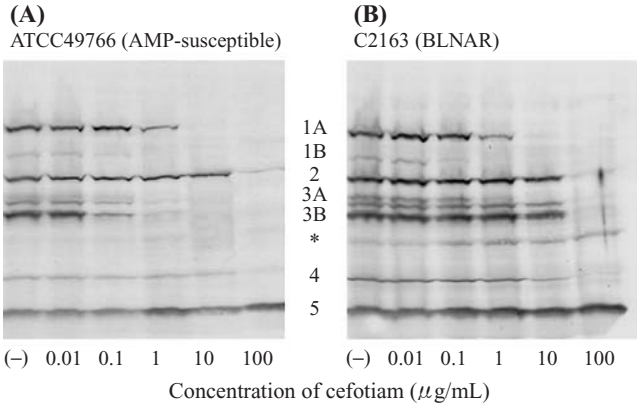

Fig. 2. PBP profiles of *H. influenzae* ATCC49766 (AMP susceptible) (A) and C2163 (BLNAR phenotype) (B) obtained in competition assays with cefotiam. Membrane fractions were first treated with the unlabeled competing drug, cefotiam. The fractions were then labeled with biotinylated AMP. Proteins were separated by SDS-PAGE before the detection of PBPs by a chemiluminescence reaction. The PBPs of *H. influenzae* are indicated in the center.

important for resistance to CTM. Furthermore, BLNAR strains that were resistant to CZX and CPDX, third-generation cephems, had three residues (Met-377, Ser-385, and Leu-389) in *ftsI* replaced with Ile, Thr, and Phe, respectively. In addition, changes of Asp-350 to Asn-350 and Ser-357 to Asn-357 were found in almost all of these BLNAR strains. These substitutions found in BLNAR strains were located around the penicillin-binding sites. According to the classification of the amino acid substitution of PBP3A/3B (7), most BLNAR clinical strains tested were classified into group III (Table 3). Multiple substitutions in amino acid sequence seemed to result in extended resistance against β-lactams, including third-generation cephems.

RAPD fingerprints: The genomic DNAs prepared from 34 isolates of BLNAR *H. influenzae* were amplified with the arbitrary primer AP2. All the major and minor bands that were reproducible by repeated experiments were scored. RAPD fingerprints of 34 BLNAR strains were classified into 31 groups (Figure 3). Two of three pairs of BLNAR strains with the same RAPD pattern showed a high homology of the deduced amino acid sequence of the *ftsI* gene, suggesting

Table 3. Substitutions in deduced amino acid sequences of *ftsI* gene of *Haemophilus influenzae* strains and MICs of four β-lactams for the strains

Rd	Amino acid substitution												MIC			
	350 Asp	352 Ser	357 Ser	377 Met	385 Ser	389 Leu	511 Val	526 Asn	547 Val	562 Val	569 Asn	586 Ala	AMP	CTM	CZX	CPDX
ATCC49766													0.5	1	≦0.063	≦0.063
ATCC49247								Lys	Ile		Ser		4	2	0.5	0.5
C1396													0.25	0.5	≦0.063	0.125
A3490													1	0.5	≦0.063	0.125
B4161													1	0.5	≦0.063	0.125
A0674											Ser		0.25	0.5	≦0.063	0.125
YD32									Ile				1	1	≦0.063	0.125
B4040								Lys	Ile		Ser	Ser	2	16	0.25	0.25
B3844								Lys	Ile		Ser	Ser	2	16	0.25	0.25
B0120	Asn							Lys			Ser		2	8	0.25	0.25
B4230	Asn							Lys	Ile		Ser		2	16	0.125	0.25
C1138	Asn		Asn					Lys	Ile		Ser	Ser	2	4	0.25	0.25
B0023	Asn		Asn					Lys	Ile		Ser	Ser	2	≧16	0.25	0.5
C5258	Asn		Asn				Ala	Lys	Ile		Ser	Ser	2	≧16	0.5	1
A3791	Asn		Asn	Ile	Thr	Phe		Lys	Ile	Leu	Ser	Ser	4	≧16	1	1
C5827	Asn		Asn	Ile	Tht	Phe		Lys	Ile	Leu	Ser	Ser	2	4	1	2
A1498	Asn		Asn	Ile	Thr	Phe		Lys	Ile	Leu	Ser	Ser	2	≧16	1	2
YG62	Asn		Asn	Ile	Thr	Phe		Lys	Ile	Leu	Ser	Ser	2	≧16	1	2
A1498	Asn		Asn	Ile	Thr	Phe		Lys	Ile	Leu	Ser	Ser	2	≧16	1	2
YG59	Asn		Asn	Ile	Thr	Phe		Lys	Ile	Leu	Ser	Ser	4	8	1	2
C6204	Asn		Asn	Ile	Thr	Phe		Lys	Ile	Leu	Ser	Ser	4	≧16	1	2
C2163	Asn	Phe	Asn	Ile	Thr	Phe		Lys	Ile	Leu	Ser	Ser	2	≧16	0.5	4
C5983	Asn		Asn	Ile	Thr	Phe		Lys	Ile	Leu	Ser	Ser	2	≧16	1	4
A2992	Asn		Asn	Ile	Thr	Phe		Lys	Ile	Leu	Ser	Ser	2	≧16	2	4
A2249	Asn		Asn	Ile	Thr	Phe		Lys	Ile	Leu	Ser	Ser	2	≧16	1	8
A2329	Asn		Asn	Ile	Thr	Phe		Lys	Ile	Leu	Ser	Ser	2	≧16	4	4
C0116	Asn		Asn	Ile	Thr	Phe		Lys	Ile	Leu	Ser	Ser	8	≧16	4	8

