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EPIDEMIOLOGY AND SURVEILLANCE

Multiclonal Expansion and High Prevalence of β-Lactamase-Negative *Haemophilus influenzae* with High-Level Ampicillin Resistance in Japan and Susceptibility to Quinolones

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ABSTRACT β-Lactam-resistant *Haemophilus influenzae* is a clinical concern. A high prevalence (>40%) of β-lactamase-negative high-level ampicillin-resistant *H. influenzae* (high-BLNAR) isolates in Japan has been reported. However, the reasons for the expansion are unknown. High-BLNAR strains possess an amino acid substitution, either Asn526Lys (group III) or Arg517His (group III-like) in addition to Ser385Thr, in penicillin-binding protein 3 (PBP3). To determine the current prevalence of high-BLNAR strains and the mechanisms behind their expansion in Japan, their prevalence, PBP3 types, multilocus sequence types, and susceptibilities to quinolones approved in Japan as alternatives were determined. Sixty percent of *H. influenzae* clinical isolates (62/104 isolates) were β-lactamase-negative ampicillin-resistant *H. influenzae* (BLNAR) strains. Among BLNAR isolates, 92% (57/62 isolates) were high-BLNAR strains. Most isolates were classified as belonging to group III, which contained many genotypes (11 PBP3 types and 25 sequence types). These results indicated that the expansion of high-BLNAR isolates was multiclonal and such strains are still predominant in Japanese clinical settings. One high-BLNAR isolate harbored the novel amino acid substitution Asn526Met in addition to Ser385Thr in PBP3, suggesting a new group (group IV). No quinolone-resistant *H. influenzae* isolates were identified. The MICs for the quinolones (moxifloxacin, garenoxacin, and tosufloxacin) were similar to that for levofloxacin, whereas sitafloxacin exhibited a lower MIC. However, we obtained 4 *H. influenzae* isolates with decreased quinolone susceptibility with the amino acid substitution Ser84Leu in GyrA, and 3 of those isolates were high-BLNAR isolates. In summary, this study shows that multiclonal high-BLNAR strains predominate in a Japanese university hospital. Isolates remain sensitive to quinolones, but vigilance is required to prevent the development of fluoroquinolone resistance in high-BLNAR strains.

KEYWORDS antimicrobial resistance, penicillin-binding proteins, *Haemophilus influenzae*

Haemophilus influenzae is a causal pathogen of community-acquired infections such as pneumonia, otitis media, sinusitis, and meningitis (1, 2). Recent 16S rRNA-targeted analysis of the microbiota of bronchoalveolar lavage fluid samples from

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patients with community-acquired pneumonia revealed that *H. influenzae* was the most prevalent bacterium detected, along with *Streptococcus pneumoniae* (3).

The increasing prevalence of β-lactam-resistant *H. influenzae* is a clinical concern worldwide. Two mechanisms are involved in the acquisition of β-lactam resistance in *H. influenzae*. One mechanism is the acquisition of β-lactamase genes such as bla_{TEM-1} and bla_{ROB-1}, and such strains are referred to as β-lactamase-positive ampicillin-resistant *H. influenzae* (BLPAR) strains (4, 5). BLPAR isolates with bla_{TEM-1} are predominant in Japan (6). The other mechanism is a mutation in *ftsI*, which encodes penicillin-binding protein 3 (PBP3), and strains are called β-lactamase-negative ampicillin-resistant *H. influenzae* (BLNAR) strains. Currently, BLNAR strains are more prevalent than BLPAR strains in many countries (6–11).

BLNAR strains are further classified into 4 groups, I, II, III, and III-like, as defined by the nature of amino acid substitutions in PBP3 (7, 12, 13). Isolates harboring the amino acid substitutions Arg517His and Asn526Lys near the conserved Lys-Thr-Gly (KTG) motif of PBP3 are classified as group I and group II, respectively. These are called β-lactamase-negative low-level ampicillin-resistant *H. influenzae* (low-BLNAR) isolates and exhibit decreased susceptibility to ampicillin, for which the MIC is typically 1 mg/liter, compared with β-lactamase-negative ampicillin-susceptible (BLNAS) isolates, with an ampicillin MIC of 0.25 mg/liter (12).

Isolates harboring amino acid substitutions near the Ser-Ser-Asn (SSN) motif, such as Ser385Thr, Ser357Asn, Met377Ile, and Leu389Phe, in addition to the amino substitution Asn526Lys, are classified as group III (12). Garcia-Cobos et al. reported a subgroup of group III, designated group III-like (7). Group III-like isolates possess the amino acid substitution Arg517His instead of the Asn526Lys substitution in group III (7). Group III and group III-like isolates are called β-lactamase-negative high-level ampicillin-resistant *H. influenzae* (high-BLNAR) strains and exhibit slightly higher ampicillin MICs (2 mg/liter) and >2-fold higher MICs for some cephems, such as cefuroxime, cefotaxime, and cefpodoxime, compared to low-BLNAR strains (7, 12).

A recent study indicated that BLNAR clinical isolates are largely classified as group III or group III-like through acquisition of Ar526His or Asn517His with Ser385Thr, respectively, because these substitutions are the main contributors of β-lactam resistance (14). In addition, BLNAR isolates with β-lactamase genes are referred to as β-lactamase-producing amoxicillin-clavulanate-resistant *H. influenzae* (BLPACR) strains (15–17).

In the past, low-BLNAR isolates were clinically predominant (10, 11, 15, 18, 19). However, high-BLNAR isolates have emerged since 1998 (19). Whereas low-BLNAR isolates constitute the majority of BLNAR strains in the United States and many European countries (7, 9–11, 14, 15), high-BLNAR isolates have significantly increased in prevalence in Japan and South Korea since 2005 (6, 8, 13, 20–23). According to the latest 2011 data in Japan, the prevalence of high-BLNAR isolates was 42.6% of *H. influenzae* isolates overall and 91.2% of all BLNAR isolates (22). These results reflect a serious issue in Japanese clinics with respect to the use of β-lactam antimicrobials for the treatment of *H. influenzae* infections. However, the situation with respect to the expansion of high-BLNAR strains in Japanese clinical settings has not been evaluated since 2011 (22), and the reasons for the high prevalence of high-BLNAR strains in Japan remain unknown.

