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ORIGINAL ARTICLE

Diverse mutations in the *ftsI* gene in ampicillin-resistant *Haemophilus influenzae* isolates from pediatric patients with acute otitis media

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Abstract To clarify molecular changes in β-lactamase-nonproducing, ampicillin-resistant (BLNAR) *Haemophilus influenzae*, which is increasing in pediatric patients with acute otitis media (AOM) in Japan, we identified amino acid (aa) substitutions in penicillin-binding protein 3 for the BLNAR strains. Of 191 *H. influenzae* strains isolated from middle ear fluid of pediatric AOM patients between October 2005 and March 2008, BLNAR strains determined by PCR accounted for 49.2%. Of the BLNAR strains, 91.5% possessed 4 aa substitutions: Met377Ile, Ser385Thr, Leu389Phe, and either Asn526Lys or Arg517His. Additionally, the emergence of BLNAR strains possessing a new aa substitution of Val329Ala in the conserved aa motif of Ser327-Thr-Val-Lys, or Val511Ala adjacent to the conserved aa motif of Lys512-Thr-Gly, was noted. Transformation of the *ftsI* gene into the Rd reference strain (ATCC 51907) demonstrated that these two aa substitutions reduced susceptibility to amoxicillin more than to cephalosporins. Pulsed-field gel electrophoretic profiles of BLNAR strains were highly diverse. These results suggested that inadequate antibiotic use may increase BLNAR strains by selecting mutations in the *ftsI* gene and that such use may have favored the new aa substitutions.

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Introduction

Haemophilus influenzae is an important pathogen causing respiratory tract infection, pneumonia, acute otitis media (AOM), and meningitis. Two well-known mechanisms are implicated in the resistance of *H. influenzae* to ampicillin (AMP). One is the enzymatic hydrolysis of β-lactam agents resulting from the production of TEM-1 and ROB β-lactamases [1–4]; the other is decreased affinity of penicillin-binding protein (PBP) 3 for β-lactam antibiotics reflecting amino acid substitutions in the enzyme [3, 5]. Strains with alterations in PBP3 are termed β-lactamase-nonproducing, AMP-resistant (BLNAR) *H. influenzae*. In addition, strains demonstrating both mechanisms are termed β-lactamase-producing, amoxicillin-clavulanic acid-resistant (BLPACR) *H. influenzae*.

Amino acid substitutions in PBP3 surrounding the conserved Lys512-Thr-Gly (KTG) and Ser379-Ser-Asn (SSN) motifs are responsible for β-lactam resistance [5–9]. Single substitution of Asn526Lys or Arg517His was commonly found in BLNAR isolates with intermediate resistance to AMP (low-BLNAR). Additional amino acid substitutions, Met377Ile, Ser385Thr, and/or Leu389Phe, were characterized by higher than intermediate resistance to AMP (BLNAR).

In the United States, the prevalence of BLNAR strains was reported to be less than 5% in a recent study [10]. Another recent study reported a relatively constant prevalence of BLNAR strains, about 9%, in most of Europe [11]. In Japan and Spain, however, a marked increase in the number of BLNAR isolates has been observed [12–14].

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Currently, an increase in intractable or recurrent AOM caused by BLNAR has become a great concern in pediatrics and otolaryngology in Japan [15, 16]. Despite the high reported prevalence of BLNAR strains from AOM, their evolutionary molecular changes in the *ftsI* gene have not been clarified. In this study, we characterized amino acid substitutions in PBP3 and their correlation with antibiotic susceptibilities in *H. influenzae* isolated from pediatric patients with AOM.

Materials and methods

Strains

In Japanese medical institutions, 191 clinical *H. influenzae* strains were isolated from middle ear fluid collected from pediatric patients with AOM by puncture or incision of the tympanic membrane between October 2005 and March 2008. The middle ear fluid samples were sent to our laboratory, where we immediately carried out bacterial culture and species identification based on the requirement of β-NAD (V factor) and hemin (X factor). Additionally, PCR was performed on all isolates, as described below, to identify species, β-lactamase genes, and *ftsI* mutations.

Polymerase chain reaction (PCR)

We performed PCR for *H. influenzae* using six sets of primers described previously [7]. The six targets were the 16S rRNA gene identifying species [17], the TEM-1 β-lactamase gene [18], the ROB-1 β-lactamase gene [19], an amino acid substitution of Asn526Lys in the *ftsI* gene [20], an amino acid substitution of Ser385Thr in the *ftsI* gene [5], and the Hib-specific *capB* locus [21]. PCR cycling conditions using lysates extracted from colonies of isolates included 35 cycles at 94°C for 15 s; at 53°C for 15 s; and at 72°C for 15 s. On the basis of the PCR results, the resistance class was described by attaching "g" to indicate genetic identification as opposed to other biologic assays, yielding designations such as gBLNAR, gLow-BLNAR, gBLPAR, and gBLNAS.

Serotyping

Serotypes of *H. influenzae* strains, except for type b, were determined by the agglutination test using antiserum purchased from Becton–Dickinson (Franklin Lakes, NJ, USA).

Antibiotic susceptibility

Susceptibility testing was performed by an agar dilution method [22]. Antibiotics used in this study were AMP, amoxicillin (AMX), cefditoren (CDN), and tebipenem (TBM, a new oral carbapenem; Meiji Seika Kaisha, Tokyo, Japan); cefdinir (CDR; Astellas Pharma, Tokyo, Japan); cefotaxime (CTX; Aventis Pharma, Tokyo, Japan); and meropenem (MEM; Dainippon Sumitomo Pharma, Osaka, Japan). *H. influenzae* ATCC 49247 and ATCC 49766 were used as quality control strains.

Sequencing

The 1.0-kb DNA region of the *ftsI* gene corresponding to the transpeptidase domain of PBP3 was amplified from the chromosomal DNA of *H. influenzae* by PCR using a sense primer, 5′-GTTGCACATATCTCCGATGAG-3′, and a reverse primer, 5′-CAGCTGCTTCAGCATCTTGC-3′, as described previously [5]. Amplified DNA fragments were purified using a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and used as templates. Sequencing reactions were carried out using a BigDye Terminator cycle sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA, USA). DNA sequencing was performed with an ABI Prism 3130/3130xl genetic analyzer (Applied Biosystems).

Transformation

The Rd strain (ATCC 51907) was transformed with an open reading frame corresponding to the *ftsI* gene, which was PCR-amplified. Transformation was carried out using a cuvette with a 0.1-cm electrode gap and a MicroPulser electroporation apparatus (Bio-Rad Laboratories, Hercules, CA, USA), as described previously [5]. Conditions for electroporation were 1.8 kV/cm with time constants of 5.8 to 5.9 ms. Colonies grown on selective agar plates containing CTX at 0.016, 0.063, and 0.25 mg/l were selected at random, and antibiotic susceptibilities for the colonies were determined by the agar dilution method as described above. The *ftsI* gene of the colonies was sequenced to confirm gene transfer.

Pulsed-field gel electrophoresis (PFGE)

PFGE was carried out according to the method described previously, with some modifications [23]. Chromosomal DNAs extracted from each *H. influenzae* strain were digested with *Sma*I. Electrophoresis was performed using CHEF Mapper (Bio-Rad Laboratories). Separation of DNA fragments was achieved at 6 V/cm at 14°C for 20 h and 18 min. Pulse time, which changed in a lineal manner, was 0.47 to 63.08 s.

Results

Resistance classes and susceptibility

The resistance classes of 191 *H. influenzae* isolates were identified by PCR. AMP-resistant strains were extremely

common, representing 60.2% of all isolates; the proportion of each resistance class was 49.2% for gBLNAR, 6.8% for gLow-BLNAR, 3.7% for gBLPACR II, and 0.5% for gBLPAR. Strains without any resistance genes, i.e., gBLNAS, AMP-susceptible strains, represented 39.8%.

Of all strains tested, only 6 (3.1%) were serotyped as type b ($n = 5$) or type f ($n = 1$); the remaining strains were nontypable (NT).