AMP, ampicillin; CTM, cefotiam; CZX, ceftizoxime; CPDX, cefpodoxime.
All strains shown in Table 3 were β-lactamase negative. Strains of which MIC of AMP was ≥2 mg/L were BLNAR phenotype.

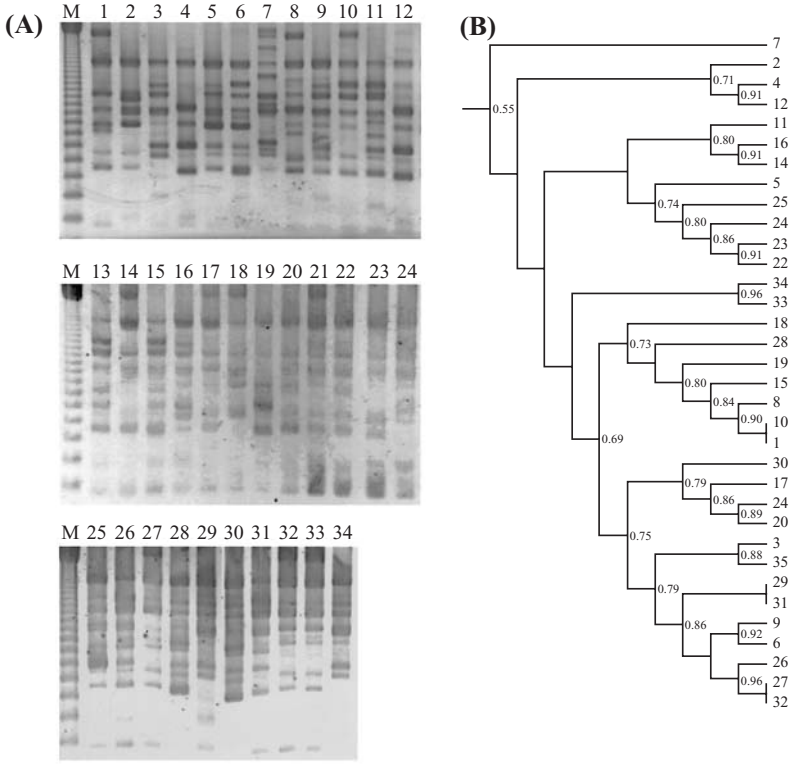

Fig. 3. Demonstration of RAPD fingerprints of representative BLNAR strains isolated in Okinawa Prefecture. (A) RAPD fingerprints of 34 BLNAR strains. (B) Dendrogram of RAPD fingerprints of BLNAR strains. The clustering pattern was generated using the UPGMA method. The 34 strains were classified into 31 groups.

that the strains were almost identical. For other strains, no apparent relationship between RAPD patterns and *ftsI* mutations was noted (data not shown).