Macrolides and quinolones (especially fluoroquinolones such as ciprofloxacin and levofloxacin) are used as alternative antimicrobials for the treatment of β-lactam-resistant *H. influenzae* infections. In addition to these agents, quinolones such as moxifloxacin and garenoxacin have been approved for treatment of respiratory infections and tosufloxacin for pediatric use in Japan. Furthermore, sitafloxacin is an approved quinolone in Japan for the treatment of severe bacterial infections, because it has greater antimicrobial activity against various bacteria than do other quinolones (24). It is important to understand the susceptibilities to the series of quinolones when making a secondary choice of antimicrobials for the treatment of infections caused by high-BLNAR isolates. In this study, we collected *H. influenzae* clinical isolates in a

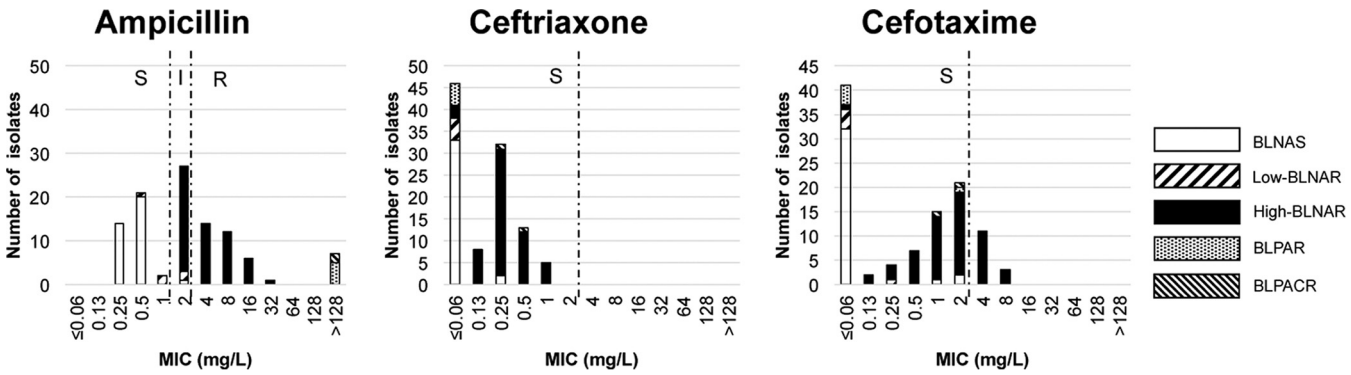


FIG 1 Susceptibilities to β-lactams and the genetic types of β-lactam resistance in *H. influenzae* clinical isolates. S, susceptible; I, intermediate; R, resistant.

Japanese university hospital from 2016 to 2018 and investigated (i) the current prevalence of high-BLNAR strains, (ii) the clonality of high-BLNAR strains by genotypic analysis, and (iii) the susceptibility to various quinolones, to reveal the effectiveness of secondary-choice antimicrobials for treatment of high-BLNAR infections.

RESULTS

β-Lactam susceptibilities, PBP3 genogroups, and possession of β-lactamase genes. In total, 104 *H. influenzae* clinical isolates were obtained, all of which were nontypeable by serotyping. The ampicillin MIC_{50} and MIC_{90} values were 2 and 8 mg/liter, respectively, and the prevalence of ampicillin-resistant isolates was 37.5% (Fig. 1). The ceftriaxone MIC_{50} and MIC_{90} values were 0.13 and 0.5 mg/liter, respectively, and ceftriaxone-nonsusceptible isolates were not observed. The cefotaxime MIC_{50} and MIC_{90} values were 0.5 and 4 mg/liter, respectively, and the prevalence of cefotaxime-nonsusceptible isolates was 13.5%.

The 104 isolates were classified by BLNAS/BLNAR genotype based on amino acid substitutions in PBP3 (Table 1). Sixty-two *H. influenzae* isolates (59.6%) were identified as being BLNAR because they possessed either Arg517His or Asn526Lys in PBP3. There were no significant differences in data on patient gender, patient age, and specimen type between sources of BLNAS and BLNAR isolates (see Table S1 in the supplemental material).

The BLNAR isolates were classified into 4 groups (groups I, II, III, and III-like) on the basis of the presence of the amino acid substitutions Arg517His, Asn526Lys, and Ser385Thr in PBP3 (Table 2). Five isolates (4.8% of the total *H. influenzae* isolates) were low-BLNAR strains, and they belonged to group II, possessing Asn526Lys. There were no isolates belonging to group I, possessing Arg517His. In contrast, 57 isolates (54.8% of all *H. influenzae* isolates and 91.9% of BLNAR isolates) were high-BLNAR strains (Table 1). The high-BLNAR isolates were further divided into group III, possessing Ser385Thr and Asn526Lys, and group III-like, possessing Ser385Thr and Asn517His. Group III accounted for 45.2% of all *H. influenzae* isolates and 75.8% of BLNAR isolates, and group

TABLE 1 Classification of BLNAS, low-BLNAR, and high-BLNAR isolates and their susceptibilities to β-lactams[a]

			MIC (mg/liter)											
			Ampicillin				Ceftriaxone				Cefotaxime			
Genotype	Group	No. of strains	Geometric mean	MIC_{50}	MIC_{90}	Range	Geometric mean	MIC_{50}	MIC_{90}	Range	Geometric mean	MIC_{50}	MIC_{90}	Range
BLNAS		35	0.4	0.5	0.5	0.35–0.5	≤0.06	≤0.06	≤0.06	≤0.06–0.25	≤0.06	≤0.06	≤0.06	≤0.06–2
Low-BLNAR	II	5	1.15	1	2	0.5–2	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06–0.25
High-BLNAR	III	47	3.77	4	8	2–16	0.28	0.25	1	≤0.06–1	1.34	2	4	≤0.06–8
High-BLNAR	III-like	9	5.44	8	16	2–16	0.16	0.25	0.25	≤0.06–0.5	2.59	4	8	0.25–8

[a]Five BLPAR isolates and 2 BLPACR isolates possessed bla_{TEM-1} and were excluded from this table. The PBP3 type of the BLPACR isolates was group III. The high-BLNAR isolate SMHi90 was excluded because of its unique *ftsI* sequence (see Fig. S1 and S2 in the supplemental material).