Table 1 shows the MIC ranges, MIC_{50}s, and MIC_{90}s of seven β-lactam antibiotics for *H. influenzae* strains classified into four resistance groups, excluding gBLPAR, which consisted of 1 strain. The MIC_{90} of the standard antibiotic AMP for gBLNAR, 8 mg/l, was 16 times higher than the value for gBLNAS, 0.5 mg/l. In contrast, the MIC_{90}s of most cephalosporin antibiotics (CDR, CDN, and CTX) for gBLNAR were markedly increased; the MIC_{90} values of CDR, CDN, and CTX were 64, 8, and 32 times higher than the value for gBLNAS, respectively. The *ftsI* gene mutations affected the MICs of cephalosporin antibiotics more than those of AMP. The MICs of TBM and MEM for AMP-resistant strains were affected slightly by *ftsI* gene mutations. The MIC_{90}s of TBM and MEM for gBLNAR were increased 4 to 8 times relative to those for gBLNAS.

Amino acid substitutions in PBP3

Table 2 shows the deduced amino acid substitutions in PBP3 in gLow-BLNAR ($n = 13$), gBLNAR ($n = 94$), and gBLPACR II ($n = 7$) strains. These strains were classified into groups based on the eight amino acid substitutions reported by Hasegawa et al. [24]. Of these eight amino acid substitutions, three substitutions (Arg517His, Asn526Lys, and Ser385Thr) were considered to importantly affect resistance.

All gLow-BLNAR strains commonly possessed Asn526Lys without Ser385Thr, which had been identified frequently among AMP-resistant *H. influenzae* strains isolated in the late 1990 s. On the other hand, all gBLNAR and gBLPACR II strains possessed Ser385Thr and either Asn526Lys or Arg517His. Furthermore, 91.1% (92/101) of the gBLNAR and gBLPACR II strains possessed Met377Ile and Leu389Phe as well.

The emergence of gBLNAR with a Val329Ala substitution in the conserved amino acid motif of Ser327-Thr-Val-Lys (STVK) and gBLNAR, with a Val511Ala substitution adjacent to the KTG motif, which had not been identified in the early 2000 s in Japan, was noted.

Correlation between Val329Ala and Val511Ala and antibiotic susceptibilities

To investigate the effects of Val329Ala in the STVK motif and Val511Ala adjacent to the KTG motif in the *ftsI* gene upon antibiotic susceptibilities, the AMP-susceptible strain Rd was transformed with PCR-amplified *ftsI* gene fragments from gBLNAR with Val329Ala (KU007) and gBLNAR with Val511Ala (KU026). As controls, PCR-amplified *ftsI* gene fragments from gBLNAR without Val329Ala (KU001), gBLNAR without Val511Ala (KU002), and gLow-BLNAR (KU046) were introduced to Rd as well.

Table 1 MIC distributions and resistance classes of *Haemophilus influenzae* strains

Antimicrobial agent and Resistance class	MIC (mg/l) Range	MIC_{50}	MIC_{90}
Ampicillin			
gBLNAS[a] ($n = 76$)	0.063–1	0.25	0.5
gLow-BLNAR ($n = 13$)	0.5–2	1	1
gBLNAR ($n = 94$)	0.5–32	2	8
gBLPACR-II ($n = 7$)	2–>64	16	>64
Amoxicillin			
gBLNAS	0.125–1	0.5	0.5
gLow-BLNAR	0.5–4	2	4
gBLNAR	0.25–64	8	32
gBLPACR-II	2–>64	64	>64
Cefdinir			
gBLNAS	0.031–1	0.25	0.5
gLow-BLNAR	0.5–4	0.5	2
gBLNAR	2–32	8	32
gBLPACR-II	8–16	16	16
Cefditoren			
gBLNAS	0.002–0.063	0.016	0.031
gLow-BLNAR	0.016–0.063	0.031	0.063
gBLNAR	0.031–1	0.25	0.25
gBLPACR-II	0.125	0.125	0.125
Cefotaxime			
gBLNAS	0.004–0.063	0.016	0.031
gLow-BLNAR	0.016–0.125	0.031	0.125
gBLNAR	0.063–4	0.5	1
gBLPACR-II	0.25–0.5	0.5	0.5
Meropenem			
gBLNAS	0.008–0.125	0.063	0.125
gLow-BLNAR	0.063–0.5	0.125	0.25
gBLNAR	0.031–0.5	0.25	0.5
gBLPACR-II	0.063–0.25	0.063	0.25
Tebipenem			
gBLNAS	0.008–0.25	0.063	0.125
gLow-BLNAR	0.031–0.5	0.25	0.5
gBLNAR	0.031–1	0.25	1
gBLPACR-II	0.25–0.5	0.5	0.5

gBLNAS, gLowBLNAR, low-β-lactamase-nonproducing, ampicillin (AMP)-resistant; gBLPACR-II, β-lactamase-producing, amoxicillin-clavulanic acid-resistant-II

[a] g in the strain name denotes genetic identification

Table 2 Amino acid substitutions identified in the *ftsI* genes from gBLNAR and gBLPACR II *H. influenzae* strains

Resistance class	Subgroup	No. of strains	Amino acid substitution								MIC_{90} (mg/l)						
			STVK[a]	SSN motif[b]			KTG motif[c]										
			Val-329	Met-377	Ser-385	Leu-389	Ala-502	Val-511	Arg-517	Asn-526	AMP	AMX	CDR	CDN	CTX	MEM	TBM
gBLNAS[d]			–	–	–	–	–	–	–	–	0.25	0.5	0.25	0.008	0.008	0.063	0.125
gLow-BLNAR ($n = 13$)		6	–	–	–	–	–	–	–	Lys	1	4	1	0.063	0.063	0.5	0.5
		3	–	–	–	–	Thr	–	–	Lys							
		3	–	–	–	–	Val	–	–	Lys							
		1	–	Ile	–	–	Val	–	–	Lys							
gBLNAR ($n = 94$)	i	1	–	–	Thr	–	–	–	His	–	4	4	32	0.25	1	0.25	0.25
	ii	1	–	Ile	Thr	–	–	–	His	–							
	iii	8	–	Ile	Thr	Phe	–	–	His	–							
	iv	1	–	–	Thr	–	–	–	–	Lys	4	32	32	0.25	1	0.5	1
	v	2	Ala	–	Thr	–	–	–	–	Lys							
	vi	3	–	–	Thr	–	Thr	–	–	Lys							
	vii	75	–	Ile	Thr	Phe	–	–	–	Lys	16	32	32	0.5	1	0.5	1
	viii	1	–	Ile	Thr	Phe	–	Ala	–	Lys							
	ix	2	–	Ile	Thr	Phe	Val	–	–	Lys							
gBLPACR-II ($n = 7$)	ii	1	–	Ile	Thr	–	–	–	His	–	16	64	8	0.125	0.5	0.25	0.25
	iii	1	–	Ile	Thr	Phe	–	–	His	–							
	vii	5	–	Ile	Thr	Phe	–	–	–	Lys	>64	>64	16	0.125	0.5	0.25	0.5

AMP ampicillin, *AMX* amoxicillin, *CDR* cefdinir, *CDN* cefditoren, *CTX* cefotaxime, *MEM* meropenem, *TBM* tebipenem

[a] STVK, Ser327-Thr-Val-Lys

[b] SSN, Ser379-Ser-Asn

[c] KTG, Lys512-Thr-Gly

[d] Control, ATCC49766

Table 3 MICs of *H. influenzae* strains transformed with a *ftsI* DNA fragment and donor strains

