Table 1 Association of serotypes with multilocus sequence typing profiles and genetic *pbp* patterns among *S. pneumoniae* strains

Serotype	ST	CC	Genotype	No. of strains	Allele gene							First reported	
					aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*	Year	Country (city)
1	**5239**	306	gPSSP	1	12	8	13	5	16	**336**	20		
3	**5234**	180	*2x*	1	7	15	2	10	6	1	**383**		
4	246	246	gPSSP	4	16	13	4	5	6	10	18	1997	UK
6A	3115	3115	gPRSP	2	7	32	6	1	6	14	14	1989	Korea
	2756	3787	gPRSP	1	8	8	19	16	77	1	68	2004	China
	5243	3787	gPRSP	1	8	8	4	16	77	1	26		
	3787	3787	*2x*	1	8	8	19	16	6	1	68	UN	Singapore
6B	90	156	gPRSP	7	5	6	1	2	6	3	4	1986	Spain
	3387	156	gPRSP	1	5	6	1	2	6	3	26	2002	Korea
	2983	156	*2x* , *1a+2x*	3	5	6	1	2	6	1	271	2003	Japan (Okayama)
	902	490	gPRSP	1	2	13	2	1	6	121	121	2000	Singapore
	2923	490	*2x* , *1a+2x*	2	2	13	2	5	6	121	29	2003	Japan (Kurume)
	5233	490	*2x+2b*	1	2	13	2	5	6	121	14		
	5232	Singleton	gPRSP	1	2	29	4	1	6	121	121		
	5238	Singleton	gPRSP	1	2	5	2	**149**	6	121	121		
	5244	Singleton	gPRSP	1	2	168	2	5	6	121	**385**		
	5245	Singleton	*2x*	1	2	13	19	5	6	124	29		
	2224	2224	gPRSP, *1a +2x*	2	7	12	7	1	116	14	29	1996	UK
	5235	2224	*1a+2x*	1	7	12	7	1	116	20	29		
	5230	180	gPRSP	1	7	15	2	10	6	1	**382**		
	2924	2924	*2x*	1	1	5	2	6	6	1	14	2003	Japan (Hyogo)
6C	2924	2924	*2x*	1	1	5	2	6	6	1	14	2003	Japan (Hyogo)
	5247	156	*2x*	1	1	29	8	6	6	6	14		
	2923	490	*2x*	1	2	13	2	5	6	121	29	2003	Japan (Kurume)
	5241	Singleton	*2x+2b*	3	7	9	8	6	1	6	**384**		
7C	2758	2758	gPSSP	1	10	5	1	1	9	220	8	2005	China
9V	280	280	*2x* , *2x+2b*	3	15	17	4	16	6	1	17	1998	Vietnam
	5231	280	*2x*	3	15	17	4	**148**	6	1	17		
10A	1263	280	*2x*	1	15	13	4	16	6	1	17	1998	USA
	5236	Singleton	gPSSP	1	7	12	1	1	10	1	11		
12F	4846	1527	*2b*	4	12	32	111	1	13	48	6	UN	Japan (Osaka)
14	343	554	gPRSP	2	8	8	4	15	39	12	14	1998	Norway
	236	320	gPRSP	1	15	16	19	15	6	20	26	1993	Taiwan
	5240	230	gPRSP, *1a+2x*	4	5	19	2	17	6	22	14		
	13	15	*1a+2x*	1	1	5	4	5	5	27	8	1997	Australia,USA
	2922	15	*1a+2x*	3	1	5	4	5	5	20	8	2003	Japan (Hyogo)
15A	63	63	*1a+2x*	1	2	5	36	12	17	21	14	1992	Sweden
15B	199	199	*1a+2x*	1	8	13	14	4	17	4	14	1987	Netherlands
15C	199	199	*2x*	1	8	13	14	4	17	4	14	1987	Netherlands
18C	3594	3594	gPSSP	1	10	13	34	16	6	1	31	2007	South Korea
19F	236	320	gPRSP	16	15	16	19	15	6	20	26	1993	Taiwan
	115	115	gPRSP	1	15	16	19	15	30	20	39	1994	Taiwan
19A	3111	3111	*2x* ,*1a+2x*	2	61	60	67	16	10	104	14	1989	USA
	2331	2331	*2x*	1	10	16	150	1	17	1	29	1999	Czech
	5237	2331	*1a+2x*	1	10	16	150	1	30	1	29		
22F	433	433	*2x*	1	1	1	4	1	18	58	17	1997	Poland
23F	242	242	gPRSP	7	15	29	4	21	30	1	14	1996	Taiwan
	1437	1437	gPRSP	1	1	32	6	6	6	1	14	2000	Japan
	63	63	gPRSP	1	2	5	36	12	17	21	14	1992	Sweden
	338	156	gPRSP, *2x+2b*	3	7	13	8	6	1	6	8	1995	Colombia
	5246	156	*2x+2b*	1	7	13	8	6	1	138	8		
	5242	156	*2x+2b*	1	7	13	8	6	1	**337**	8		
23A	338	156	gPRSP, *2x+2b*	3	7	13	8	6	1	6	8	1995	Colombia
24	4982	4982	gPSSP	1	7	120	8	8	25	28	14	2001	USA
34	3116	Singleton	*2x* , gPSSP	3	10	8	6	1	9	1	279	2004	Japan (Okayama)
35	558	558	gPRSP	1	18	12	4	44	14	77	97	1998	South Korea
	2755	2755	*2x*	1	10	12	2	1	152	28	14	2004	China
38	393	393	gPSSP	1	10	43	41	18	13	49	6	1998	USA

New sequence types (STs) and alleles are shown in bold face and underlined. Country, first country of isolation for the same ST clone referred from the MLST database

CC clonal complex, *year*, first isolation year of the same ST clone referred from the MLST database

Table 2 Associations of isolation areas with serotypes and sequence types

Serotype	MLST type (n)	Area					
		Hokkaido Tohoku	Kanto	Chubu	Kinki	Chugoku Shikoku	Kyushu
4	246(4)		246(1)	246(2)			246(1)
6A	3115(2)		3115(1)			3115(1)	
6B	2923(2)				**2923(2)**		
	90(7)	90(3)	90(1)		90(2)		90(1)
	2983(3)		2983(3)				
	2224(2)				2224(1)		2224(1)
	2924(1)				**2924(1)**		
6C	2923(1)	**2923(1)**					
	2924(1)				**2924(1)**		
	5241(3)		5241(1)		5241(1)		5241(1)
9V	280(3)	280(1)		280(2)			
	5231(3)		5231(1)	5231(1)		5231(1)	
12F	4846(4)		4846(1)		4846(2)		4846(1)
14	343(2)			343(2)			
	5240(4)		5240(1)				5240(3)
	2922(3)		2922(1)		2922(1)		2922(1)
	236(1)		**236(1)**				
15A	63(1)		**63(1)**				
15B	199(1)						**199(1)**
15C	199(1)		**199(1)**				
19A	3111(2)		3111(2)				
19F	236(16)	**236(2)**	**236(4)**		**236(6)**	**236(1)**	**236(3)**
23A	338(3)		**338(2)**	**338(1)**			
23F	338(3)		**338(1)**		**338(2)**		
	63(1)				**63(1)**		
	242(7)	242(1)	242(2)	242(1)	242(1)	242(1)	242(1)
34	3116(4)	3116(2)		3116(1)	3116(1)		

Sequence types (STs) with two or more strains were selected. Novel STs are shown in bold face and underlined

The STs corresponding to the multiple serotypes are indicated in red bold face

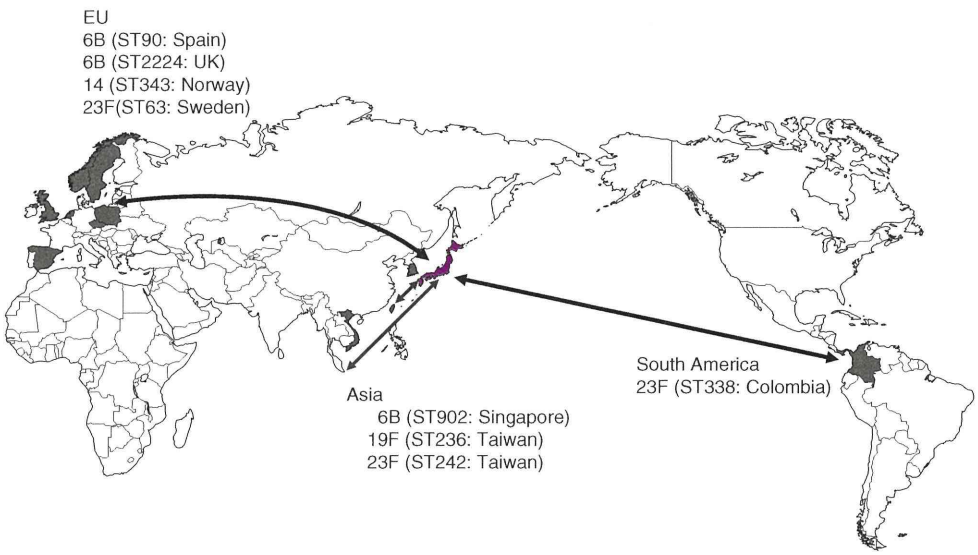

Fig. 3 Possible spread of prevalent serotypes, such as 6B, 19F, 23F, and 14, and corresponding sequence types between foreign countries and Japan

in serotype 19F, and ST63/ST242/ST338 in serotype 23F. As shown in the figure, pneumococcal strains now present in Japan and also other areas may have spread between Japan and European, Asian, or South American countries. In the future, clonal expansion is likely to result from human population drift.