TABLE 2 Amino acid substitutions in PBP3 in BLNAR and BLPACR isolates[a]

Genotype	Group	No. of strains	MIC (mg/liter)			Amino acid substitution(s) in PBP3																							
			AMP	CTR	CTX	D350	S357	M377	S385	L389	V436	A437	A502	R517	T532	N526	A530	V547	A554	G555	Y557	V562	N569	A586	S594	A595	I601	E603
Low-BLNAR (n = 5)	II	1	1	≤0.06	≤0.06											**K**												
		1	2	≤0.06	0.25											**K**	S	I									L	G
		1	2	≤0.06	≤0.06	N		I					V			**K**		I					S	S	T	T		
		1	1	≤0.06	≤0.06	N		I					V			**K**		I				L	S	S	T	T		
		1	0.5	≤0.06	≤0.06	N						S	V			**K**		I				L	S	S	T	T		D
High-BLNAR (n = 47)	III	19	3.2[b]	0.4[b]	1.8[b]	N	N	I	**T**	F						**K**		I				L	S					
		15	5[b]	0.4[b]	2.4[b]	N	N	I	**T**	F						**K**		I				L	S	S	T	T		D
		3	3.2[b]	0.1[b]	0.5[b]	N	N	I	**T**	F			V			**K**												
		3	4[b]	0.2[b]	0.6[b]	N	N	I	**T**	F						**K**	S											
		1	2	0.25	0.5	N	N	I	**T**							**K**		I				L	S					
		1	4	0.13	0.5	N	N	I	**T**	F			T			**K**		I					S					
		1	2	0.25	2	N	N	I	**T**	F						**K**		I									L	G
		1	8	1	2	N	N	I	**T**	F	A					**K**		I				L	S					
		1	8	0.25	1	N	N	I	**T**	F						**K**		I				L	S					D
		1	2	≤0.06	0.13	N	N	I	**T**	F			V			**K**		I					S					D
		1	2	0.13	0.25	N	N		**T**				V			**K**		I					S	S	T	T		D
High-BLNAR (n = 9)	III-like	1	2	≤0.06	0.25	N	N	I	**T**					**H**	S			I									L	N
		4	5.7[b]	0.3[b]	5.7[b]	N	N	I	**T**	F				**H**	S			I			H		S					
		1	2	0.13	0.5	N	N		**T**					**H**				I		E	H		S	S	T	T		
		1	16	0.25	2	N	N	I	**T**	F				**H**				I	T		H						L	D
		1	16	0.5	2	N	N	I	**T**	F				**H**				I		E	H		S	S	T	T		
		1	4	0.25	1	N	N	I	**T**	F				**H**				I		E	H		S	S	T	T		D
BLPACR (n = 2)	III	2	>128	0.25 or 0.5	1 or 2	N	N	I	**T**	F						**K**		I				L	S					

[a]Amino acid substitutions in the region from D350 to E603 in PBP3 were examined. Bold letters indicate amino acid substitutions classifying low- and high-BLNAR isolates and their groups. Amino acids at positions 379 to 381 and 512 to 514 were the Ser-Ser-Asn (SSN) motif and the Lys-Thr-Gly (KTG) motif, respectively. The high-BLNAR isolate SMHi90 was excluded because of its unique *ftsI* sequence (see Fig. S1 and S2). AMP, ampicillin; CTR, ceftriaxone; CTX, cefotaxime.

[b]Geometric mean.

TABLE 3 Characteristics of isolates with decreased susceptibility to quinolones[a]

Isolate	Genotype	Group	ST	Patient age (yr)	Patient sex	Specimen type	MIC (mg/liter)								Mutation(s) in QRDR	
							AMP	CTR	CTX	LVX	MFX	GNX	TFX	SFX	GyrA	ParC
SMHi67	BLNAS		ND	66	M	Sputum	0.5	0.25	1	0.5	1	0.5	0.5	0.03	Ser84Leu	Asn138Ser, Ser230Ala
SMHi9	High-BLNAR	III	156	69	M	Sputum	2	0.25	1	0.13	0.25	0.13	0.13	0.03	Ser84Leu, Glu142Lys	
SMHi18	High-BLNAR	III	1218	5	M	Pharyngeal mucus	8	0.5	1	0.13	0.25	0.25	0.13	0.03	Ser84Leu	
SMHi90	High-BLNAR	IV	57	64	F	Sputum	32	0.25	2	0.13	0.25	0.25	0.13	0.015	Ser84Leu	

[a]No amino acid substitutions in the QRDRs of GyrB and ParE were observed. M, male; F, female; AMP, ampicillin; CTR, ceftriaxone; CTX, cefotaxime; LVX, levofloxacin; MFX, moxifloxacin; GNX, garenoxacin; TFX, tosufloxacin; SFX, sitafloxacin; ND, not determined because of the failure of *fucK* amplification by PCR.

III-like accounted for 8.7% of all *H. influenzae* isolates and 14.5% of BLNAR isolates. One of the high-BLNAR isolates, SMHi90, was not classified into either group because it harbored Asn526Met, in addition to Ser385, in PBP3, resulting from a TGA insertion between nucleotide positions 1576 and 1578 of *ftsI* (Fig. S1); it exhibited a higher ampicillin MIC and similar cephalosporin MICs, compared to group III and group III-like isolates (Table 3).