Strains	Amino acid substitution								MIC (mg/l)						
	STVK[a]	SSN motif[b]			KTG motif[c]										
	Val-329	Met-377	Ser-385	Leu-389	Ala-502	Val-511	Arg-517	Asn-526	AMP	AMX	CDR	CDN	CTX	MEM	TBM
Recipient Rd	-	-	-	-	-	-	-	-	0.125	0.5	1	0.008	0.016	0.063	0.063
Donor KU 046	-	-	-	-	-	-	-	Lys	1	2	1	0.016	0.031	0.25	0.25
Donor KU 001	-	-	Thr	-	-	-	-	Lys	2	2	16	0.125	0.5	0.5	0.5
Donor KU 007	Ala	-	Thr	-	-	-	-	Lys	1	64	64	0.25	4	0.5	0.25
Donor KU 002	-	Ile	Thr	Phe	-	-	-	Lys	2	4	16	0.25	2	0.25	0.5
Donor KU 026	-	Ile	Thr	Phe	-	Ala	-	Lys	2	64	64	0.25	2	0.5	0.5
Rd^{KU046}	-	-	-	-	-	-	-	Lys	0.5	1	1	0.008	0.031	0.125	0.125
Rd^{KU001}	-	-	Thr	-	-	-	-	Lys	1	2	16	0.063	0.5	0.25	0.25
Rd^{KU007}	Ala	-	Thr	-	-	-	-	Lys	1	32	64	0.25	2	0.25	0.25
Rd^{KU002}	-	Ile	Thr	Phe	-	-	-	Lys	1	2	16	0.125	2	0.25	0.25
Rd^{KU026}	-	Ile	Thr	Phe	-	Ala	-	Lys	1	32	64	0.125	2	0.25	0.5

AMP ampicillin, *AMX* amoxicillin, *CDR* cefdinir, *CDN* cefditoren, *CTX* cefotaxime, *MEM* meropenem, *TBM* tebipenem

[a] STVK, Ser327-Thr-Val-Lys

[b] SSN, Ser379-Ser-Asn

[c] KTG, Lys512-Thr-Gly

Table 3 shows the MICs of seven β-lactam antibiotics for each transformant and donor strains. The MICs of AMX, CDR, CDN, and CTX for transformant Rd^{KU007} were higher than those for transformant Rd^{KU001}. Especially, the AMX MIC of transformant Rd^{KU007} was 16 times higher than that for transformant Rd^{KU001}. On the other hand, the MICs of AMP, MEM, and TBM for transformant Rd^{KU007} were same as those for transformant Rd^{KU001}. The MICs of AMX and CDR for transformant Rd^{KU026} were 16 and 4 times higher than those for transformant Rd^{KU002}. In contrast, the MICs of AMP, CDN, CTX, MEM, and TBM for transformant Rd^{KU026} were equal or almost equal to those for transformant Rd^{KU002}.

Electrophoretic profiles

Figure 1 shows the PFGE profiles of 20 gBLNAR and 7 gBLPACR II strains selected randomly from each amino acid substitution subgroup. The profiles were extremely diverse and could not be classified into groups. Interestingly, the strains that shared an amino acid substitution subgroup and had the same resistance type differed in PFGE profiles. This diversity represents evidence that the resistant strains were clonally different from each other.

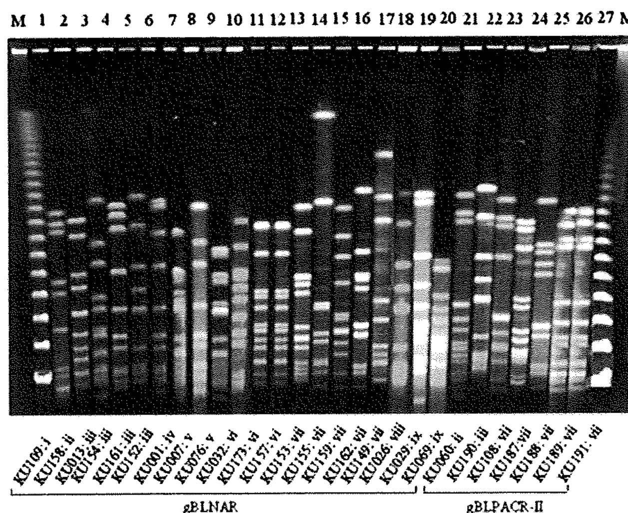

Fig. 1 Pulsed-field gel electrophoresis (PFGE) profiles of chromosomal DNA from gLow-β-lactamase-nonproducing, ampicillin-resistant (*BLNAR*), *gBLNAR*, and g β-lactamase-producing, amoxicillin-clavulanic acid-resistant II (*gBLPACR II*) strains ($n = 27$) digested with *Sma*I restriction enzyme ('g' in the strain name denotes genetic identification). *Lanes M*, λ Ladder molecular size marker; *1*, gBLNAR subgroup i (KU109); *2*, gBLNAR subgroup ii (KU158); *3–6*, gBLNAR subgroup iii (KU013, 154, 161, and 152); *7*, gBLNAR subgroup iv (KU001); *8–9*, gBLNAR subgroup v (KU007 and 076); *10–12*, gBLNAR subgroup vi (KU032, 173, and 157); *13–17*, gBLNAR subgroup vii (KU153, 155, 159, 162, and 149); *18*, gBLNAR subgroup viii (KU026); *19–20*, gBLNAR subgroup ix (KU029 and 069); *21*, gBLPACR-II subgroup ii (KU060); *22*, gBLPACR-II subgroup iii (KU190); *23–27*, gBLPACR-II subgroup vii (KU108, 187, 188, 189, and191)

Discussion

In 2001, Ubukata et al. [5] found three amino acid substitutions near the SSN motif: Met377Ile, Ser 385Thr, and Leu389Phe; and two amino acid substitutions near the KTG motif: Asn526Lys and Arg517His; in PBP3 of BLNAR strains isolated in Japan. They reported that these amino acid substitutions affected the MICs of cephalosporin antibiotics more than those of AMP. Additional new amino acid substitutions of Val329Ala in the STVK motif and Val511Ala adjacent to the KTG motif were identified in 2006 in Japan [14]. However, their effects upon antibiotic susceptibilities have remained to be clarified. In the present study, we demonstrated that Val329Ala affected the MICs of AMX and the cephalosporin antibiotics, CDR, CDN, and CTX, while Val511Ala affected the MICs of AMX and CDR. In particular, we found that either of these amino acid substitutions increased the MIC of AMX by 16 times. AMX is the antibiotic most often used to treat community-acquired respiratory infections in the United States and Europe [25]. Similarly, since around 2005, AMX has commonly been used to treat pediatric outpatients with respiratory infections and AOM in Japan. In the present study, three patients from whom the BLNAR strains with the new amino acid substitutions were isolated had not been treated with AMX for 7 days before the strains were isolated. However, AMX may have been used previously to treat their AOM or respiratory infections, because they were aged 3, 4, and 6 years, i.e., they were older than the pediatric patients commonly seen with AOM, who are usually age 1 and under. We suggest that the change of antibiotic use in Japan, from oral cephalosporin to AMX, may have promoted the emergence of these two amino acid substitutions, Val329Ala and Val511Ala, which influenced the MICs of AMX.

The AMP-resistant strains tested in the present study showed incredible diversity in their PFGE profiles, although some of them had the same amino acid substitution subgroup and the same resistance type. The diversity is equivalent to that in *H. influenzae* as normal flora [26, 27]. From the above finding, we suppose that *H. influenzae* as normal flora in healthy children acquired resistance in some way and turned out to be a causative strain. Horizontal transfer of the mutated *ftsI* gene may be one of the ways of acquiring resistance. Takahata et al. [28, 29] reported horizontal transfer of the mutated *ftsI* gene from clinical isolates of BLNAR to the Rd strain of BLNAS. When we examined this phenomenon using clinically isolated *H. influenzae* as a recipient, horizontal transfer of the mutated *ftsI* gene was not identified (data not shown). The spread of resistant *H. influenzae* by horizontal transfer of the mutated *ftsI* gene is a reasonable possibility, because *H. influenzae* in nature is capable of transformation [30, 31]. However, β-lactam antibiotics at doses that provide inadequate concentrations may more likely favor mutations in the *ftsI* gene and select gBLNAR.

In summary, we have described evolutionary molecular changes in the *ftsI* gene involving the reduced antibiotic susceptibilities of *H. influenzae* isolated from pediatric patients with AOM. These molecular changes seem to be related to a change in antibiotic use. Thus, strict control of antibiotic use, based on evidence such as trends concerning resistant strains and their molecular changes, is important to prevent increases of resistant strains.