Our manuscript represents the first report regarding associations of serotypes with MLST data and genotypic resistance classes based on PBP alterations in pneumococcal strains from children with meningitis in Japan. Pneumococcal MLST results have already been described in isolates from Japanese adults with community-acquired pneumonia [16]. Invasive pneumococcal disease is an important concern in Japan, and differences in serotype distributions between children and adults should be noted [21].

Continuous, accurate molecular epidemiologic surveillance regarding pneumococcal strains continues to be important in terms of global issues including vaccination and new antibiotic development.

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Application of the Real-Time PCR Method for Genotypic Identification of β-Lactam Resistance in Isolates from Invasive Pneumococcal Diseases

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We sought to identify genotypic resistance classes by real-time PCR in 300 *Streptococcus pneumoniae* isolates from invasive pneumococcal diseases. Primers and molecular beacon probes were designed for the *lytA* gene, 3 *pbp* genes, and the *mef*A/*erm*B genes. Targeted sequences of *pbp1a*, *pbp2x*, and *pbp2b* genes in susceptible strain R6 corresponded to those of penicillin G-nonsusceptible strains, including sites within or adjacent to conserved amino acid motifs. If amplification did not occur, the corresponding penicillin-binding protein (PBP) was considered to possess amino acid substitution(s) affecting minimal inhibitory concentrations (MICs) of β-lactam antibiotics. Real-time PCR required 90 min or less. Strains were assigned to six genotypic classes: Genotypic penicillin-susceptible *S. pneumoniae* (gPSSP) with 3 normal genes (22.3%); genotypic penicillin-intermediate *S. pneumoniae* (gPISP) (*pbp2x*) with an abnormal *pbp2x* gene (25.3%); gPISP (*pbp2b*) with an abnormal *pbp2b* gene (7.3%); gPISP (*pbp1a*+*2x*) with abnormal *pbp1a*+*2x* genes (11.3%); gPISP (*pbp2x*+*2b*) with abnormal *pbp2x*+*2b* genes (4.7%); or genotypic penicillin-resistant *S. pneumoniae* (gPRSP) with 3 abnormal PBP genes (29.0%). Sensitivity and specificity of real-time PCR compared with those of conventional PCR were high, 73.7–100% and 97.7–100%, respectively. As for relationships between genotype and β-lactam MICs, 90% of MICs for every resistance class were distributed within three serial dilutions for almost all antibiotics. MICs of each β-lactam antibiotic were estimated with high probability from genotypic patterns. In conclusion, determination of genotypic classes of *S. pneumoniae* using rapid real-time PCR is useful in selecting effective therapeutic agents for patients with pneumococcal infection.

Introduction

Streptococcus pneumoniae is a leading etiologic agent in children and adults with severe invasive infections that contribute importantly to morbidity and mortality.[20,32] Strains resistant to penicillin G (PEN) have emerged and spread rapidly worldwide.[1,15]

In Japan, clinical isolates of PEN-resistant *S. pneumoniae* (PRSP) and PEN-intermediate *S. pneumoniae* (PISP) have increased rapidly since the late 1990s among school and preschool children as well as patients aged 65 years or older with either respiratory tract infections (RTI) or invasive pneumococcal diseases (IPD).[28,30] The mortality rate reportedly is higher in elderly IPD patients than in pediatric patients.[8]

Characteristically, PRSP and PISP strains show simultaneous resistance to cephalosporin antibiotics used in ambulatory practice.[30] The resistance mechanism for β-lactam antibiotics in PRSP and PISP is a decrease in affinities of three PEN-binding proteins (PBP) involved in peptidoglycan synthesis. These three enzymes, PBP1A, PBP2X, and PBP2B, are encoded by the *pbp1a*, *pbp2x*, and *pbp2b* genes, respectively. Among PEN-nonsusceptible strains (PRSP and PISP), abnormal genetic mosaic patterns of *pbp1a*, *pbp2x*, and/or *pbp2b* were found to differ from those of PEN-susceptible *S. pneumoniae* (PSSP).[9,14] Although a variety of mosaic regions have been detected in each gene, the main contributors to β-lactam resistance are amino acid substitutions identified within or adjacent to conserved amino acid motifs such as Ser-Thr-Met-Lys (STMK), Ser-Ser-Asn (SSN), and Lys-Ser-Gly (KSG).[2,3,13,23,24]

Therefore, we established a conventional PCR method to determine whether or not a pneumococcal isolate is PEN-susceptible according to molecular evidence.[31] This PCR was completed within 2.5 hr from selection of a colony for testing by amplification and gel electrophoresis. The resistance pattern based on the results of conventional PCR

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was defined as genotypic (g) resistance and represented by designations such as gPRSP (*pbp1a+pbp2x+pbp2b*), gPISP(*pbp1 a+pbp2x*), gPISP(*pbp2x*), and gPSSP. Currently, the prevalence of gPRSP possessing three abnormal *pbp* genes exceeds 46% among pediatric patients and 17% among adults in Japan.[8]

Given this situation, therapeutic choices for Japanese IPD patients have gradually eroded, with empirical first-line therapy shifting from penicillins or third-generation cephalosporins to carbapenem antibiotics. At the same time, numbers of adults and elderly persons with various underlying diseases posing high risk of IPD have increased rapidly.

In the present study, we aimed to construct a novel assay using real-time PCR that eliminates the need for gel electrophoresis, allowing completion of all procedures within 90 min. We describe sensitivity and specificity of our real-time PCR compared with conventional PCR and efforts to estimate MICs of therapeutic agents against various strains belonging to different PBP genotypic classes.

Materials and Methods

Strains and serotyping

Clinical isolates of *S. pneumoniae* obtained from IPD patients were collected from 186 clinical laboratories at medical institutions participating in our program of active nationwide surveillance for emerging and re-emerging of infectious diseases. We randomly selected 300 strains as follows: Blood ($n=218$), cerebrospinal fluid ($n=56$), pleural fluid ($n=14$), joint fluid ($n=6$), and others ($n=6$). These strains were sent to our laboratory from August, 2006, to July, 2007, accompanied by application form with a similar format as the Active Bacterial Core Surveillance (ABCs) case report.

The serotypes of all strains were determined in real time by the Quellung reaction using antiserum purchased from the Statens Serum Institute (Copenhagen, Denmark). The serotypes of these strains were mainly 6B ($n=47$), 12F ($n=28$), 14 ($n=27$), 3 ($n=26$), 4 ($n=22$), 9 ($n=20$), 19F ($n=19$), 23F ($n=18$), 6A/6C ($n=16$), 19A ($n=14$), 15 ($n=12$), and others ($n=51$).

Real-time PCR primers and molecular beacon probes

Sequences of six sets of primers and molecular beacon (MB) probes and amplicon sizes (bp) applied for our real-time PCR are shown in Table 1. Target genes and the DNA amplification positions were the *lytA* gene encoding the autolysin enzyme specific to *S. pneumoniae*[12]; the *pbp1a* gene detected in susceptible strains,[18] located in the region including a conserved amino acid motif, STMK, corresponding to that of resistant strains; the *pbp2x* gene detected in susceptible strains,[16] located in the region surrounding the STMK motif corresponding to divergent sequences of resistant strains; the *pbp2b* gene detected in susceptible strain,[10] located in the region adjacent the SSN motif; the *mef*A gene

Table 1. Primers and Molecular Beacon Probes for Real-Time PCR

Target gene	*Sequence (5′ to 3′)*	*Position*	*Amplicon size (bp)*	*Target amino acid substitution*
Autolysin (*lytA*)				
Sense primer	CAGAATTAGGTTTTTTCTCGC	723–743	188	—
Reverse primer	TAAGAGTTCGATATAAAGGCG	890–910		
Probe	FAM-CGCGATCAGGTCTCAGCA TTCCAACCGCCGATCGCG-BHQ1	809–830		
PBP 1A (*pbp1a*)				
Sense primer	AAACCGCGACTGGGGATCAAC	2037–2057	239	S(T)MK
Reverse primer	GGTTGAGTCCGACCTTGTTT	2275–2256		↓
Probe	FAM-CGCGATCACTGGGATAGGGG CTACTTTGGCGATCGCG-BHQ1	2174–2196		A or S
PBP 2X (*pbp2x*)				
Sense primer	CCAGGTTCCACTATGAAAGTG	1255–1275	197	S(T)(M)K
Reverse primer	ATCCCAACGTTACTTGAGTGT	1451–1431		↓ ↓
Probe	FAM-CGCGATCAGATGCCACGATTC GAGATTGGGGATCGCG-BHQ1	1353–1375		A F
PBP 2B (*pbp2b*)				
Sense primer	CCTATATGGTCCAAACAGCCT	1566–1586	147	SSN(T)
Reverse primer	GGTCAATTCCTGTCGCAGTA	1712–1693		↓
Probe	FAM-CGCGATCTCGGCACCAGCAAT CTAGAGTCTGATCGCG-BHQ1	1626–1648		A or S
Macrolide efflux (*mef*A)				
Sense primer	GGGACCTGCCATTGGTGTGC	180–199	402	—
Reverse primer	CCCAGCTTAGGTATACGTAC	581–562		
Probe	FAM-CGCGATCCCCCAGCACTCAAT GCGGTTACACGATCGCG-BHQ1	359–382		
Adenine methylase (*erm*B)				
Sense primer	CGTACCTTGGATATTCACCG	721–740	224	—
Reverse primer	GTAAACAGTTGACGATATTCTCG	944–922		
Probe	FAM-CGCGATCCCGCCATACCACAG ATGTTCCGATCGCG-BHQ1	852–872		

encoding the efflux protein for 14-membered macrolide (ML) antibiotics[25]; and the *ermB* gene encoding adenine methylase for 14- and 16-membered ML antibiotics.[27]