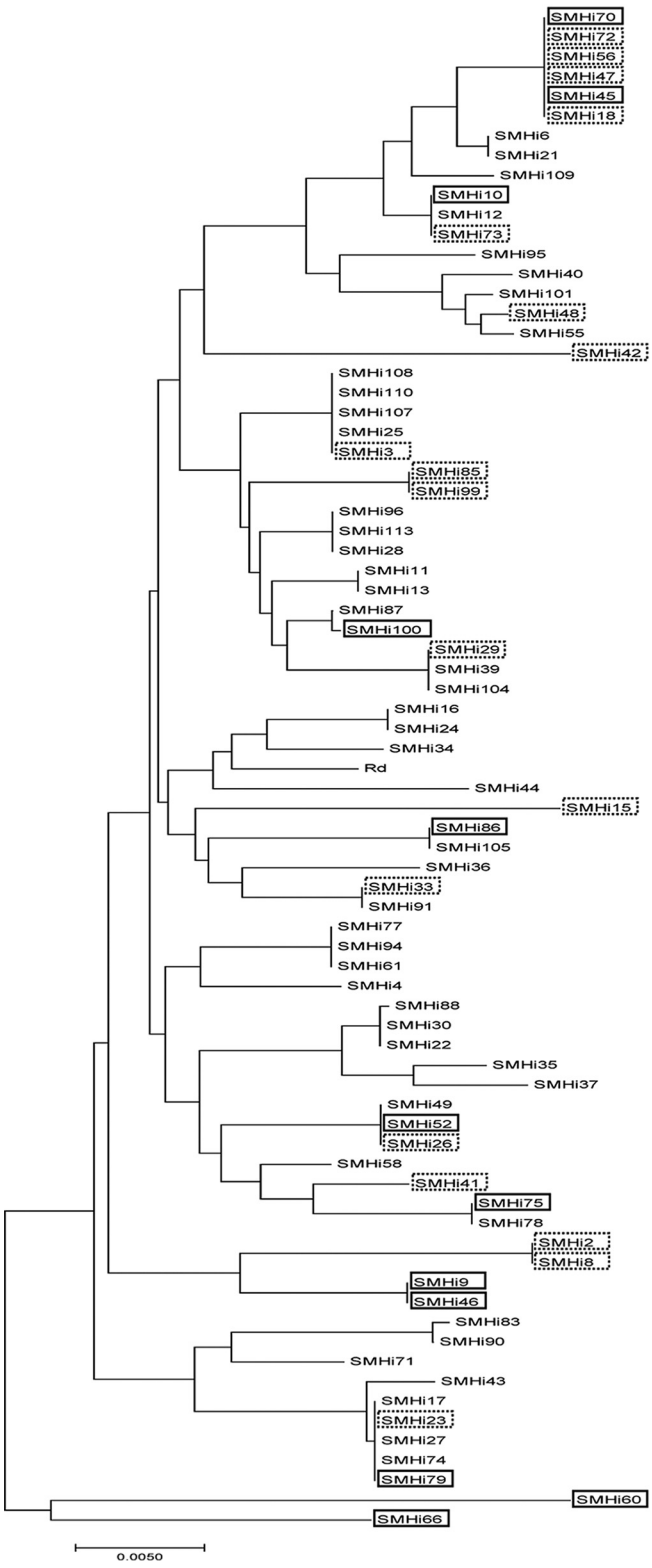
The associations between ampicillin, ceftriaxone, and cefotaxime susceptibilities and PBP3 genogroups were evaluated (Table 1 and Fig. 1). BLNAS isolates exhibited the lowest values for all parameters (MIC geometric mean, MIC_{50}, MIC_{90}, and MIC range) in the presence of the three antibiotics. The MIC parameters increased in the order of low-BLNAR isolates and then high-BLNAR isolates. The MICs of ampicillin and cefotaxime, but not ceftriaxone, for group III-like isolates were slightly higher than those for group III isolates. Isolate SMHi90, which harbored Asn526Met, exhibited MICs of 32, 0.25, and 2 mg/liter for ampicillin, ceftriaxone, and cefotaxime, respectively.

The gene bla_{TEM-1} was detected in 7 isolates (5 BLPAR isolates and 2 BLPACR isolates), and their ampicillin MICs were >128 mg/liter. BLPAR isolates exhibited MICs of ≤0.06 mg/liter and ≤0.06 or 2 mg/liter for ceftriaxone and cefotaxime, respectively, while BLPACR isolates exhibited corresponding values of 0.25 or 0.5 mg/liter and 2 or 1 mg/liter. None of the isolates possessed bla_{ROB-1}.

Amino acid sequence typing of PBP3, multilocus sequence typing, and phylogenetic analysis. BLNAR isolates were classified in detail by PBP3 typing based on the partial amino acid sequences of the main region of PBP3, Asp350 to Glu611 (Table 2). Isolates of group II were divided into 5 PBP3 types. Isolates of groups III and III-like were divided into 11 and 6 PBP3 types, respectively. The high-BLNAR isolate SMHi90 was not classified into any genotype because it was found to possess multiple unique amino acid substitutions in PBP3, including Asn526Met, compared with other BLNAR isolates (Fig. S1A).

Multilocus sequence typing (MLST) analysis was performed for all BLNAR, BLPAR, and BLPACR isolates and 11 randomly selected BLNAS isolates. Four isolates could not be typed, because their *fucK* genes could not be amplified using the specific primer pair. The most prevalent sequence type (ST) was ST1218 (6 isolates), followed by ST422 and ST183 (5 isolates each). We observed 4 new STs, in 2 BLNAS isolates (ST1878 and ST1879), 1 high-BLNAR isolate (ST1880), and 1 BLPAR isolate (ST1881).