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Capsular Type and Antibiotic Resistance in *Streptococcus agalactiae* Isolates from Patients, Ranging from Newborns to the Elderly, with Invasive Infections[▽]

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***Streptococcus agalactiae* isolates (n = 189) from patients with invasive infections were analyzed for capsular type by PCR, for antimicrobial susceptibility, and for the presence of resistance genes. In contrast to the predominance of capsular type III in children, types Ib and V were most common among adults. All 45 levofloxacin-resistant strains had two amino acid substitutions, $Ser_{81}Leu$ in the *gyrA* gene and $Ser_{79}Phe$ in the *parC* gene, and showed similar pulsed-field gel electrophoresis patterns.**

Streptococcus agalactiae (a group B streptococcus [GBS]) is the main microorganism causing meningitis and sepsis in infants and also sepsis in nonpregnant adults (12, 14).

GBS infection in infants is classified as early onset, occurring in newborns within the first week of life, or late onset, developing in infants more than 1 week old, with most infections arising in the first 3 months and only extremely rarely in older infants (18). In the 1970s, morbidity and mortality from these GBS infections were high (3, 4, 9). In 1996, however, recommendations for the prevention of perinatal GBS infection were issued by the American College of Obstetricians and Gynecologists (2), the Centers for Disease Control and Prevention (7), and later also the American Academy of Pediatrics (1). As a result, preventive efforts increased and the incidence of early-onset disease decreased substantially (6, 23). A more detailed revised guideline, based on prenatal bacterial cultures and epidemiologic studies, was recommended in 2002 (17).

Recently, Phares et al. (15) reported on a 7-year epidemiologic survey of invasive GBS disease in the United States that demonstrated a significant decline in the incidence of early-onset disease in infants, contrasting with an increase in GBS disease among adults ≥65 years old.

In the present paper, we describe details concerning patient age, disease, and underlying diseases associated with invasive GBS infection, as well as the capsular types, antimicrobial susceptibilities, and resistance genes of isolates in Japan.

Between August 2006 and July 2007, our laboratory received 189 GBS strains from the bacteriologic laboratories of 97 medical institutions participating in the Invasive Streptococcal Disease Working Group at the 19th Annual Meeting of the Japanese Society for Clinical Microbiology. All isolates were from sterile sites: blood (n = 124), cerebrospinal fluid (n = 54), pustule fluid (n = 7), joint fluid (n = 3), and tissue (n = 1).

To identify the capsular type of GBS by PCR, we used nine sets of primers from types Ia to VIII as reported by Poyart et al. (16). We also applied our newly designed *dltS* primers for the identification of GBS (Table 1).

One colony was picked up from each agar plate and placed in 30 μl of lysis solution containing 1 U of mutanolysin. The

TABLE 1. Primers for PCR and sequencing for FQ resistance in *S. agalactiae*

Gene and primer	Sequence (5′–3′)	Length (mer)	Amplicon size (bp)
dltS			
dlts-F	CTGTAAGTCTTTATCTTTCTCG	22	199
dlts-R	TCCATTCGCTTAGTCTCC	18	
gyrA			
gyrA-F	GGTTTAAAACCTGTTCATCGTCGT	24	407
gyrA-R	GCAATACCAGTTGCACCATTGACT	24	
gyrB			
gyrB-F	CGAAGCTTTCAATCGATTCCTATT	24	495
gyrB-R	GGTCGCATAAAACGATAAATCAGAG	25	
parC			
parC-F	CCGGATATTCGTGATGGCTT	20	403
parC-R	TGACTAAAAGATTGGGAAAGGC	22	
parE			
parE-F	GCAAAGCAACTTCGATATGAAATTC	25	368
parE-R	CGGAGCTATTTACAGACAACGTTTT	25	

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TABLE 2. Correlation of capsular types of strains with 189 invasive GBS infections

Patient group and infection	Capsular type (no. of cases)									Total
	Ia	Ib	II	III	IV	V	VI	VII	VIII	
Children										
Meningitis	3	5		39			3			50 (76.9)[a]
Sepsis	5	2	2	5						14 (21.5)
Other		1								1 (1.5)
Subtotal	8 (12.3)	8 (12.3)	2 (3.1)	44 (67.6)			3 (4.6)			65 (100)
Adults										
Meningitis		1	1	2						4 (0.8)
Sepsis	9	31	12	6		20	6	1	8	93 (75.0)
Other	2	7	2	7		3	3		3	27 (21.8)
Subtotal	11 (8.9)	39 (31.5)	15 (12.1)	15 (12.1)		23 (18.5)	9 (7.3)	1 (0.8)	11 (8.9)	124 (100)

[a] Values in parentheses are percentages.

lytic reaction was carried out for 20 min at 60°C, followed by 5 min at 94°C. The lysate was added to each of five tubes containing PCR mixtures for individual capsular types: types Ia and Ib in tube A, types II and III in tube B, types IV and *dltS* in tube C, types V and VII in tube D, and types VI and VIII in tube E. The reaction mixture (25 μl) consisted of 20 pmol of each primer, 0.625 U of Ampli*Taq* Gold polymerase (Applied Biosystems, Tokyo, Japan), 2.5 μl of 10× PCR Gold buffer, 2.5 μl of 25 mM $MgCl_2$, 2 μl of a 2 mM deoxynucleotide triphosphate mixture, and 16.875 μl of DNase- and RNase-free distilled water. DNA amplification was carried out with 40 cycles of 94°C for 1 min, 53°C for 2 min, and 72°C for 2 min.

We measured the antimicrobial susceptibilities of GBS strains to 14 antibiotics including oral and parenteral agents by agar plate dilution methods using blood agar.

Three genes for macrolide (ML) resistance, *erm*(A), *erm*(B), and *mef*(A), were identified with the three sets of primers and PCR conditions described previously (21).

To identify fluoroquinolone (FQ) resistance, four sets of primers were designed based on the sequences of the *gyrA*, *gyrB*, *parC*, and *parE* genes (Table 1). The PCR mixture (50 μl) consisted of 20 pmol of each primer, 0.625 U of TaKaRa *Ex Taq* polymerase (Takara Bio, Kyoto, Japan), 5 μl of 10× *Ex Taq* buffer, 4 μl of the 2.5 mM deoxynucleotide triphosphate mixture, and 38.25 μl of DNase- and RNase-free distilled water. Amplified and purified DNA samples were sequenced with a BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems, Foster City, CA). The *pbp2x* gene encoding the PBP2X enzyme, which mediates septum formation during cell wall synthesis, was also sequenced with primers reported previously (11).

We performed pulsed-field gel electrophoresis (PFGE) on the 45 GBS strains determined to have FQ resistance according to mutations in the *gyrA* and *parC* genes. Plug-embedded GBS cells were lysed with lysozyme (5,000 U/3 ml) and mutanolysin (20 U/ml) at 50°C for 3 h by a modification described previously (5, 8). Chromosomal DNA was digested at 37°C for 18 h with ApaI (100 U/ml). PFGE was performed with 1% agarose and 0.5× TBE buffer (1× TBE is 90 mM Tris base, 88 mM boric acid, and 2 mM EDTA) at pulse times of 2.91 to 17.33 s, at an angle of 120°, at 6.0 V/cm, and at 14°C for 20 h with the CHEF Mapper (Bio-Rad Laboratories, Hercules, CA).

Table 2 shows relationships between capsular types of GBS pathogens and diagnoses, separately considering children ≤17 years old (n = 65) and adults (n = 124). Diseases were classified into meningitis, sepsis, and other infection groups. In children including newborns (10.8%) with early-onset disease and neonates (70.8%) with late-onset disease, capsular type III predominated at 67.7%, with small numbers of other types. Among adults, those at least ≥50 years old accounted for 83.1% of the cases; capsular type Ib predominated at 31.5%, followed by V (18.5%), II (12.1%), and III (12.1%). In addition to sepsis (75.0%), a variety of diseases were noted: cellulitis, arthritis, necrotizing fasciitis, meningitis, and bacterial endocarditis. Importantly, 88.7% of the affected adults had underlying disease such as diabetes, liver dysfunction, or immune compromise. Instances of death and neurologic sequelae included one of each among children, and eight (6.4%) and two (1.6%) among adults, respectively.