Primers and MB probes corresponding to *pbp1a*, *pbp2x*, and *pbp2b* genes were designed to amplify the DNA only in susceptible strains. All MB probes were labeled with a fluorescent reporter of 6-carboxyfluorescein (FAM) at the 5′ end and also with a black hole quencher 1 (BHQ-1) at the 3′ end. Reporters and quenchers were connected to stem oligonucleotides.

Real-time PCR conditions

The real-time PCR reaction mixture consisted of 15 μl of 2× real-time PCR Master Mix (Toyobo, Tokyo, Japan), each primer at 0.2 μM, and each MB probe at 0.3 μM. The final volume of the mixture was adjusted to 30 μl by addition of DNase- and RNase-free H_2O. After each reaction mixture was pipetted into a 96-well plate, plates were stored at −30°C until use.

One colony grown on a sheep blood agar plate was picked up and suspended in 30 μl of lysis solution.[29] The tube then was placed in a thermal cycler (Gene Amp PCR System 9600R; Perkin-Elmer Cetus, Norwalk, CT) and heat-treated for 5 min at 60°C and for 5 min at 94°C to obtain template DNA. Next, after wells of the frozen real-time PCR reagent were thawed on ice, 2 μl of each template DNA was added to each well. Real-time PCR was performed immediately with a Stratagene Mx3000P (Stratagene, La Jolla, CA). The PCR conditions included an initial DNA denaturation step of 95°C for 30 sec, followed by 40 cycles of 95°C for 15 sec, 50°C for 20 sec, and 75°C for 15 sec. The time required from the lyses reaction to completion of real-time PCR was 90 min.

Conventional PCR

Conventional PCR was performed as a control assay for the real-time PCR in the same strains, using a commercially available kit (Wakunaga Pharmaceuticals, Hiroshima, Japan). The right of commercial production for this kit had been transferred to the company from Ubukata et al.[19,30]

Sequencing of pbp *genes with discrepancies between the two PCR methods*

Both the *pbp1a* and *pbp2x* genes in *S. pneumoniae* strains for which a discrepancy in the PCR data was recognized between the conventional and real-time methods were sequenced to identify the amino acid substitution. PCR primers used for analysis were a sense primer for *pbp1a*, 5′-TGGGA TGGATGTTTACACAAATG-3′; a reverse primer for *pbp1a*, 5′-TGTGCTGGTTGAGGATTCTG-3′; a sense primer for *pbp2x*, 5′-TATGAAAAGGATCGTCTGGG-3′; and a reverse primer for *pbp2x*, 5′-AGAGAGTCTTTCATAGCTGAAGC-3′, as described previously.[2,3]

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). DNA sequencing was performed with an ABI 3130/3130xl genetic analyzer (Applied Biosystems).

Susceptibility testing

MICs of the five β-lactam antibiotics PEN, ampicillin (AMP), cefotaxime (CTX), meropenem (MEM), and panipenem (PAM) were determined by an agar dilution method using Mueller–Hinton II agar (MH, Becton Dickinson, Franklin Lakes, NJ) supplemented with 5% defibrinized sheep blood. Bacterial inoculum size and culture conditions were in accordance with a previously described method.[28]

S. pneumoniae ATCC49619 and R6 reference strains were used as quality controls.

Multilocus sequence typing and eBURST analysis

Multilocus sequence typing (MLST) performed for *S. pneumoniae* strains recognized discrepancy in the data of the two PCR methods. MLST and eBURST analysis was performed according to the MLST site (http://spneumoniae.mlst.net/).

Results

Resistant genotypes determined by real-time PCR

Figure 1 shows four patterns from a computer display connected to the real-time PCR instrument shown just after the PCR reaction was completed. Each tested strain was identified as follows: A, as gPSSP by DNA amplification corresponding to *lytA* (a), *pbp1a* (b), *pbp2x* (c), and *pbp2b* (d) genes; B, as gPISP (*pbp2x*), with only the *pbp2x* gene not amplified; C, as gPISP (*pbp1a*+*pbp2x*), with *pbp1a* and *pbp2x* genes not amplified; and D, as gPRSP (*pbp1a*, *pbp2x*, and *pbp2b*), with all 3 *pbp* genes not amplified. With regard to ML resistance, a strain showing DNA amplification for *mef*A and/or *erm*B genes was identified as ML resistant.

Genotypic classification of β-lactam and macrolide resistance

All strains tested were classified into six genotypic categories by real-time PCR for 3 *pbp* genes: gPSSP with three normal genes ($n=67$, 22.3%); gPISP (*pbp2x*) with an abnormal *pbp2x* gene ($n=76$, 25.3%); gPISP (*pbp2b*) with an abnormal *pbp2b* gene ($n=22$, 7.3%); gPISP (*pbp1a*+2*x*) with abnormal *pbp1a*+2*x* genes ($n=34$, 11.3%); gPISP (*pbp2x*+2*b*) with abnormal *pbp2x*+2*b* genes ($n=14$, 4.7%); and gPRSP with three abnormal PBP genes ($n=87$, 29.0%). Strains examined included 106 from pediatric patients (35.3%) and 194 from adult patients (64.7%). Percentages of the strains representing gPRSP accounted for 50.5% of isolates from children and 17.5% of those from adults.

Although detailed data are not shown, ML resistance in these strains was classified into four genotypic categories based on presence or absence of resistance genes: ML-susceptible strains ($n=58$, 19.3%); 14-membered ML-resistant strains possessing an *mef*A gene ($n=79$, 26.3%); 14- and 16-membered ML-resistant strains possessing an *erm*B gene ($n=150$, 50.0%); or an ML-resistant strain possessing *mef*A and *erm*B genes ($n=13$, 4.3%). Proportions of strains showing ML resistance were 85.9% of isolates from children and 77.9% of the isolates from adults.

Sensitivity and specificity of real-time PCR

As shown in Table 2, sensitivity and specificity for all strains were compared between conventional and real-time