DISCUSSION

We surveyed the antimicrobial susceptibilities of clinical isolates of *H. influenzae* from Okinawa Prefecture. The ratio of strains showing the BLNAR phenotype (MIC of AMP ≥2 μg/mL and β-lactamase negative) was extremely high. A recent study in the United States describes an unusually high level of AMP-resistant BLNAR strains (15), but our survey did not show such a strain in this region. These BLNAR strains were more resistant to cephems than non-BLNAR strains, suggesting that the same mechanism was responsible for BLNAR and resistance to these cephems. PIPC is reported to have potent activity against BLNAR strains (16), and our study confirmed that finding. However, we should be note that β-lactamase-producing strains were completely resistant to PIPC. It is noteworthy that several BLPACR strains were identified in this study. BLPACR strains were resistant to ABPC/CVA, PIPC, and possibly to third-generation cephem, and its increase may cause the clinical practice regarding respiratory infections to deteriorate. Matic et al. (17) reported that two BLPACR strains had TEM-1 type β-lactamase and multiple mutations within the *ftsI* gene. The resistant mechanisms of BLPACR strains found in this study should be studied in the near future.

The competitive affinity assay of CTM, a second-generation cephem, using biotinylated AMP (BIO-AMP) for PBP showed that bindings of CTM to PBP 3A and 3B were decreased in BLNAR strain C2163, while bindings of CTM to other PBPs were not decreased. Our data supported previous reports suggesting that the reduced affinity of PBP 3A/3B is responsible for the BLNAR phenotype (6,7). Our study showed that the BIO-AMP method was more suitable for detecting PBP 3A/3B of BLNAR *H. influenzae* than was the [^{3}H]-PEN method. Subsequently, we determined the sequence of the *ftsI* gene, which encodes PBP3A and/or PBP3B, of collected BLNAR strains. According to the classification proposed by Dabernat et al. (7), most strains belonged to class III, which corresponds to class VI in the classification proposed by Ubukata et al. (6). Our study, taken together with other reports, demonstrated that the point mutations within the *ftsI* gene were the cause of the emergence of the BLNAR phenotype. Recently, it was shown using site-directed mutagenesis and gene recombinants that the PBP 3 mutations R517H, N526K, S385T, and L389F are responsible for the β-lactam resistance (18). To the contrary, no mutation in R517 was noted in β-lactam-resistant strains in our study. Instead, additional mutations in β-lactam-resistant strains such as S357N and M376I were shown. The significance of each amino acid substitution requires further investigation.

In a previous study, genetic and phenotypic diversity of BLNAR nontypeable *H. influezae* strains from geographically diverse regions were shown (4). We questioned whether the outbreak of BLNAR in a relatively small area was due to a few clone expansions or simultaneous occurrence of mutations in multiple clones. Therefore, RAPD fingerprinting of the BLNAR isolates was analyzed, and multiple clones of BLNAR *H. influenzae* were discovered among the isolates. To the contrary, we also found three pairs of the same RAPD pattern from different patients, which suggested the possible transmission of BLNAR within a small population, such as family members, as previously reported (19). These findings imply that selection pressure such as antimicrobial use may be the impetus for BLNAR prevalence in this area. Considering that several AMP/CVA-resistant strains were also identified, it is possible that overuse of cephems is the selection pressure. Recently, clonal expansion of BLNAR phenotype *H. influenzae* type b strains among isolates from patients with meningitis in Japan has been suggested (8), but it should be considered that type b *H. influenzae* strains themselves show limited genetic diversity (20). Serotypes of encapsulated *H. influenzae* are closely related to genetic relationship (21).

In summary, BLNAR has been rapidly increasing in Okinawa Prefecture. These BLNAR strains are more resistant to second- and third-generation cephems than are non-BLNAR strains. Genetic analysis revealed that the molecular mechanism of BLNAR in this region was similar to the molecular mechanisms of BLNAR isolates in other places. Our findings show the genetic heterogeneity of BLNAR strains of *H. influenzae* from Okinawa Prefecture, suggesting that the prevalence of BLNAR in this region is a result of the emergence of multiple clones of this phenotype.

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