TABLE 3. Susceptibilities of 189 *S. agalactiae* isolates to 14 antimicrobial agents

Delivery route and antibiotic	MIC range[a]	MIC_{50}[a]	MIC_{90}[a]
Oral			
Penicillin G	0.016–0.125	0.063	0.063
Ampicillin	0.031–0.25	0.125	0.125
Amoxicillin	0.031–0.25	0.063	0.125
Cefdinir	0.016–0.125	0.031	0.063
Cefditoren	0.016–0.063	0.031	0.031
Erythromycin	0.016–≥64	0.032	≥64
Clarithromycin	0.031–≥64	0.125	≥64
Clindamycin	0.031–≥64	0.063	≥64
Levofloxacin	0.5–≥64	2	≥64
Intravenous			
Cefazolin	0.063–0.5	0.125	0.25
Cefotiam	0.125–2	0.5	0.5
Cefotaxime	0.016–0.125	0.031	0.063
Panipenem	0.008–0.031	0.016	0.031
Meropenem	0.031–0.125	0.063	0.063

[a] Values are in micrograms per milliliter.

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TABLE 4. Correlation of capsular types with FQ and ML resistance

Patient group and resistance pattern	No. of strains of serotype:									Total no. (%)
	Ia	Ib	II	III	IV	V	VI	VII	VIII	
Children										
FQ^r		6								6 (9.2)
ML^r [*erm*(A)]				2						2 (3.1)
ML^r [*erm*(B)]	1			6						7 (10.8)
Susceptible	7	2	2	36			3			50 (76.9)
Subtotal	8	8	2	44	0	0	3	0	0	65
Adults										
FQ^r		32	1	1			1			35 (28.2)
FQ^r ML^r [*erm*(A)]				1						1 (0.8)
FQ^r ML^r [*erm*(B)]		2		1[a]						3 (2.4)
ML^r [*erm*(B)]			1	4		4		1		10 (8.1)
Susceptible	11	5	13	8		19	8	0	11	75 (60.4)
Subtotal	11	39	15	15	0	23	9	1	11	124

[a] This strain showed three amino acid substitutions in PBP2X. The MICs of ampicillin and cefotiam for the strain were 0.25 and 2.0 μg/ml, respectively.

Table 3 shows the MIC ranges and MICs for 50 and 90% of the strains tested (MIC_{50}, and MIC_{90}, respectively) of oral and intravenous antibiotics for GBS strains. The MIC range of β-lactam agents was narrow, and penicillin-resistant strains were not recognized. Notably, in a strain where cefotiam susceptibility was reduced to 2 μg/ml, four amino acid substitutions, Gly_{398} to Ala, Gln_{412} to Leu, His_{438} to Tyr, and Ile_{600} to Val, were identified in the *pbp2x* gene.

Table 4 shows relationships between ML and FQ resistance and capsular type, separately considering children and adults. Of 23 strains showing ML resistance (12.2%), 3 possessed the *erm*(A) gene and 20 possessed the *erm*(B) gene. The M type was not recognized. ML-resistant strains detected in both children and adults were mostly type III, but a few strains showed other capsular types.

In 45 strains showing high levofloxacin resistance (23.8%), two amino acid substitutions, Ser_{81} to Leu encoded by the *gyrA* gene and Ser_{79} to Phe encoded by the *parC* gene, were identified simultaneously. The capsular type of these strains, including six isolated from children, was predominately Ib, which was observed in 34 strains; other types (II, III, and VI) were each seen in a few strains.

The PFGE patterns of 45 FQ-resistant strains are shown in Fig. 1. These strains included 40 strains of type Ib and 5 strains representing other types. All type Ib strains showed highly homologous restriction patterns that differed clearly from those of type II or III strains.

In Japan, the proportion of the elderly population with underlying diseases has increased rapidly. As a consequence, invasive infections caused not only by GBS, but also *S. dysgalactiae* subsp. *equisimilis* and *S. pneumoniae*, are expected to increase gradually and to become serious problems (19, 20).

The capsular type in isolates from newborns was mostly type

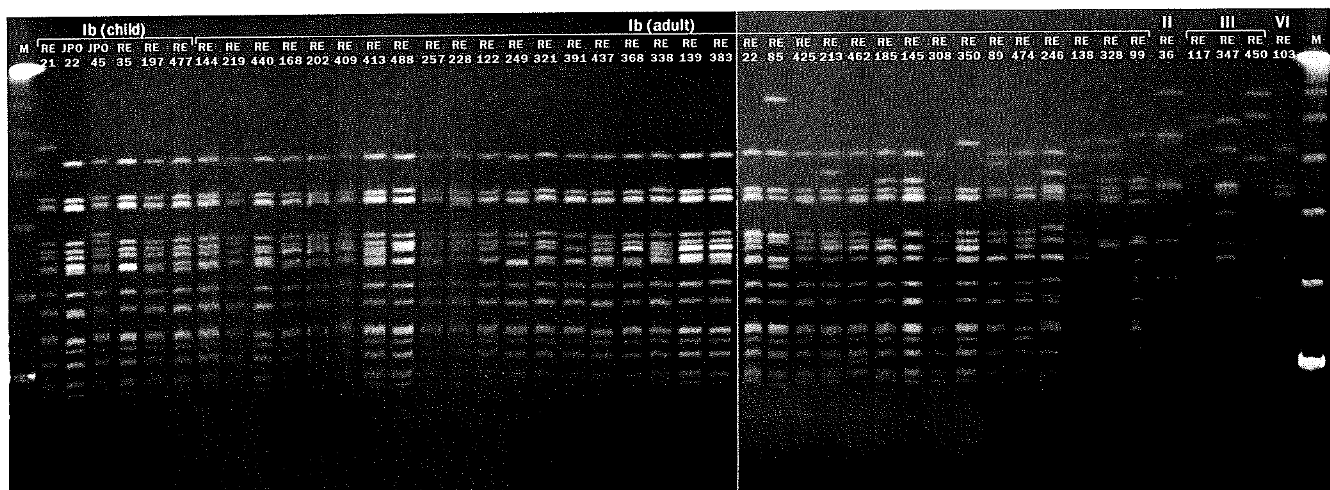

FIG. 1. PFGE patterns of levofloxacin-resistant *S. agalactiae* isolates. Each DNA sample was digested with the ApaI restriction enzyme. Lanes M, lambda ladder.

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III, in agreement with previous results. In most cases involving adults at least 50 years old, however, type Ib was predominant, followed by type V. These findings differ from previous epidemiologic data from the United States; the reason for this disparity is not known.

The percentage of ML resistance was not particularly high compared with that in other countries. Much attention has been drawn to the emergence of GBS with reduced susceptibility to penicillin and cephalosporin antibiotics arising from mutations in the *pbp2x* gene (11). One of our collected strains had mutations of the *pbp2x* gene; this was a type III strain with multiple-antibiotic resistance to ML and FQ. FQ-resistant strains have been reported previously (10, 13, 22) but at extremely low rates. In our results, however, strains resistant only to FQ accounted for 23.8% of the isolates, and most of these were type Ib. FQ-resistant GBS from newborns, who had not been exposed to the agent, showed a PFGE pattern very similar to type Ib from adults. The observations suggest that a single clone acquired FQ resistance and spread rapidly throughout Japan.

Antimicrobial use in Japan favors oral cephalosporins as the drugs of first choice for children, while oral FQ and ML, as well as cephalosporins, are often prescribed for adults. Notably, the size of individual doses of antimicrobials typically is small in Japan compared with that in other countries. These factors will expand the mutant selection window for many pathogens, including GBS, and thus may cause an increase in resistant microorganisms.

To control the emergence of resistant organisms, continuous molecular epidemiologic surveillance for pathogens is needed.