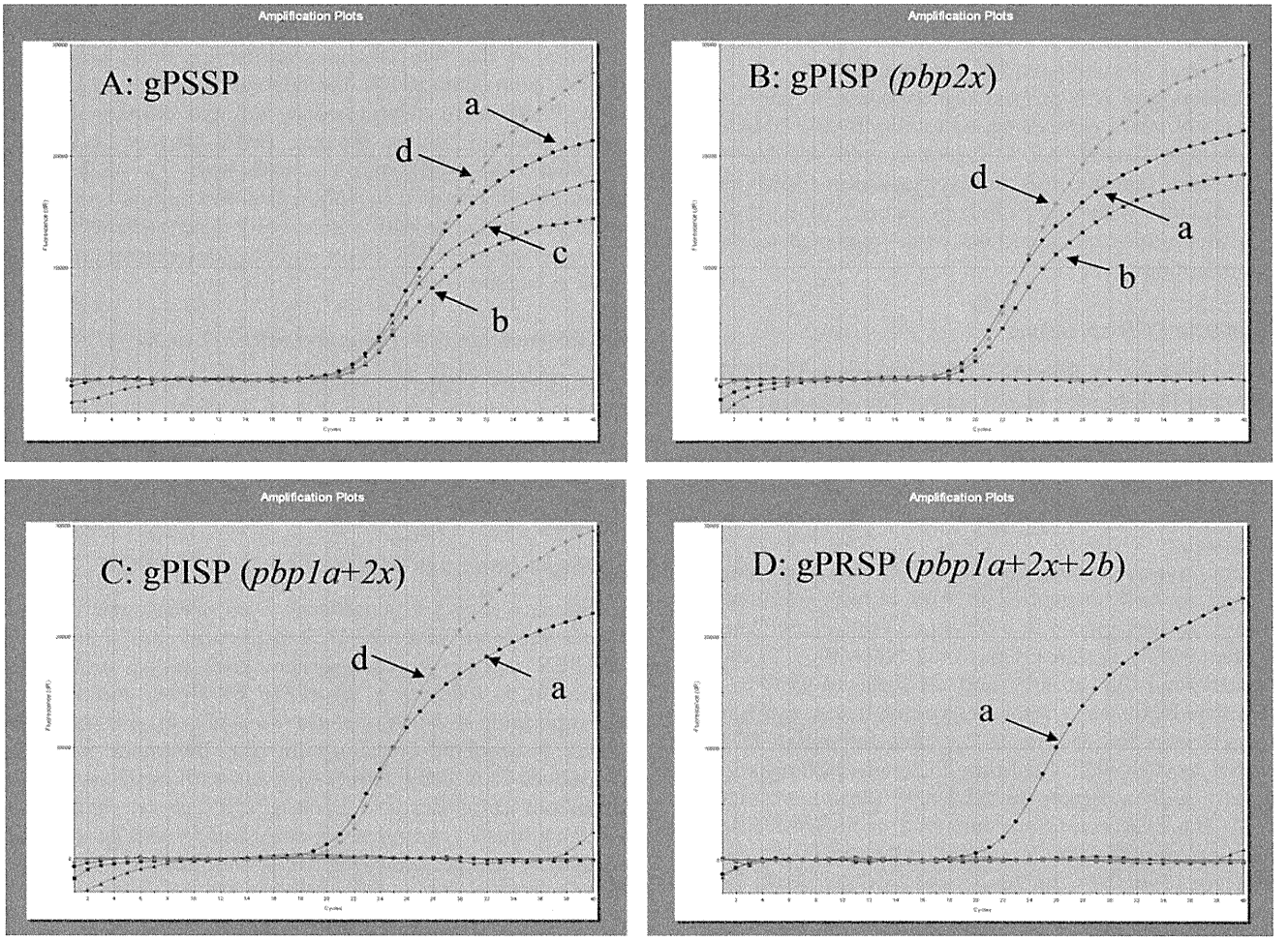

FIG. 1. Four genotypic resistance patterns from a computer display connected to the real-time PCR instrument, seen just after the PCR reaction was completed. (**A**) Genotypic penicillin-susceptible *Streptococcus pneumoniae* (gPSSP) by DNA amplification corresponding to *lytA* (a), *pbp1a* (b), *pbp2x* (c), and *pbp2b* (d) genes. (**B**) Genotypic penicillin-intermediate *S. pneumoniae* (gPISP) (*pbp2x*), with only the *pbp2x* gene not amplified. (**C**) gPISP (*pbp1a*+*pbp2x*), with *pbp1a* and *pbp2x* genes not amplified. (**D**) Genotypic penicillin-resistant *S. pneumoniae* (gPRSP) (*pbp1a*, *pbp2x*, and *pbp2b*), with 3 *pbp* genes not amplified.

PCR. The sensitivity and specificity for the *mefA* gene and the *ermB* gene were calculated to be 100%.

Table 3 shows detailed information for the nine strains (3.0%) showing a discrepancy between real-time PCR and conventional PCR. In these strains, DNA amplification for the *pbp1a* or *pbp2x* gene corresponding to the susceptible strain occurred weakly in conventional PCR but not at all in real-time PCR. According to susceptibility testing for AMP, and CTX, results of real-time PCR proved more accurate than those of conventional PCR. Overall, our new real-time PCR method showed to have excellent sensitivities and specificities compared with those of conventional PCR.

Relationships between PBP gene alterations and MIC of β-lactam agents

Figure 2 shows relationships between MICs of five β-lactam agents and results of real-time PCR for *pbp1a*, *pbp2x*, and *pbp2b* genes in the tested strains. MICs of PEN, MEM, and PAM were affected by *pbp2b* alterations rather than those in *pbp2x*. On the other hand, the MIC of CTX was 4–8 times lower than that of PEN due to *pbp2x* alterations. Notably, 90% of MICs in each genotype resistance class were distributed essentially within three serial dilution concentrations (for instance, gPRSP in PEN, from 0.5 to 2 mg/L) for almost all antibiotics. However, eight gPSSP strains with a CTX MIC ranging from 0.125 to 0.25 mg/L possessed substitutions of Thr550Ala adjacent to a KSG motif in PBP2X that could not be detect with the real-time PCR constructed in this study.

Estimated MIC_{50} values and corresponding ranges for 90% of β-lactam antibiotics among six PBP genotypic categories are listed in Table 4. On the basis of these data, MIC estimation for parenteral β-lactam antibiotics associated with clinical efficacy could be made with high probability.

Discussion

The ultimate global public health goal in the 21st century is to develop and disseminate vaccination to prevent infectious diseases caused by various viruses and bacteria more effectively. For immunity against pneumococcal infections, development of 23-valent pneumococcal polysaccharide

TABLE 2. SENSITIVITES AND SPECIFICITIES OF REAL-TIME PCR COMPARED WITH THOSE CONVENTIONAL PCR

Genotype	*Real-time PCR*	*Conventional PCR (%)*		*Total no. of samples*
		Positive	*Negative*	
gPSSP	Positive	67 (98.5)[a]	0 (0.0)	67
	Negative	1 (1.5)	232 (100.0)[b]	233
	Total	68	232	300
gPISP (*pbp2x*)	Positive	75 (96.2)	1 (0.5)	76
	Negative	3 (3.8)	221 (99.5)	224
	Total	78	222	300
gPISP (*pbp2b*)	Positive	22 (100.0)	0 (0.0)	22
	Negative	0 (0.0)	278 (100.0)	278
	Total	22	278	300
gPISP (*pbp1a+2x*)	Positive	31 (100.0)	3 (1.1)	34
	Negative	0 (0.0)	266 (98.9)	266
	Total	31	269	300
gPISP (*pbp2x+2b*)	Positive	14 (73.7)	0 (0.0)	14
	Negative	5 (26.3)	281 (100.0)	286
	Total	19	281	300
gPRSP (*pbp1a+2x+2b*)	Positive	82 (100.0)	5 (2.3)	87
	Negative	0 (0.0)	213 (97.7)	213
	Total	82	218	300

[a]Sensitivity.
[b]Specificity.

vaccine (PPV23)[5] began in the early 1980s in the United States, and this vaccine was introduced in Japan in 1988. In Japan, 7-valent pneumococcal conjugate vaccine (PCV7) has just been approved on a voluntary basis to prevent IPD among children with immunologic immaturity.

In countries where PCV7 has been introduced into the vaccine schedule, incidence of pediatric IPD caused by vaccine-type strains has decreased significantly,[4,6,22] while a related decrease of IPD among adults also has been reported.[17] However, prevalence of IPD caused by serotypes 19A and 6A (nonvaccine types) has increased, accompanied by a shift from PEN-susceptible to PEN-resistant strains.[7,11,21] Some investigators also have reported that overall incidence of IPD is little changed.[26]

In Japan, great clinical attention has been paid to the increase of PRSP and PISP among *S. pneumoniae* isolates from IPD,[8] which strongly reflects the difference in use of oral antibiotics between pediatricians and internists. Specifically, in pediatric practice, oral cephalosporins are favored over penicillins for outpatients, although a recent shift back toward amoxicillin and AMP has been noted. On the other hand, in internal medicine, ML and fluoroquinolone agents rather than β-lactam antibiotics are preferred. This might contribute significantly to rates of

TABLE 3. DETAILS OF 9 STRAINS SHOWING A DISCREPANCY IN RESULTS IN BETWEEN REAL-TIME PCR AND CONVENTIONAL PCR

No of strain	*Genotype*		*MIC (mg/L)*					*Serotype*	*ST*	*CC*
	Conventional PCR	*Real-time PCR*	*PEN*	*AMP*	*CTX*	*MEM*	*PAM*			
Ref R6	gPSSP	gPSSP	0.016	0.016	0.016	0.008	0.004	—	—	—
RS-009	gPISP(*pbp2x*)	gPISP(*pbp1a+2x*)	0.125	0.5	1	0.031	0.008	14	13	15
RS-027	gPISP(*pbp2x*)	gPISP(*pbp1a+2x*)	0.125	0.5	1	0.031	0.008	6B	385	156
RS-083	gPISP(*pbp2x*)	gPISP(*pbp1a+2x*)	0.125	0.5	1	0.031	0.004	6B	2983	156
RS-046	gPISP(*pbp2x+2b*)	gPRSP	0.5	1	2	0.063	0.016	14	343	554
RS-101	gPISP(*pbp2x+2b*)	gPRSP	0.5	2	2	0.063	0.016	14	343	554
RS-193	gPISP(*pbp2x+2b*)	gPRSP	0.5	1	0.5	0.063	0.016	14	343	554
RS-311	gPISP(*pbp2x+2b*)	gPRSP	0.5	1	2	0.125	0.016	14	343	554
RS-065	gPISP(*pbp2x+2b*)	gPRSP	1	4	1	0.25	0.031	6B	6939	None[a]
RS-208	gPSSP	gPISP(*pbp2x*)	0.063	0.125	0.125	0.016	0.004	6A	4542	156