Isolates of the same ST mostly belonged to the same PBP3 genogroup. However, some STs (ST155, ST160, and ST183) contained both group III and group III-like isolates. ST147 contained a high-BLNAR isolate and 2 BLNAS isolates. Low- and high-BLNAR isolates were clustered into 34 STs and high-BLNAR isolates into 20 STs. We created a phylogenetic tree based on MLST data for 76 isolates, including BLNAR, BLPAR, and BLPACR isolates and 10 randomly selected BLNAS isolates, and the isolates grouped into many clusters (Fig. 2). A phylogenetic tree based on *ftsI* sequences (Asp350 to Asn611) of 80 isolates, including all BLNAR, BLPAR, and BLPACR isolates and 11 randomly selected BLNAS isolates, was also created. Most of the group III isolates belonged to 4 clusters (clusters A, B, C, and F), and group III-like isolates belonged to



ST	CG	genotype	group	MIC (mg/L)		
				AMP	CTR	CTX
1218	8	High-BLNAR	III	4	0.5	2
1218	8	High-BLNAR	III	2	0.5	2
1218	8	High-BLNAR	III	4	0.5	1
1218	8	High-BLNAR	III	4	0.25	4
1218	8	High-BLNAR	III	8	0.5	2
1218	8	High-BLNAR	III	8	0.5	1
159	8	BLNAS		0.25	≤0.06	≤0.06
159	8	BLNAS		0.5	≤0.06	≤0.06
107	8	High-BLNAR	III	2	0.13	4
155	36	High-BLNAR	III	4	0.25	1
155	36	High-BLNAR	III-like	2	≤0.06	0.25
155	36	High-BLNAR	III	4	0.25	1
196	-*	Low-BLNAR	II	0.5	≤0.06	≤0.06
1197	15	High-BLNAR	III	2	0.13	0.25
388	15	BLPAR		>128	≤0.06	2
395	15	High-BLNAR	III	8	0.5	2
253	15	High-BLNAR	III	4	0.13	0.5
161	-*	High-BLNAR	III	4	0.25	1
183	4	High-BLNAR	III-like	8	0.25	4
183	4	High-BLNAR	III-like	8	0.25	8
183	4	High-BLNAR	III-like	8	0.25	8
183	4	High-BLNAR	III-like	2	0.5	4
183	4	High-BLNAR	III	2	0.25	0.13
143	2	High-BLNAR	III	2	1	4
143	2	High-BLNAR	III	2	0.5	4
836	14	BLPACR	III	>128	0.5	2
836	14	BLPACR	III	>128	0.25	1
836	14	High-BLNAR	III	2	0.25	2
404	2	BLNAS		0.25	≤0.06	≤0.06
404	2	BLNAS		0.25	≤0.06	≤0.06
367	3	Low-BLNAR	II	1	≤0.06	≤0.06
137	3	High-BLNAR	III	4	0.25	2
147	14	High-BLNAR	III	16	0.5	4
147	14	BLNAS		0.25	≤0.06	≤0.06
147	14	BLNAS		0.5	≤0.06	≤0.06
1653	-*	BLNAS		0.25	≤0.06	≤0.06
1653	-*	BLNAS		0.25	≤0.06	≤0.06
1415	23	High-BLNAR	III-like	16	0.5	2
		BLNAS		0.25	≤0.06	≤0.06
1418	23	High-BLNAR	III	8	0.25	1
834	25	High-BLNAR	III	4	0.25	1
208	-*	High-BLNAR	III	16	0.25	4
208	-*	High-BLNAR	III	2	0.25	1
986	143	High-BLNAR	III-like	2	0.13	0.5
262	49	High-BLNAR	III	4	0.25	2
262	49	High-BLNAR	III	2	0.25	0.5
321	66	High-BLNAR	III	4	0.13	0.5
321	66	High-BLNAR	III	4	0.25	0.5
321	66	High-BLNAR	III	4	0.25	1
142	56	Low-BLNAR	II	2	≤0.06	0.25
898	7	BLPAR		>128	≤0.06	≤0.06
103	7	BLPAR		>128	≤0.06	≤0.06
103	7	BLPAR		>128	≤0.06	≤0.06
1878	-*	BLNAS		0.25	≤0.06	≤0.06
1879	-*	BLNAS		0.25	≤0.06	≤0.06
160	106	High-BLNAR	III-like	16	0.25	2
160	106	High-BLNAR	III	8	1	8
160	106	High-BLNAR	III	2	0.5	4
14	29	Low-BLNAR	II	2	≤0.06	≤0.06
408	3	High-BLNAR	III	2	0.25	1
1414	19	High-BLNAR	III	8	1	2
1414	19	High-BLNAR	III	8	1	2
34	35	High-BLNAR	III	2	0.25	2
34	35	High-BLNAR	III	2	0.25	2
156	16	High-BLNAR	III	2	0.25	1
156	16	High-BLNAR	III	2	0.25	4
1884	-*	BLPAR		>128	≤0.06	≤0.06
57	9	High-BLNAR	IV	32	0.25	2
1324	9	Low-BLNAR	II	1	≤0.06	≤0.06
411	22	High-BLNAR	III-like	4	0.25	1
422	22	High-BLNAR	III	8	≤0.06	0.25
422	22	High-BLNAR	III	2	0.25	2
422	22	High-BLNAR	III	2	0.13	0.5
422	22	High-BLNAR	III	2	0.13	0.5
422	22	High-BLNAR	III	8	0.25	2
425	21	High-BLNAR	III	2	0.5	1
1880	-*	High-BLNAR	III	16	1	4

FIG 2 Phylogenetic tree based on MLST of *H. influenzae* clinical isolates. A neighbor-joining dendrogram of BLNAS ($n = 10$), low-BLNAR ($n = 5$), high-BLNAR ($n = 54$), BLPAR ($n = 5$), and BLPACR ($n = 2$) isolates investigated in this study was constructed. Four isolates (SMHi5, SMHi67, SMHi97, and SMHi102) were excluded because *fucK* was not amplified by PCR. Dotted rectangles indicate the most common type of amino acid substitution in group III, and solid rectangles indicate the second most common type. Asterisks indicate singletons.