This study was planned at one of the workshops at the 19th Annual Meeting of the Japanese Society for Clinical Microbiology, aiming to determine the molecular epidemiology and clarify background factors in invasive *S. agalactiae* infection. We express our thanks to staff members at all participating institutions.

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原　著

溶連菌感染症を反復した児から検出された A 群溶血連鎖球菌における *emm* 型別と PFGE パターン解析

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Key words: group A *streptococcus*, recurrent infection, carrier, streptococcal infection, *Emm* type

要　旨

2006 年 6 月から 2008 年 12 月までに，A 群溶血連鎖球菌（GAS）感染症が疑われ，咽頭培養から GAS が検出されることを 2 回以上認めた例を GAS 反復感染例とし，それらの児から得られた GAS について T 血清型と *emm* 遺伝子型別，PFGE 解析を実施して，その相同性を検討した．21 名の小児で，43 回の GAS 感染症を確認した．2 回感染が 20 名，3 回感染が 1 名であった．3 回感染の 1 名では 2 回目と 3 回目も比較したが，菌の *emm* 型および PFGE パターンが一致した例は 11 名，一致しなかった例も 11 名であった．初回と 2 回目（1 名は 2 回目と 3 回目）の感染の期間は一致例が 29.1±17.1 日，不一致例が 215.2±187.8 日で有意差を認めた（p＝0.0013）．初回感染後 8 週以内の再発例 12 名中一致例は 10 例であり，8 週以降では 10 名中 1 名であり，有意差を認めた（p＝0.0028）．

〔感染症誌　83：647～651，2009〕

はじめに

A 群溶血連鎖球菌（Group A *Streptococcus*, GAS）による咽頭炎・扁桃炎は小児科では日常的に診療する機会が多い疾患である．しかし，GAS 感染後に非化膿性合併症であるリウマチ熱や糸球体腎炎が発症することもあり，その対応には注意が必要とされている．

GAS 感染症を反復する児は少なくないが，医師によって対応が異なるのが現状である．今回，著者は GAS 感染症を反復した児から検出された GAS について T 血清型，*emm* 遺伝子解析による型別および pulsed-field gel electrophoresis（PFGE）を実施して，分離菌が同一であったか否かを検討したので報告する．

対象および方法

1. 対象

2006 年 6 月から 2008 年 12 月までに，旭川厚生病院小児科，カケハシ小児科医院，丘のうえこどもクリニックを受診し，発熱・咽頭痛の症状と咽頭の所見から，GAS 感染症が疑われ，咽頭培養から GAS が検出された例を GAS 感染例とした．対象期間中に，同様の所見が得られた児を GAS 反復感染例とした．無症状で検出された例は，抗菌薬による除菌失敗例，あるいは保菌者として除外した．

2. T 型別および *emm* 型別

GAS 感染反復例から得られた GAS について T 血清型別と *emm* 遺伝子解析による型別を実施した．T 血清型別は A 群溶血レンサ球菌 T 型別用免疫血清「生研」（デンカ生研）を用いて測定した．*emm* 型別は Beall ら[1]の方法に準じ，M 蛋白をコードする遺伝子を PCR で増幅し，その増幅産物の 5'末端側の塩基配列を決定した後，300bp の配列を CDC レファレンスセンターへ送信し，CDC のデータベースとのマッチングにより型別した．

3. PFGE 解析

PFGE 解析は 37℃ で一夜培養した被験液の 1mL 遠心後，その沈渣を PettIV solution（1M NaCl, 10mM EDTA（pH8.0））に懸濁した後，等量の 2% 低融点アガロースを加え混和し，ただちにプラグモールドに注入して寒天を固化させサンプルプラグを作成した．サンプルプラグはマイクロチューブに移し，achromopeptidase (4mg/mL) および lysozyme (1mg/mL) 添加 lysis solution [1M NaCl, 0.1M EDTA (pH8.0),

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平成21年11月20日

Table 1 Properties of isolates from children with recurrent Group A streptococcal infection

not-coincident								coincident							
Case No	Gender	Age (years)	Duration between 1st and 2nd episodes (days)	T type in 1st episode	T type in 2nd episode	*Emm* type in 1st episode	*Emm* type in 2nd episode	Case No	Gender	Age (years)	Duration between 1st and 2nd episodes (days)	T type in 1st episode	T type in 2nd episode	*Emm* type in 1st episode	*Emm* type in 2nd episode
1	F	8	185	12	6	12	6	3	F	6	58	12	12	12	12
2	M	6	221	4	1	4	1	4	M	6	29	28	28	28	28
5	M	4	212	12	B3264	12	89	6	M	7	31	12	12	12	12
7	M	11	79	12	28	12	28	8	M	9	41	12	12	12	12
10	M	7	19	no typable	1	102	1	9	M	6	47	1	1	1	1
11	M	6	32	1	28	1	28	12	F	5	46	1	1	1	1
13	M	6	451	B3264	1	89	1	16*	M	5	16	4	4	4	4
14	F	5	500	4	28	4	28	17	M	6	22	12	12	12	12
15	M	4	510	1	4	1	4	20	F	8	14	25	25	75	75
18	M	7	66	3	11	3	11	21	F	7	9	B3264	B3264	89	89
19	M	9	92	28	1	77	1	22	M	10	7	25	25	75	75
Average		6.6 ± 2.1	215.2 ± 187.8#					Average		6.8 ± 1.6	29.1 ± 17.1				

*: same case with No 15 (2nd and 3rd episodes)
#: p = 0.013

10mM Tris-HCl, 0.5% Briji58,0.2% sodium deoxycholate, 0.5% sodium N-dodecanoyl-sarcosinata] 中にて35℃で一晩反応させた．マイクロチューブから lysis solution を除去し，次いで proteinase K (1mg/mL) 添加 ES solution [0.25M EDTA (pH8.0), 1% sodium N-dodecanoyl- sarcosinate] を加え，50℃で一晩反応させた．マイクロチューブから ES solution を除去し，phenylmethyl sulphonyl fluoride 添加 TE [10mM Tris-HCl, 1mM EDTA (pH8.0)] を加え，室温で約3時間反応させた後，サンプルプラグを TE で洗浄した．制限酵素処理を行ったサンプルは CHEF-DRIII (Bio-Rad) を用いて，PFGE を行った．電気泳動後のアガロースゲルはゲルスター（タカラバイオ）を用いて染色した後，トランスイルミネーターを用い写真撮影を行った．症例番号1～19のサンプルは制限酵素 *SmaI* を用いて30℃で8時間処理を行い，initial time 3.0, final time 60.0 agarose 濃度1.0%，電圧 6V/cm の条件下で18時間電気泳動を行った．症例番号20から22は上記の方法では，鮮明な DNA パターンが得られなかったため，制限酵素 *Sfi* I を用いて50℃，8時間処理を行い，initial time 20.0, final time 70.0, agarose 濃度1.0%，電圧 6V/cm の条件下で22時間電気泳動を行った．

4. 有意差検定

有意差検定は StatMateIII for Macintosh（アトムス）により Mann-Whitney 検定，またはカイ二乗検定を行い，p<0.05 を有意差ありとした．

成　績

1. 21症例から分離された GAS 株の T 型別と *emm* 型別からみた同一性

対象期間中に，小児21名における合計43回の GAS 感染症を確認した．Table 1には症例の年齢，性別，初回感染（初回エピソード）と2回目感染（2回目エピソード）との期間，T 型別と *emm* 型別との成績を示した．3回の感染エピソードがみられた症例については，初回と2回目，2回目と3回目の成績をそれぞれ示した．

2回の感染エピソード例が20名，3回の感染がみられた例が1名認められた．このうち，T 型別および *emm* 型別が2回のエピソードとも一致していた症例は11例，不一致であった症例は11例であった．Emm 型が一致していた症例の内訳は，*emm*12型が4例，*emm*1型と *emm*75型がそれぞれ2例，その他に *emm*4型，*emm*28型，および *emm*89型が各1例ずつであった．