When DNA amplification occurred, the corresponding *pbp* gene showed the same sequences as the susceptible strain; for example, a strain showing amplification of *pbp1a* and *pbp2b* genes was designation gPISP(pbp2x).
[a]ST6939 is not present in any group of clonal complexes.
MIC, minimum inhibitory concentration; PEN, penicillin; AMP, ampicillin; CTX, cefetaxime; MEM, meropenem; PAM, panipenem; ST, sequence type; CC, clonal complex; gPSSP, genotypic penicillin-susceptible *Streptococcus pneumoniae;* gPISP, genotypic penicillin-intermediate *S. pneumoniae;* gPRSP, genotypic penicillin-resistant *S. pneumoniae.*

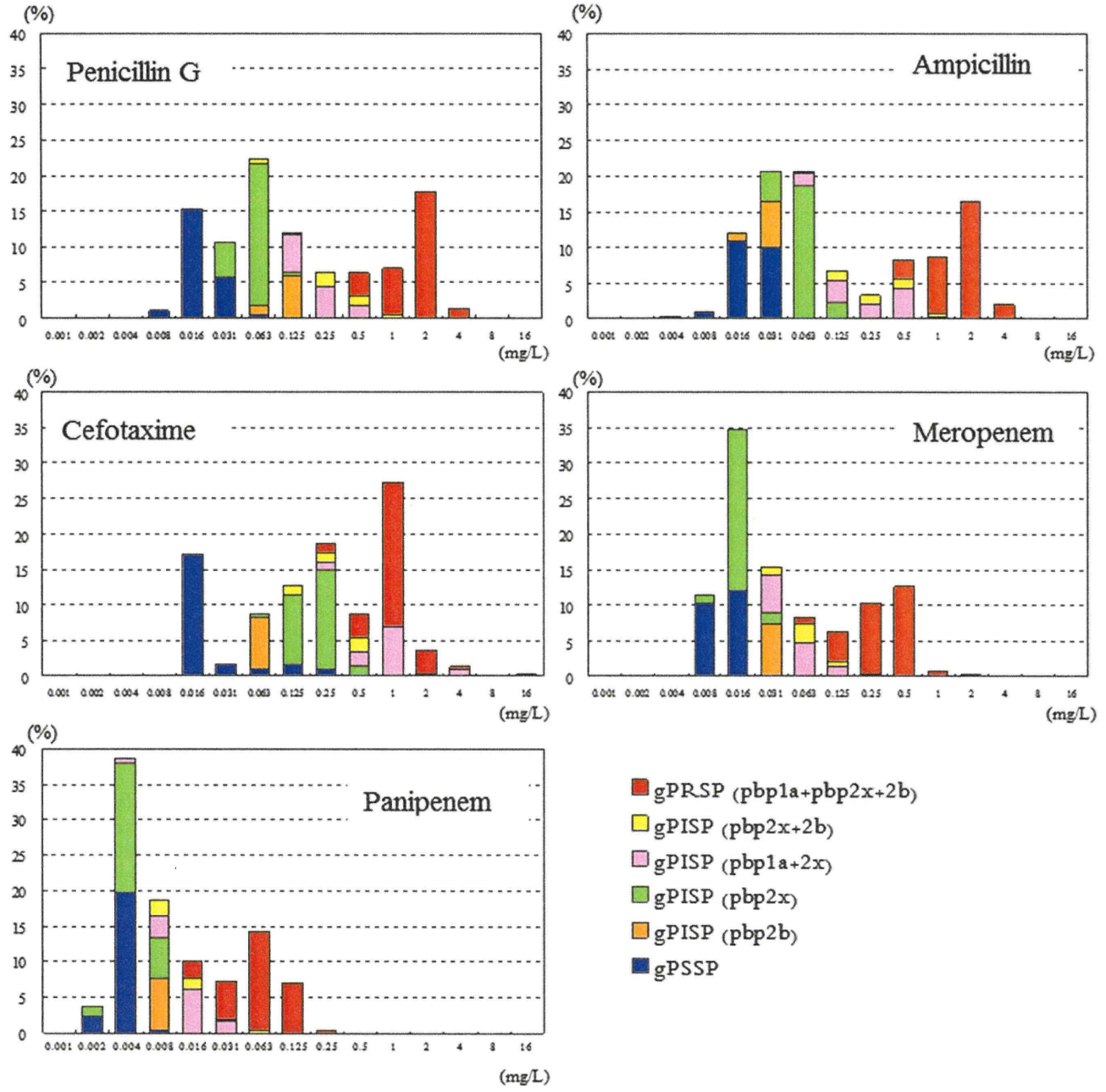

FIG. 2. Correlation between minimal inhibitory concentrations (MICs) of five β-lactam antibiotics and penicillin-binding protein (PBP) gene alterations for 300 *Streptococcus pneumoniae* isolates from invasive infections.

gPRSP isolated from pediatric patients and gPISP isolated from adult patients.

These situations concerning antibiotic resistance, in addition to the present state of pneumococcal vaccination, show that a need for rapid and accurate determination of resistance in clinical isolates is necessary for appropriate selection of chemotherapeutic agents in pneumococcal infections.

We initially identified species and antibiotic resistance using colony samples likely to be *S. pneumoniae* from blood agar plate using a conventional PCR method completed within 2.5 hr using gel electrophoresis.[30] Intrinsically, three primer sets designed on *pbp1a*, *pbp2x*, and *pbp2b* genes detect the most important amino acid substitutions affecting β-lactam susceptibilities, all positioned within or adjacent to conserved amino acid motifs in each PBP—substitutions from STMK to SAMK or SSMK in PBP1A, substitutions from STMK to SAMK or SAFK and from (L)KSG to (V)KSG in PBP2X, and substitution from SSN(T) to SSN(A) or SSN(S) in PBP2B. The genotypic resistance pattern based on the *pbp* gene analysis was divided into six categories: gPSSP, gPISP(*pbp2x*), gPISP(*pbp2b*), gPISP(*pbp1a*+*pbp2x*), gPISP (*pbp2x*+*pbp2b*), and gPRSP (*pbp1a*+*pbp2x*+*pbp2b*).

This was not shown in the results, but each class of resistance genes was not of a single clone. For example, gPRSP was divided into 11 serotypes with various clonal complexes (CCs). The major serotypes and CCs were serotype 6B with

TABLE 4. ESTIMATED MIC_{50s} AND FITTING RANGES OF 90% OF β-LACTAM ANTIBIOTICS FOR 6 PBP GENOTYPE CLASSES

Genotype	n	Estimated MIC (mg/L)				
		PEN	*AMP*	*CTX*	*MEM*	*PAM*
gPSSP	67	0.016 (0.016–0.031)	0.016 (0.016–0.031)	0.016 (0.016–0.125)	0.016 (0.008–0.016)	0.004 (0.002–0.004)
gPISP (*pbp2b*)	22	0.125 (0.063–0.125)	0.031 (0.016–0.031)	0.063 (0.063)	0.031 (0.031)	0.008 (0.008)
gPISP (*pbp2x*)	76	0.063 (0.031–0.063)	0.063 (0.031–0.063)	0.25 (0.125–0.25)	0.016 (0.016–0.031)	0.004 (0.002–0.008)
gPISP (*pbp1a+2x*)	34	0.25 (0.125–0.5)	0.25 (0.063–0.5)	1 (0.25–2)	0.063 (0.031–0.125)	0.016 (0.008–0.031)
gPISP (*pbp2x+2b*)	14	0.25 (0.063–0.5)	0.25 (0.063–0.5)	0.25 (0.125–0.5)	0.063 (0.031–0.125)	0.016 (0.008–0.031)
gPRSP (*pbp1a+pbp2x+2b*)	87	2 (0.5–2)	2 (0.5–2)	1 (0.5–2)	0.5 (0.125–0.5)	0.063 (0.031–0.125)

MIC, minimum inhibitory concentration; PEN, penicillin; AMP, ampicillin; CTX, cefetaxime; MEM, meropenem; PAM, panipenem; gPSSP, genotypic penicillin-susceptible *Streptococcus pneumoniae*; gPISP, genotypic penicillin-intermediate *S. pneumoniae*; gPRSP, genotypic penicillin-resistant *S. pneumoniae*.

CC156 and CC490, serotype 19F with CC320, serotype 23F with CC156, CC242 and CC1437, serotype 6A with CC3115, CC3787 and CC81, and serotype 14 with CC320 and CC554.

As stated in the Results section, real-time PCR yielded satisfactory sensitivity and specificity compared with conventional PCR. Accurate estimation of MICs of each β-lactam antibiotic on the basis of genotypic patterns is highly important. Our novel real-time PCR assay also can be completed within 90 min after selection of colony samples, with elimination of gel electrophoresis, saving both time and labor.

Another merit of this assay is possible direct testing of usually sterile specimens (such as cerebrospinal fluid, joint fluid, and pleural fluid) from IPD patients, because primers and MB probes for amplification of the *lytA* gene are included in the real-time PCR. In the future, simultaneous performance of speciation and identification of resistance gene(s) by real-time PCR should optimize cost and benefit in clinical settings.