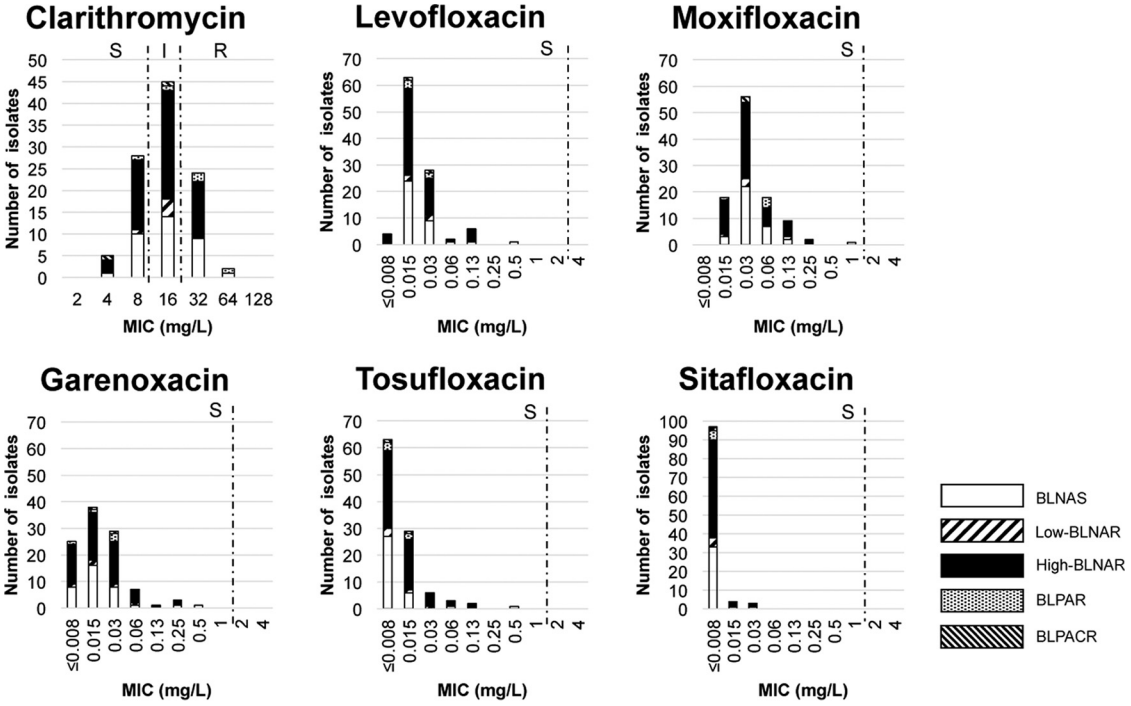


FIG 3 Susceptibilities to clarithromycin and various quinolones and the genetic patterns of β-lactam resistance in *H. influenzae* clinical isolates. S, susceptible; I, intermediate; R, resistant.

a single cluster (cluster D). Low-BLNAR, BLNAS, and BLPAR isolates constituted another cluster (cluster E) (Fig. S2).

Clarithromycin and quinolone susceptibilities and amino acid substitutions in quinolone-resistance-determining regions of GyrA, GyrB, ParC, and ParE. Susceptibility to clarithromycin and various quinolones was examined for the *H. influenzae* clinical isolates (Fig. 3). For clarithromycin, the MIC_{50} and MIC_{90} values were 16 and 32 mg/liter, respectively; 45 isolates (43.3%) were of intermediate resistance, and 26 isolates (25.0%) were resistant. For the quinolones, the MIC_{50} values were 0.015, 0.03, 0.015, ≤0.008, and ≤0.008 mg/liter and the MIC_{90} values were 0.03, 0.13, 0.06, 0.03, and ≤0.008 mg/liter for levofloxacin, moxifloxacin, garenoxacin, tosufloxacin, and sitafloxacin, respectively. No quinolone-nonsusceptible isolates were identified. Four isolates exhibited higher quinolone MICs than did the quinolone-susceptible isolates (Table 3). These 4 isolates exhibited 1 to 3 amino acid substitutions in the quinolone-resistance-determining regions (QRDRs) of GyrA and ParC but none in GyrB or ParE (Table 3).

DISCUSSION

In this study, we revealed that the high prevalence of BLNAR isolates obtained in a Japanese university hospital between 2016 and 2018 could be attributed to an increase in high-BLNAR isolates. Although this study was performed in a single Japanese university hospital, the antimicrobial usage and the prevalence of β-lactam-resistant *H. influenzae* strains in this facility were similar to those determined in national surveillance in Japan (25, 26). Thus, this study should illustrate the status of expansion of β-lactam-resistant *H. influenzae* strains in Japanese clinical settings. The prevalence of BLNAR strains was similar to that in a previous study conducted at another site in Japan in 2011 (Table 4), although the clinical background was different from that in the present study (i.e., isolates used in the previous study were collected from pediatric acute otitis media cases) (22). This prevalence is distinct from that in other Asian and European countries, as well as the United States (Table 4). These results suggest that high-BLNAR isolates have been established in Japanese clinical settings since around 2005 (Table 4). Although a primary reason for the expansion of high-BLNAR isolates in

TABLE 4 Prevalence of high-BLNAR isolates in Japan and other countries

Country	Year(s) of isolation	No. (%) of *H. influenzae* isolates			High-BLNAR strains among BLNAR strains (%)	Reference
		Total	BLNAR[a]	High-BLNAR[b]		
Japan	2016–2018	104	62 (59.6)	57 (54.8)	91.9	This study
	2011–2012	122	57 (46.7)[c]	52 (42.6)	91.2	22
	2004–2005	172	61 (35.5)[c]	43 (25.0)	70.5	23
	2003	264	172 (65.2)[c]	74 (28.0)	43.0	13
	2002–2004	457	211 (46.2)[c]	121 (26.5)	57.3	42
	2000–2009	508	247 (48.6)[c]	159 (31.3)	55.3	20
	1999	296	78 (26.3)[c]	39 (13.1)	50.0	15
	1995–2003	621	229 (36.9)	77 (12.4)	33.6	6
	1987–2000	162	22 (13.6)[c]	3 (1.9)	13.6	19
Korea	2012	122	49 (40.2)	18 (14.8)	36.7	8
Norway	2007	808	116 (14.4)	3 (0.4)	2.6	14
Korea	2005–2006	540	33 (6.1)	8 (1.5)	33.3	21
Portugal	2001–2008	240	94 (39.1)	2 (0.8)	2.1	9
Spain	2001–2006	354	198 (55.9)	12 (3.4)	6.1	7
France	2000–2008	2,206	354 (16.0)	3 (0.14)	0.9	11
Spain	2000–2009	95	27 (28.4)	0 (0)	0	10
Korea	2000–2005	229	67 (29.3)	0 (0)	0	18
United States	1999	95	13 (13.7)[c]	0 (0)	0	15

[a]Isolates with Arg517His or Asn526Lys substitutions in PBP3.
[b]Isolates with Ser385Thr in addition to Arg517His or Asn526Lys substitutions in PBP3.
[c]Defined by PCR.