2. 21症例から分離された GAS 株の PFGE パターン解析

Fig. 1には症例番号1～19より分離された GAS 株を *Sma*I酵素で処理したサンプルの PFGE による DNA パターン，Fig. 2には症例番号20～22より分離された GAS 株を *Sfi* I 酵素で処理したサンプルの同様の成績を示す．それぞれのサンプルは初回エピソードと2回目エピソードの菌はペアで泳動されている．

2回のエピソードから菌が分離され，*emm* 型が一致

Fig. 1 PFGE patterns of Group A *Streptococcus* strains isolated from children (Cases 1 to 19) with recurrent infection at initial (left) and recurrent (right) visit M: marker

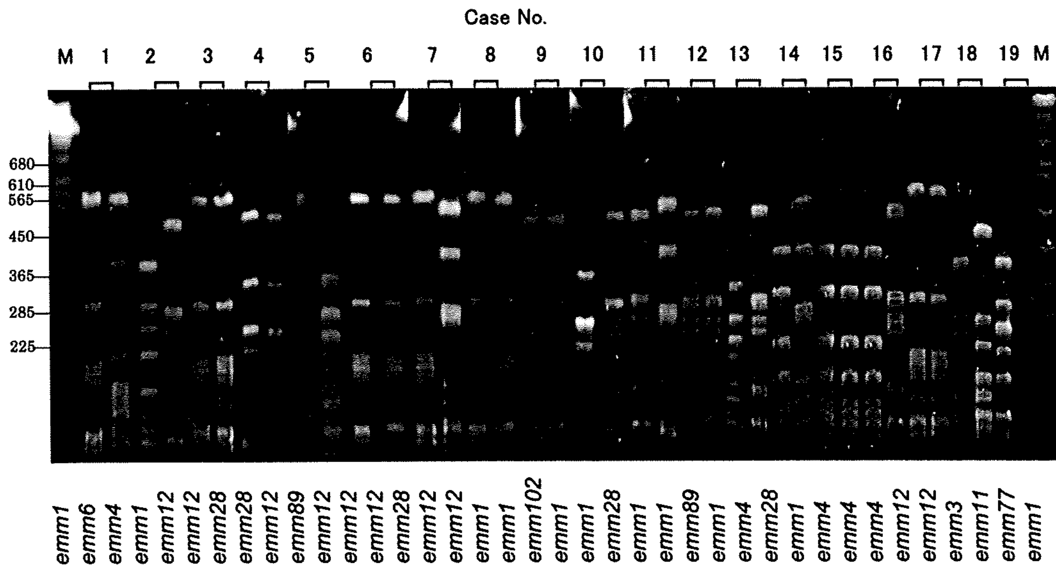
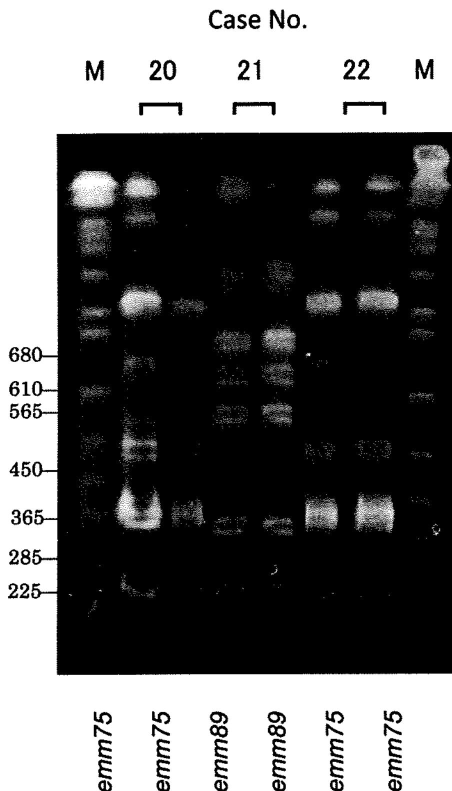


Fig. 2 PFGE patterns of Group A *Streptococcus* strains isolated from children (Cases 20, 21 and 22) with recurrent infection at initial (left) and recurrent (right) visit. M: marker



していた症例サンプルのDNAパターンは明らかに同一で11例認められた．これに対し，*emm*型が不一致であった症例では，DNAパターンも明らかに異なっており，11例が認められた．

なお，異なった症例間においても，同一の*emm*型を示すGAS株が分離されていた際のDNAパターンは，ほぼ同一と思われるパターンを示していた．一致例の平均年齢は6.8±1.6歳，不一致例は6.6±2.1歳で有意差を認めなかった．

3. エピソード間隔と菌の*emm*型およびPFGEパターンの関連

初回発症のエピソードと2回目エピソードに分離された菌の*emm*型あるいはPFGEの成績が同一であった場合は「同一菌株による再燃，あるいは一度消失した菌もよる再感染（菌株一致例）」，それらが異なっていた場合には「異なった菌株による新たな感染（再感染：菌株不一致例）とみなし，それぞれの症例におけるエピソード間隔（期間）とそれらの関係を比較した．

Fig. 3にはそれらの成績を示したが，初回8週以内に再び発症した例では，12症例中10例が一致例であった．これに対し，8週以降に再び発症した例では一致例は10症例中1例のみであり，両者間には有意な差が認められた（$p=0.0028$）．

なお，初回エピソードと2回目エピソードとの間隔期間を日数換算すると，一致例の平均は29.1±17.1日，不一致例の平均は215.2±187.8日となり，明らかな有意差を認めた（$p=0.013$）．

考　案

GAS感染症を反復する場合，抗菌薬投与によっても除菌されなかった株が感染を起こす再燃と新たに獲得した株が感染を起こす再感染に分けられる．それに加えて，保菌しているだけの例が，なんらかの感染症に罹患して，咽頭の検査が行われて，GASが検出されるという，みかけ上のGAS感染症がある．これら

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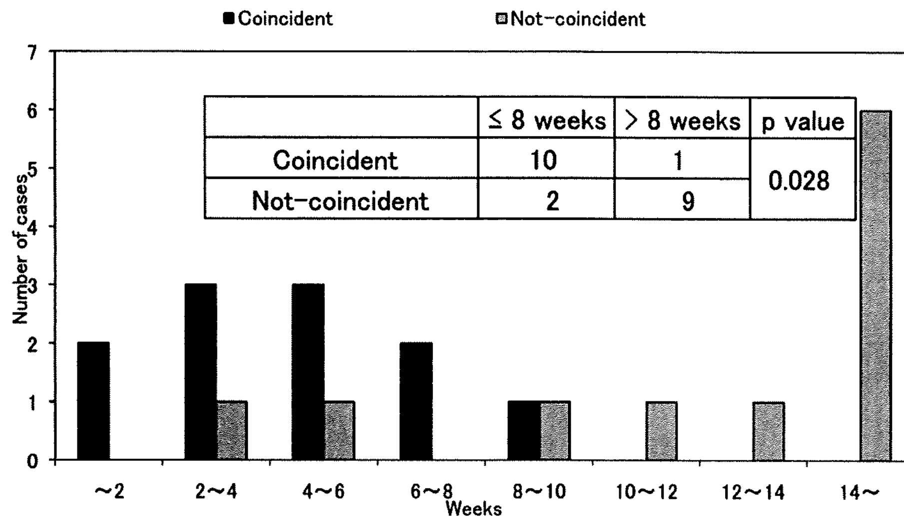
Fig. 3 Relationship between duration of recurrent Group A streptococcal infection and strain properties



	≦8 weeks	＞8 weeks	p value
Coincident	10	1	0.028
Not-coincident	2	9	

を厳密に識別することは困難であるが，それぞれの株の性状が異なれば再感染である．同一の場合では再燃と考えられるが，GAS 感染症が流行している集団に属していると，同一の株を再獲得することもあるので，厳密に再燃と再感染とを分けることはできない．