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REVIEW ARTICLE

Invasive infection caused by *Streptococcus dysgalactiae* subsp. *equisimilis*: characteristics of strains and clinical features

Takashi Takahashi · Kimiko Ubukata · Haruo Watanabe

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Abstract Among clinically isolated β-hemolytic streptococci, *Streptococcus pyogenes* and *S. agalactiae* were considered the main pathogens in humans until recently. In 1996, *S. dysgalactiae* subsp. *equisimilis* (SDSE) was proposed as a novel taxon among human-derived streptococcal isolates. SDSE has Lancefield group C or G antigens, exhibits strong β-hemolysis, and exerts streptokinase activity upon human plasminogen and proteolytic activity upon human fibrin. Similarly to group A streptococci, SDSE possesses virulence factors including M protein, streptolysin O, streptolysin S, streptokinase, hyaluronidase, C5a peptidase, and others. SDSE may exist among the normal flora of the skin, oropharynx, and gastrointestinal and genitourinary tracts. In the twenty-first century, invasive SDSE infection (i.e., cellulitis, urosepsis, and pneumonia) leading to various disseminated diseases is being diagnosed increasingly in Japan, elsewhere in Asia, in Europe, and in America. Particularly, among elderly patients, these invasive diseases are encountered increasingly in Japanese hospital emergency departments. Analysis of the part of the *emm* gene encoding the amino acid sequence at the N-terminal end of the M protein is used to determine the molecular epidemiology of SDSE. The distribution of *emm* types from patients with invasive or noninvasive infections differs between surveillance results from different countries. In this review, we summarize the characteristics of phenotypes and virulence factors in SDSE strains; the review also focuses on emerging SDSE infectious disease and future vaccination research.

Keywords *Streptococcus dysgalactiae* subsp. *equisimilis* · *Streptococcus pyogenes* · *Streptococcus agalactiae* · Invasive infection · Clinical features · Emerging infectious disease

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Introduction

The β-hemolytic streptococci are common pathogens causing community-acquired infections. To distinguish between species of β-hemolytic streptococci, clinical laboratories usually use Lancefield typing, which is based on sero-agglutination involving C-polysaccharide antigens on the bacterial cell surface. In general, β-hemolytic streptococci isolated from humans may possess any of Lancefield group A, B, C, G, F, or L antigens. Of these isolates, only group A streptococci (GAS; *Streptococcus pyogenes*) [1] and group B streptococci (GBS; *S. agalactiae*) [2, 3] were considered important pathogens in clinical settings until recently. Other streptococci belonging to groups C or G were thought to show extremely low pathogenicity, except in patients with certain predisposing medical conditions.

In 1996, Vandamme et al. [4] proposed that a novel subspecies, *S. dysgalactiae* subsp. *equisimilis* (SDSE), was a clinical pathogen. This microorganism possesses group C or G antigens (rarely, A antigen), and exhibits strong β-hemolysis. Distinct categories of C or G streptococci

include the *S. anginosus* group, consisting of *S. anginosus*, *S. constellatus* subspecies *constellatus*, *S. constellatus* subsp. *pharyngis*, and *S. intermedius*. The *S. anginosus* group is easily distinguished from SDSE on the basis of biologic characteristics and colony size on sheep blood agar plates.

Beginning around 2000, invasive infections such as bacteremia caused by SDSE have been reported increasingly worldwide, as well as those caused by GAS and GBS [5–8].

In this review, we summarize the characteristics of phenotypes and virulence factors in SDSE strains, and describe the emergence of invasive SDSE infection in Japan and other countries. Our review also provides perspectives on SDSE as an emerging infectious disease and on future vaccination research.

The novel taxon and its biologic characteristics

Phenotypic characteristics including Lancefield antigen, hemolysis type, colony size, hosts, and biochemical properties are summarized for the important β-hemolytic streptococci in Table 1 [9]. Lancefield serologic differentiation [10] does not correspond to individual species of streptococci.

For example, streptococci having group C antigen include the following taxa; β-hemolytic *Streptococcus dysgalactiae* isolated from human specimens, β-hemolytic *S. dysgalactiae* from porcine specimens, α-hemolytic *S. dysgalactiae* from bovine specimens [11], 2 β-hemolytic subspecies of *Streptococcus equi* (subsp. *equi* and subsp. *zooepidemicus*) from equine specimens, and a β-hemolytic small-colony *Streptococcus anginosus* group from human specimens [12, 13].

Analogously, streptococci having group G antigen include *S. dysgalactiae* isolated from human specimens, an *S. anginosus* group from human specimens, and β-hemolytic *Streptococcus canis* from bovine, canine, and feline specimens [14]. The β-hemolytic streptococci with group L antigen also belong to *S. dysgalactiae* [15]. Thus, *S. dysgalactiae* consists of at least 5 distinct subgroups based on the findings of serogroup, host, and hemolysis type.

In 1996, two subspecies of *S. dysgalactiae* were proposed as new taxa by Vandamme and colleagues [4]: SDSE and *S. dysgalactiae* subspecies *dysgalactiae* (SDSD). The former, isolated from humans and animals, showed strong β-hemolysis, and the latter, isolated only from animals, showed α-, β-, or no hemolysis.

SDSE commonly possesses group G or C antigens and rarely group A antigen [16–19]. This pathogen forms large, glossy colonies that produce strong β-hemolysis on sheep blood agar plates after CO_2 incubation at 37°C, as opposed to hemolysis from GAS. However, rarely, SDSE may show α- or no hemolysis [20–22], similar to GAS.

The main biologic characteristics of SDSE are resistance to bacitracin, negativity to the pyrrolidonyl-arylamidase (PYR) test, negativity to the Voges–Proskauer test, positivity to the β-D-glucuronidase test, and negativity to the β-galactosidase test [23]. The PYR test is very useful for distinguishing SDSE from GAS, which uniformly shows positivity.

Figure 1 shows typical features of colonies of GAS and SDSE isolated from human or porcine specimens, and SDSD isolated from a porcine specimen, as well as *S. anginosus* from a human specimen.

Table 1 Phenotypic characteristics of β-hemolytic streptococci

Species	Lancefield group	Hemolysis type	Colony size	Hosts	BA	PYR	CAMP	VP	β-GUR	β-GAR
S. pyogenes	A	β	Large	Humans	+	+	−	−	−	v
S. agalactiae	B	β	Large	Humans, cows	−	−	+	−	v	−
S. dysgalactiae subsp. *equisimilis*	A, C, G	β	Large	Humans	−	−	−	−	+	−
S. dysgalactiae subsp. *dysgalactiae*	C, L	None, α, (β)	Large	Animals (pigs, cows)	−	−	−	−	+	−
S. equi subsp. *equi*	C	β	Large	Horses	−	−	−	−	+	−
S. equi subsp. *zooepidemicus*	C	β	Large	Animals (horses, pigs), humans	−	−	−	−	+	−
S. canis	G	β	Large	Animals (cows, dogs, cats)	−	−	+	−	v	−
S. anginosus group[a]	A, C, G, F, none	β, α, none	Small	Humans	−	−	−	+	−	−

BA bacitracin, *PYR* pyrrolidonyl-arylamidase test, CAMP factor reaction (synergistic hemolysis in presence of *S. aureus* β-hemolysin), *VP* Voges–Proskauer test, *β-GUR* β-D-glucuronidase test, *β-GAR* β-galactosidase test, + positive, − negative, *v* variable

[a] *S. anginosus* group includes *S. anginosus*, *S. constellatus*, and *S. intermedius*

S. intermedius alone gives a positive result for the β-galactosidase test

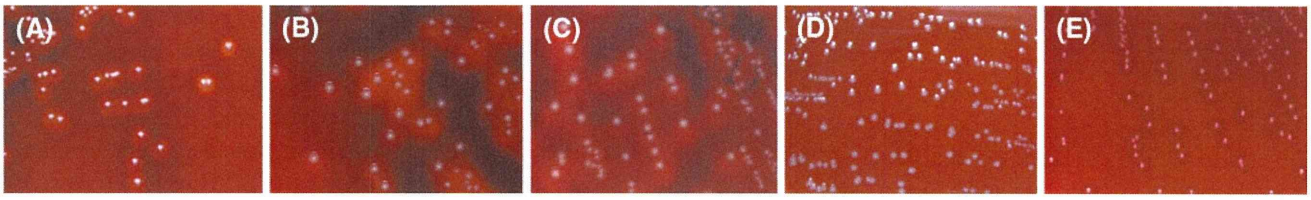

Fig. 1 Morphologic and hemolytic features of colonies of *Streptococcus pyogenes* from a human specimen (**a**), *S. dysgalactiae* subsp. *equisimilis* from a human specimen (**b**), *S. dysgalactiae* subsp. *equisimilis* from a porcine specimen (**c**), *S. dysgalactiae* subsp. *dysgalactiae* from a porcine specimen (**d**), and *S. anginosus* from a human specimen (**e**). These microorganisms were cultured on sheep blood agar plates in a 5% CO_2 atmosphere at 37°C for 20 h

Molecular characteristics of SDSE

SDSE possesses many virulence factors that are similar to those of GAS [24]. These factors may be divided into 3 categories, including bacterial products contributing to cell attachment or escape from host immunity (i.e., resistance to phagocytosis), toxins and proteases, and regulatory factors controlling expression of the products.