Japan had not been elucidated, the relatively high levels of the use of β-lactams, including cephalosporins, in Japan could be a contributing factor (25).

To explore the mechanism underlying the increase in high-BLNAR isolates in Japan, we characterized the genotypes of the isolates. Most high-BLNAR isolates (82.5%) were group III or III-like, and the majority (72.3%) of the group III isolates fell into 2 PBP3 types. However, MLST analysis revealed that the 2 PBP3 types consisted of 20 STs with 15 clonal groups (CGs) and 3 singletons. Thus, we conclude that the high prevalence of high-BLNAR strains is due to expansion of multiple lineages and limited contribution by specific lineages.

In a 2014 study conducted in Norway, Skaare et al. classified 116 BLNAR isolates according to PBP3 type and MLST analyses (14). There were only 3 high-BLNAR isolates, 1 in group III (ST1197) and 2 in group III-like (ST160), reflecting the low prevalence of high-BLNAR isolates in that study. The authors showed that the majority (74.3%) of low-BLNAR isolates (most in group II) were classified into 3 PBP3 types (types A, B, and D, in that study) (14). In MLST analysis, approximately 71.6% of the low-BLNAR isolates were classified into 7 STs, and PBP3 types A, B, and D corresponded to ST14/ST367, ST369, and ST201, respectively (14). These results suggest that the expansion of low-BLNAR isolates was caused by a few specific lineages in Norway. This conclusion is in contrast to the multiclonal expansion of high-BLNAR strains in Japan shown in the present study.

In a previous study in South Korea, Park et al. performed pulsed-field gel electrophoresis (PFGE) and phylogenetic analyses of the entire *ftsI* gene in 29 low-BLNAR isolates and 20 high-BLNAR isolates obtained from pediatric nasopharyngeal specimens in three tertiary-care hospitals in 2012 (8). The isolates clustered into 26 groups, and there were 18 singletons in the *ftsI* phylogeny. There were no specific PFGE patterns among the BLNAS, low-BLNAR, or high-BLNAR isolates (8). The phylogenetic analysis based on MLST data in the present study revealed that several STs contained BLNAS and high-BLNAR isolates or group III and group III-like isolates in the same ST (Fig. 2). These results suggest that some lineages developed their β-lactam resistance by originating as BLNAS or low-BLNAR strains (groups I and II) and evolving to express a high-BLNAR phenotype. Interestingly, ST57 and ST397, which included group III high-BLNAR isolates in this study, were identified as including group II low-BLNAR or BLNAS

isolates in a clinical setting in another country (Norway), suggesting the risk of subsequent evolution to become phenotypically high-BLNAR isolates (14).

Macrolides and quinolones are candidates for secondary choices to treat high-BLNAR infections. Clarithromycin lacks utility for such treatment because of the high prevalence (68.3%) of nonsusceptible isolates (Fig. 1). In contrast, quinolones are promising agents, because no quinolone-nonsusceptible isolates were observed. The prevalence of nonsusceptibility to quinolones is around 0.1 to 2% in Japan and other countries (27–31). These data indicate that the prevalence of quinolone-nonsusceptible *H. influenzae* strains has not increased. In addition, ceftriaxone should be considered a promising choice because no ceftriaxone-nonsusceptible isolates were detected.

We obtained 4 isolates (3.8%) of different STs that showed reduced susceptibility to quinolones (Table 3). The mechanism of the decrease in quinolone susceptibility involves amino acid substitutions in the QRDRs of GyrA (at positions Ser84 and Asp88) and ParC (at positions Gly82, Asp83, Ser84, and Glu88) in *H. influenzae* (28, 29, 32). All 4 isolates shared the amino acid substitution Ser84Leu in GyrA (Table 3). Another amino acid substitution, Glu142Lys, in GyrA was found in 1 isolate; however, this substitution is known not to contribute to quinolone nonsusceptibility (30). The Asn138Ser and Ser230Ala substitutions in ParC were also found in another isolate, and Asn138Ser is suggested not to contribute to quinolone resistance (28, 32). Although it is not known whether the Ser230Ala substitution in ParC contributes to quinolone resistance, higher quinolone MICs for the strain suggest that it is a candidate for contributing to resistance. Of note, 1 strain was isolated from a child, while *H. influenzae* isolates with decreased susceptibility to quinolones are mostly isolated from elderly people (28, 29).

Three of the isolates with decreased susceptibility to quinolones were high-BLNAR strains. This suggests that monitoring for the emergence of fluoroquinolone resistance in β-lactam-resistant *H. influenzae* is important for treatment in respiratory medicine as well as in pediatrics. It was reported that the prevalence of levofloxacin-resistant *H. influenzae* strains in Taiwan increased from 2% in 2004 to 24.3% in 2010 (33), and these data indicate that the inappropriate use of quinolones may result in the emergence of quinolone-resistant high-BLNAR isolates in the future.

One isolate, SMHi90, harbored multiple unique amino acid substitutions in PBP3 and exhibited a higher ampicillin MIC than did other high-BLNAR isolates. Thus, we propose that the *ftsI* sequence of SMHi90 represents a novel high-BLNAR group, group IV. The same *ftsI* sequence has been registered in the DNA Data Bank of Japan (DDBJ) genome database (accession number LC279277.1 [43]); it was derived from a *H. influenzae* clinical isolate from a region in Japan distinct from that in the present study. In addition, SMHi90 demonstrated decreased quinolone susceptibility. Thus, we need to be vigilant against the spread of novel emerging high-BLNAR strains.