GAS 感染症に有効な抗菌薬を投与しても，GAS が除菌できない例は，すくなからず認められる．理由として，コンプライアンスが大きな問題として考えられるが，それ以外に咽頭の常在菌叢を構成する菌が産生する β-lactamase がペニシリン薬を分解する[2]，抗菌薬によって咽頭の常在菌叢が変動し，病原菌を抑制する機能を低下している[3]，抗菌薬投与によって菌の増殖は抑制されるが，殺菌しえないため抗菌薬が中止されると再び増殖を始める tolerance 現象による[4]，集団から菌を獲得する ping-pong 感染をおこす[5]，菌が細胞内に侵入する[6]，感染ではなく保菌状態であるため抗菌薬の効果が低い[7]，などがあげられている．しかし，その機序はいまだに明らかになっていない．

Martin ら[8]は 4 年間を観察期間として，学童の咽頭からの GAS の検出状況を検討した．1 年間に 48～100 名の小児に 10 月から 5 月まで月に 2 回の定期的な咽頭スワブの採取を行い，それに加えて，上気道炎症状を呈した時にも検体を採取し，計 5,658 の検体について培養検査を行った．検出された GAS は PFGE と *emm* 型別で同一性を検討した．保菌者ではなく，GAS 感染症として抗菌薬治療を受けたのはのべ 209 名であった．全体のうちの 878（15.5%）検体から GAS が検出され，13 の *emm* 型に識別された．1 年ごとに評価していた成績であるが，4 年間で GAS 非検出者 38～46%，保菌者 27～32%，1 回感染者 18～21%，反復感染者 6～14% とほぼ一定していた．また，保菌者でも菌は常に同じ *emm* 型ではなく，同一の *emm* 型の菌を平均 10.8 週検出し，その後異なった *emm* 型を獲得していた．反復感染した 30 例では同じ *emm* 型が 15 例，違った *emm* 型が 15 例と同数であった．

Gerber ら[9]は 113 名の GAS 感染症のうち 44 名が 6 カ月以内に反復し，その多くは治療終了 4 日から 2 カ月であった．そして，同じ血清型は 15 名，異なったのが 29 名で，異なった株が多かった．著者の成績では 8 週を境にして，菌株の性状が異なり，再獲得したと思われる例が多かった．Martin ら[8]，Gerber ら[9]と著者の成績から，GAS 感染症を反復する例であっても，常に同一の株が反復して感染をきたしているのではなく，8～12 週で再獲得した GAS による炎症症状が起こっていると考えられた．GAS 感染症後 8 週間以内に，GAS 感染症を発症した場合には，同一性状の株が原因である可能性が高いと思われる．

したがって，8 週以内に GAS 感染症が発症した場合には，感染した株がなんらかの理由から投与した抗菌薬の効果が充分ではなく残存したと考えられるので，同様の治療では有効性が低いと思われるため，異なる薬剤または投与期間で治療すべきと思われる．それとは対照的に 8 週以降の発症では再獲得された GAS による可能性が大きなので，1 回目のエピソード時と同様な治療方法を選択しても問題ないと考える．

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A Study of Group A *Streptococcus* Strains Isolated from Children with Recurrent Group A Streptococcal Infection

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Recurrent Group A *Streptococcus* (GAS) infection was defined as a case in which GAS infection was suspected during June 2006～December 2008 and GAS was detected more than 2 times by throat culture. T serotype and *emn* genotype were investigated for GAS bacteria isolated from children with recurrent GAS infection. Bacteria gene homology was studied using pulsed-field gel electrophoresis (PFGE). Of the 43 cases of GAS infection in 21 children used as subjects, 20 had GAS infection 2 times and one 3 times. A comparison of the first and second infection in subjects (and the second and third for the child with 3), bacteria isolated from 11 cases had coincident properties and those from the remaining 11 not-coincident. The mean period of first and second infections (second and third for the child with 3) was 29.1±17.1 days for the coincident group and 215.2±187.8 days for the not-coincident group. A significant group difference in period was seen (p = 0.0013). Ten of 12 children with recurrent GAS infection within 8 weeks of the first infection and one of 10 who had it more than 8 weeks after that were included in the coincident group, indicating a significant difference (p = 0.0028).

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IN VIVO IL-18 SUPPLEMENTATION AMELIORATES LETHAL ACUTE LUNG INJURY IN BURN-PRIMED ENDOTOXEMIC MICE: A NOVEL ANTI-INFLAMMATORY ROLE OF IL-18

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ABSTRACT—Previously, we have found that a prior burn insult induces lethal acute lung injury (ALI) and overproduction of proinflammatory cytokines after LPS challenge in mice. The current study was aimed to determine the role of IL-18 in burn-induced LPS hypersensitivity. Except sham group, mice were subjected to a 15% total body surface area full-thickness burn and either untreated or treated with IL-18 alone, IL-18 + anti–IL-10 antibody or IL-18 + isotype immunoglobulin G. LPS was intravenously administered to all mice on the 11th day, and the mice were killed at the indicated time point, or survival was examined. We additionally examined cytokine production by splenic cells *in vitro* for the elucidation of immunologic mechanisms. Unexpectedly, the liver IL-18 decreased transiently after burn injury, and *in vivo* IL-18 supplementation improved survival and ameliorated ALI, as well as reducing the lung contents of all cytokines examined, except IL-10. Neutralization of IL-10 cancelled the protective effect of IL-18. In splenic macrophages obtained from burned mice, the production of macrophage inflammatory protein 2 (MIP-2), TNF-α, and IL-10 was augmented, whereas *in vivo* IL-18 supplementation decreased MIP-2 production, but increased IL-10 production. Furthermore, a physiological concentration of IL-18 directly attenuated MIP-2 production by splenic cells *in vitro*. Burn injury induces LPS hypersensitivity through augmented production of proinflammatory cytokines by systemic macrophages. IL-18 supplementation is protective for LPS-induced lethal ALI through the direct anti-inflammatory effect on macrophages as well as by *in vivo* acceleration of IL-10 production, and could thus be an effective prophylactic strategy against septic complications in critically ill patients.

KEYWORDS—Two-hit phenomenon, priming, systemic inflammatory response syndrome, M2 macrophage interleukin-10

INTRODUCTION

Patients with critical conditions, such as severe burns, multiple trauma, and hemorrhagic shock, become susceptible to secondary insults, including sepsis, and may develop septic shock and/or multiple organ dysfunction syndrome, especially acute lung injury (ALI)/acute respiratory distress syndrome (1). Multiple organ dysfunction syndrome was not merely derived from the direct injurious effects of insults to these organs, and LPS administration after burn injury induced progressive hypoxia in a sheep model (2). Furthermore, 38% of the severely burned patients developed lung failure even without inhalation injury (3). These complications are major determinants of prognosis at the later stages, and new therapeutic strategies based on novel pathophysiological mechanisms are currently being sought. Hyperresponsiveness to secondary stimuli after an initial insult is known as the "two-hit" phenomenon and has been confirmed in several experimental models (1, 4, 5). Previously, we have developed a murine model of burn-primed lethal septic ALI and showed that a prior burn insult induced sustained and exaggerated production of TNF-α and macrophage inflammatory protein 2 (MIP-2) in response to an LPS challenge (6). We further demonstrated that two independent cytokine synthesis inhibitors ameliorated ALI and improved survival, and concluded that inflammatory cytokines play pivotal roles in inducing lethal endotoxemic ALI after burn injury (6). However, the mechanisms responsible for the exaggerated cytokine response after burn injury were not determined.

IL-18 was initially identified as a key mediator in *Propionibacterium acnes*–induced hypersensitivity to LPS (7, 8), and its diverse immunomodulating functions have recently been clarified. IL-18 induces interferon-γ (IFN-γ) and TNF-α in the presence of IL-12, and was initially considered to be a T_H1 or proinflammatory cytokine. *In vivo* IL-18 neutralization protects wild-type mice from the effects of LPS challenge (9), and IL-18 knockout mice are resistant to LPS (10). Furthermore, exogenous IL-18 protects mice from lethal *Cryptococcus* infection (11). Thus, IL-18 seems to have *in vivo* immunostimulatory and anti-infection activities. On the other hand, although it was recently revealed that IL-18 can also induce T_H2 cytokines, such as IL-4, IL-10, and IL-13, under specific conditions, especially when IL-12 is absent (8, 12, 13), the anti-inflammatory functions of IL-18 have not been elucidated.

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