Factors contributing to cell attachment or escape from host immunity include M protein (encoded by the *emm* gene), streptokinase A (*ska*), streptococcal C5a peptidase (*scpA*), hyaluronidase (*hyl*), fibronectin binding protein (*fbp*), collagen binding protein, laminin binding protein (*lmb*), and others. Streptolysin S (*sagA*) [25, 26], streptolysin O (*slo*), and hemolysin (*hlyIII, helA1*) represent toxins or proteases, while M protein transacting positive regulation (*mga*) [27], CovR (*covR*), CovS (*covS*), and others are regulators of product expression.

Among these virulence factors, sequence analysis of the *emm* gene, which encodes the M protein on the cell surface, is often used in molecular epidemiologic studies concerning outbreaks of invasive or noninvasive SDSE infections. The first 300 highly variable bases of the 5′ end of *emm* gene sequences for the SDSE strains were sent online to the Centers for Disease Control (CDC) for comparison with those in the CDC *emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm) [3, 28, 29]. An *emm* genotype showing more than 98% homology with the CDC reference strain was identified as that particular *emm* type.

Streptokinase, which dissolves human fibrin [30], and C5a peptidase, which dissolves the complement component C5a, and also inhibits neutrophil migration [30], are the major factors associated with invasive SDSE infection.

Miyoshi-Akiyama and Murayama recently completed whole-genome analyses of the original 2 SDSE isolates [GGS_124 (GenBank accession no. AP010935) and RE378], demonstrating a 61 to 63% overlap between this subspecies and GAS genomes; overlap between the subspecies and GBS genomes was 15% (unpublished data). Ahmad and colleagues [31] have deduced genetic relationships from *emm* genotyping and multilocus sequence typing of SDSE from isolates collected in the United States, concluding that phylogenetic relationships between SDSE and GAS alleles reflected a history of interspecies recombination, with either species frequently serving as the genetic donor. Accordingly, the genomic characteristics of SDSE appear to differ from those of GBS, being somewhat more similar to those of GAS. In further investigations, virulence factors specific to SDSE as opposed to GAS should be determined to clarify the pathogenesis of SDSE infection.

Emergence of invasive SDSE infections

SDSE may sometimes be isolated as normal flora from the skin, oropharynx, and gastrointestinal and genitourinary tracts, also having been identified in respiratory tract specimens from patients with noninvasive SDSE diseases [29]. Invasive infection is defined as the isolation of SDSE from a normally sterile site (i.e., blood, cerebrospinal fluid, joint fluid, ascites, or pleural effusion) [3, 23, 29]. Since 2003, the prevalence of invasive SDSE infections, including streptococcal toxic shock syndrome (STSS) and severe soft tissue infection [26, 30, 32], has increased gradually each year in Japan. Several SDSE cases including thoracic empyema with gas formation [33], an apparently organizing pneumonia [34], gas gangrene in both legs resulting in amputation of the right leg [35], and acute peritonitis with salpingitis associated with STSS [22], have been reported in Japan.

Our group (Ubukata and colleagues) [3] conducted active laboratory-based surveillance for invasive SDSE, GAS, and GBS infections from August 2006 to July 2007, including 142 medical institutions participating in the Invasive Streptococcal Disease Working Group established at the 19th Annual Meeting of the Japanese Society for Clinical Microbiology. Clinical isolates ($n = 286$) from patients with invasive infections were identified as SDSE strains, while GAS ($n = 116$) and GBS ($n = 183$) isolates were also collected until the present time (end of 2009).

Figure 2 shows age distribution according to our original data, showing a difference between patients with

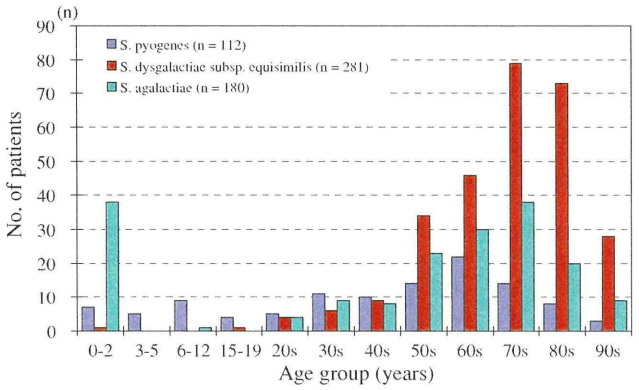

Fig. 2 Numbers of patients with invasive infections caused by *Streptococcus dysgalactiae* subsp. *equisimilis* ($n = 281$), *S. pyogenes* ($n = 112$), and *S. agalactiae* ($n = 180$), shown by age group. Patients with insufficient data were excluded. Data were modified from those of Ref. [3]

invasive SDSE disease and those with GAS or GBS infections. All patients with invasive SDSE infection were adults, who often were elderly, while GBS infected some patients 4 months old or younger, in addition to adults. Another investigation [29] also indicated that invasive SDSE infection ($n = 42$) occurred mostly in patients at least 50 years old, especially in elderly adults (60–80 years).

Underlying medical conditions in subjects with GAS infection (60.3%) were less frequent than in patients with SDSE (78.8%) or GBS (88.2%) [3]. In another study [29], severe underlying conditions (i.e., diabetes mellitus, liver or renal dysfunction, and others) were associated with 85.7% of invasive SDSE infections.

Figure 3 indicates the hospital departments at which patients with invasive SDSE, GAS, or GBS diseases presented. Some patients with SDSE infection presented to the department of orthopedics as well as to the emergency and internal medicine departments [3].

Invasive SDSE infections included sepsis with no known focus, cellulitis, septic arthritis, pneumonia, necrotizing fasciitis, meningitis, infectious endocarditis, STSS, abscesses at sites other than skin, osteomyelitis, and others [3]. Interestingly, sepsis with no known focus was more frequent among GBS-infected patients than among SDSE-infected or GAS-infected patients, while cellulitis was less frequent among GBS-infected patients than among SDSE-infected or GAS-infected patients [3] (Fig. 4). Patients infected with SDSE presented more often with septic arthritis than those infected with GBS, while patients with GAS infection had abscesses involving sites deeper than the skin more often than did patients with SDSE infection [3].

Concerning the disease outcome of SDSE infection, rates of mortality and post-infection sequelae were 12.7 and 5.3%, respectively [3]. No difference in the frequency of poor outcome (death or post-infective sequelae) was evident between SDSE, GAS, and GBS infections. In addition, poor leukocytic responses (<5000 cells/μL) and thrombocytopenia on admission were associated with a high risk of poor outcome of invasive SDSE infection [3].

The *emm* type *stG6792* confirmed most frequently in SDSE isolates ($n = 65$; 22.7%), was more strongly related to poor outcome of SDSE disease than were other SDSE *emm* types [3]. We should continue to survey invasive SDSE infections to further clarify relationships between outcome and clinical laboratory data or *emm* types in the Japanese population.

As for *emm* types of SDSE in our investigation, most strains showed *stG6792.3* ($n = 54$) and displayed similar DNA profiles with pulsed-field gel electrophoresis (PFGE), suggesting the clonal expansion of a specific subpopulation of strains rather than the spread of distinct strains [3] (Fig. 5). Judging from the CDC database concerning *emm* type sequences, the *stG6792.3* reference strain appeared to be derived from a streptococcal isolate in India, suggesting that this strain might have spread from India to Japan. In

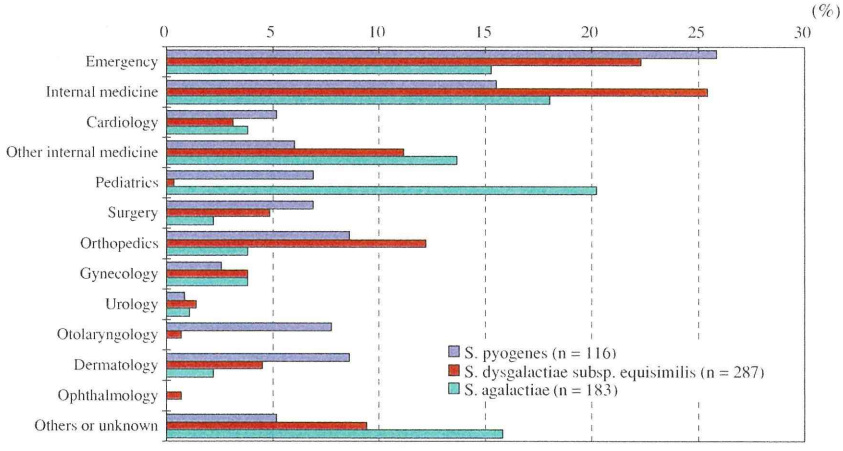

Fig. 3 Hospital departments at which patients with invasive disease caused by *Streptococcus dysgalactiae* subsp. *equisimilis* ($n = 287$), *S. pyogenes* ($n = 116$), and *S. agalactiae* ($n = 183$) presented. Patients with insufficient data were excluded. Data were modified from those of Ref. [3]