MATERIALS AND METHODS

Ethics statement. This study was approved by the institutional review board of Sapporo Medical University Hospital.

Bacterial isolates. A total of 104 clinical *H. influenzae* isolates were obtained from Sapporo Medical University Hospital between May 2016 and January 2018. For each isolate, information was obtained regarding the patient's age and sex and the clinical source. The isolates were obtained from the following types of clinical specimens: sputum ($n = 56$), pharyngeal mucus ($n = 16$), nasal mucus ($n = 9$), nasal discharge ($n = 7$), otorrhea ($n = 4$), oral cavity ($n = 3$), tonsil ($n = 3$), bronchoalveolar lavage fluid ($n = 2$), and eye, colon, pleural effusion, and abdominal cavity drainage ($n = 1$ each). All isolates were grown for 24 to 48 h on chocolate agar II (Nippon Beckton-Dickinson, Tokyo, Japan) at 37°C, in a 5% CO_2 atmosphere. These isolates were stored at −80°C using a Microbank system (Pro-Lab Diagnostics, Round Rock, TX). Identification of *H. influenzae* was achieved using a matrix-assisted laser desorption ionization (MALDI) Biotyper (Bruker Daltonics, Billerica, MA) and PCR amplification of the gene encoding outer membrane protein P6, *ompP6*, as described previously (15). Capsular serotypes were determined by PCR, as described previously (34).

Antimicrobial susceptibility. MIC determination was carried out by the microdilution method, according to Clinical and Laboratory Standards Institute (CLSI) guidelines (35). Ampicillin (Wako Pure Chemical Industry, Tokyo, Japan), ceftriaxone (Tokyo Chemical Industry, Tokyo, Japan), cefotaxime (Tokyo Chemical Industry), clarithromycin (Taisho Pharmaceutical, Tokyo, Japan), levofloxacin (Daiichi-Sankyo, Tokyo, Japan), moxifloxacin (Bayer, Osaka, Japan), garenoxacin (Toyama Chemical, Tokyo, Japan), tosufloxacin (Toyama Chemical), and sitafloxacin (Daiichi-Sankyo) were used. Geometric means of ampicillin,

ceftriaxone, and cefotaxime MICs were calculated as described previously (6). Strains with MICs exceeding 2 mg/liter for levofloxacin and/or 1 mg/liter for moxifloxacin were defined as quinolone nonsusceptible, according to CLSI guidelines (35). Because there are no CLSI definitions of breakpoints for tosufloxacin, garenoxacin, and sitafloxacin, strains with MICs exceeding 1 mg/liter were defined as being nonsusceptible, as stated by the Japanese Society of Chemotherapy (36).

Nucleotide sequences of *ftsI* genes and detection of β-lactamase genes. *Haemophilus influenzae* clinical isolates were classified into groups I to III and III-like on the basis of amino acid substitutions in PBP3 revealed by DNA sequencing of the *ftsI* gene, as described in a previous report (14). Two pairs of primers for PCR amplification and DNA sequencing of *ftsI* (nucleotide positions 1048 to 1833) were designed in this study, using the sequence of the *H. influenzae* Rd strain genome (NCBI accession number NC000907) (37), i.e., ftsI-F2 (5′-GTGGTGGGTTATACGGATATTGATGG-3′) and ftsI-R2 (5′-CATAGGCACGAG CAATTTGTAAAGGT-3′), and ftsI-F3 (5′-TAAGCGGTAAAGAAATTGTGGA-3′) and ftsI-R3 (5′-ACGATGCTGCG CCAAACCGTGTGATGAAAC-3′). Genomic DNA was isolated from cells using the DNeasy kit (Qiagen, Hilden, Germany). PCR amplification was performed with a PCR system 9700 (Applied Biosystems, Waltham, MA) using Ex *Taq* polymerase (TaKaRa Shuzo, Kyoto, Japan), as follows: 2 min of denaturation at 94°C and 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 3 min. The amplified DNA fragments were sequenced using an Applied Biosystems 3730 DNA analyzer. BLNAS, low-BLNAR (groups I and II), and high-BLNAR (group III and III-like) isolates were defined as described previously (14). Each group was further divided by amino acid substitution typing of PBP3 (PBP3 types) as deduced from the amino acid sequences of PBP3 from Asp350 to Asn611, which are associated with β-lactam resistance, using methods described previously (6–11, 14, 18, 21). Detection of the β-lactamase genes bla_{TEM-1} and bla_{ROB-1} was performed by PCR, as described previously (15).

Identification of mutations in the QRDRs of *gyrA*, *gyrB*, *parC*, and *pare*. To clarify the mechanism of decreased susceptibility to quinolones, the nucleotide sequences of the QRDRs of the quinolone target genes, *gyrA*, *gyrB*, *parC*, and *parE*, were determined. PCR amplification was performed using Quick *Taq* HS DyeMix (Toyobo, Osaka, Japan). The specific primers used for PCR amplification and DNA sequencing of the QRDRs of *gyrA*, *gyrB*, and *parE* were described previously (38). The primers for amplifying the QRDR of *parC*, i.e., HiparC-F (5′-GTGCGTTGCCTTTTATCGGTGA-3′) and HiparC-R (5′-GAAGATTGATGTGGAAGCG CTGA-3′), were designed in this study using the sequence of the H. influenzae Rd strain genome (NCBI accession number NC000907) (37). The PCR products were analyzed as described above.

MLST. STs were determined by MLST using 7 housekeeping genes (*adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi*, and *recA*), as described previously (39). Allele numbers and STs were assigned using the MLST website (http://haemophilus.mlst.net). The CGs of these STs were defined by using the eBURSTv3 database (http://eburst.mlst.net). A phylogenetic tree based on MLST data for the *H. influenzae* BLNAR isolate was constructed based on the neighbor-joining method (40), using MEGA7 (41).

Accession number(s). The nucleotide sequence of the high-BLNAR isolate SMHi90 was registered in the DDBJ database (accession number LC379877).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00851-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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We have no conflicts of interest to declare.

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