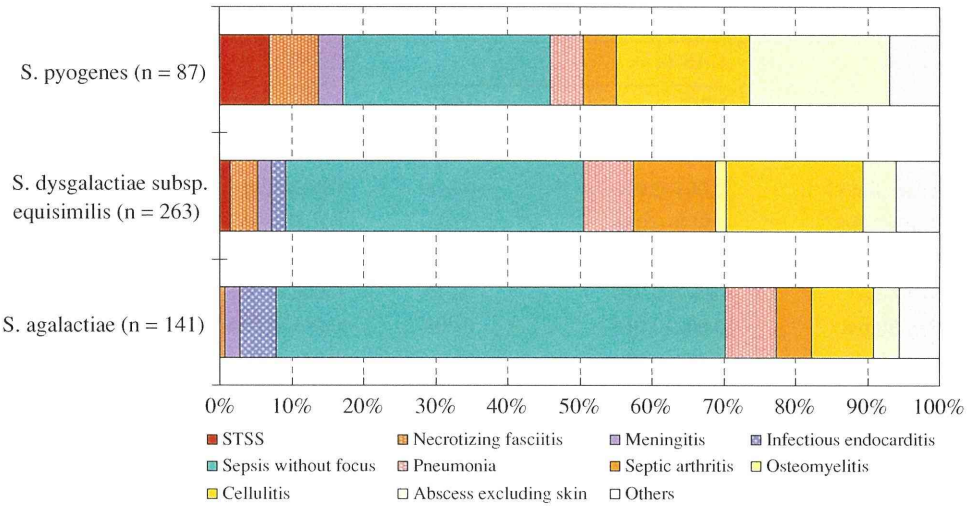

Fig. 4 Percentages of clinical syndromes among patients at least 15 years old with invasive infection caused by *Streptococcus dysgalactiae* subsp. *equisimilis* ($n = 263$), *S. pyogenes* ($n = 87$), and *S. agalactiae* ($n = 141$). Patients with insufficient data were excluded. *STSS*, Streptococcal toxic shock syndrome. Data were modified from those of Ref. [3]

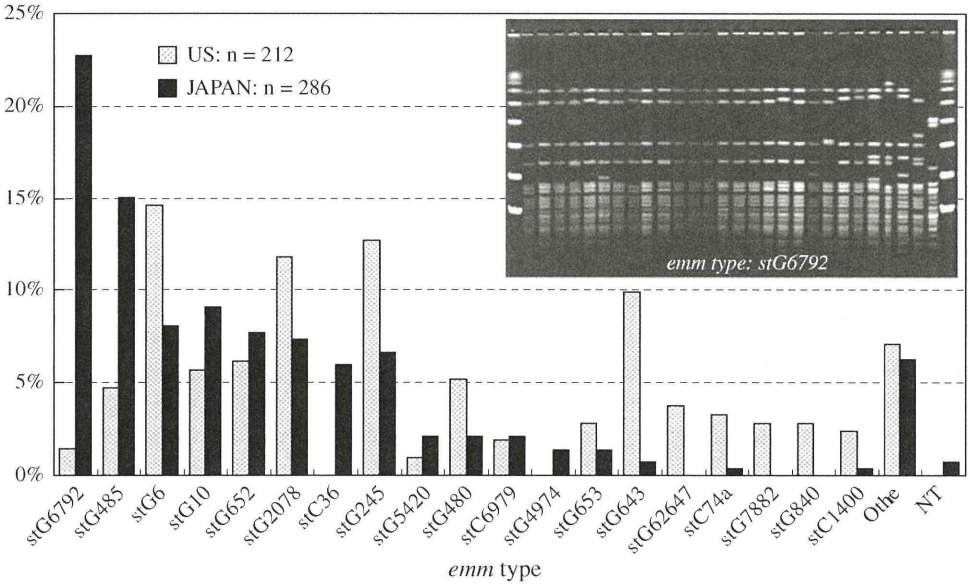

Fig. 5 Distributions of *emm* genotypes for *Streptococcus dysgalactiae* subsp. *equisimilis* strains compared between Japan ($n = 286$) and the United States ($n = 212$). *Inset* shows similar DNA profiles (ApaI digests) of *stG6792.3* ($n = 27$) and *stG6792.0* ($n = 1$, right) strains (Japan) according to pulsed-field gel electrophoresis. *NT* Non-typeable. Data were modified from those of Refs. [3, 5]

another study [29], 3 *emm* types, *stG6792*, *stG485*, and *stG2078*, predominated among the 42 invasive strains; strains with the same *emm* type showed uniform DNA profiles by PFGE. On the other hand, previous reports described *emm* types and the DNA profiles by PFGE as variable among SDSE strains [26, 30]. Person-to-person transmission routes of SDSE should be determined in detail by future surveillance.

The prevalence of invasive SDSE infection has increased over the years elsewhere in Asia [6, 36], in Europe [37–43], and in America [5, 31, 44], as well as in Japan. As for age distribution, population-based surveillance carried out by Broyles et al. ($n = 212$) [5] found that most patients with invasive diseases (59.0%) were adults less than 65 years old, while the majority in our study (74.0%) were at least 65 years old [3]. Regarding underlying diseases in United States surveillance reports, 96.2% of patients with invasive infection possessed underlying medical conditions [5], while in Japan 78.8% had an underlying illness [3]. As for the specific clinical syndromes representing invasive infection, the largest category in the United States was cellulitis (41.0%) [5], while in Japan, sepsis with no focus, at 41.4%, was the largest category [3]. Prospective surveillance studies of invasive infection in both countries carried out during the same period will be needed to better define associations between host factors and the pathogenesis of SDSE.

Concerning SDSE disease outcome, the mortality rate in our Japanese observation (12.7%) was similar to those previously described in Hong Kong and the United States (12 and 15%) [3, 5, 45]. Liao et al. [6] reported several

Table 2 Antimicrobial activity of oral and parenteral antibiotics against 3 streptococcal strains isolated from normally sterile sites

Antibiotic	GAS ($n = 97$)		SDSE ($n = 231$)		GBS ($n = 151$)	
	MIC range (μg/ml)	MIC_{90}	MIC range (μg/ml)	MIC_{90}	MIC range (μg/ml)	MIC_{90}
Oral						
Penicillin G	0.004–0.016	0.016	0.008–0.016	0.016	0.016–0.125	0.063
Ampicillin	0.008–0.031	0.031	0.016–0.031	0.031	0.031–0.25	0.125
Amoxicillin	0.008–0.031	0.031	0.016–0.031	0.031	0.031–0.25	0.125
Cefdinir	0.002–0.016	0.016	0.008–0.031	0.016	0.016–0.125	0.063
Cefditoren	0.002–0.016	0.008	0.008–0.016	0.016	0.016–0.063	0.031
Clarithromycin	0.063–>64	16	0.016–>64	0.125	0.031–>64	4
Levofloxacin	0.25–4	4	0.25–>32	1	0.5–>32	>32
Parenteral						
Cefazolin	0.063–0.125	0.125	0.063–0.25	0.125	0.063–0.5	0.25
Cefotiam	0.031–0.125	0.063	0.063–0.125	0.125	0.125–2	0.5
Cefotaxime	0.004–0.031	0.016	0.008–0.031	0.016	0.016–0.125	0.063
Panipenem	0.002–0.008	0.008	0.004–0.016	0.008	0.008–0.031	0.031
Meropenem	0.002–0.016	0.008	0.008–0.016	0.016	0.031–0.125	0.063
Vancomycin	0.25–1	0.5	0.25–1	0.5	0.25–0.5	0.5

GAS, group A streptococci (*Streptococcus pyogenes*); SDSE, *S. dysgalactiae* subsp. *equisimilis;* GBS, group B streptococci (*S. agalactiae*); MIC, minimal inhibitory concentration

Susceptibility testing was performed by agar plate dilution methods using blood agar [6]

recurrent cases (8 patients, with 1 having 4 episodes and 3 having 3 episodes) of invasive SDSE disease (primary bacteremia or cellulitis). PFGE performed upon all 12 available strains from recurrent cases indicated that 9 were identical to that responsible for the initial episode [6]. We also experienced a patient with recurrence of cellulitis caused by SDSE [46]. General practitioners should be aware of the possible recurrence of invasive SDSE infection in their patients.

The distribution of *emm* types in patients with invasive or noninvasive infections has differed between several surveillance studies in different countries. For instance, we confirmed *emm* type *stG643* in 2 SDSE isolates from a newborn and mother in our case of neonatal STSS [47]. The frequency of this detected *emm* type differed between 4 reports from different countries; prevalence was 17.2% in western Norway [48], 9.9% in the United States [5], 4.4% in Portugal [39], and 0.7% in Japan. Figure 5 compares *emm* typing patterns in Japan with those in the United States. Interestingly, the dominant *emm* types in Japan (*stG6792* and *stG485*) were different from those in the United States (*stG6*, *stG245*, *stG2078*, and *stG643*), although the study periods differed between these studies (2002–2004 vs. 2006–2009) [3, 5]. These results illustrate the importance of conducting comparative molecular epidemiologic investigations of SDSE strains in the United States and Japan by *emm* type over the same study period.

Antibiotic susceptibility

Table 2 summarizes the minimum inhibitory concentration (MIC) ranges and MIC_{90} values of oral and parenteral antimicrobial agents for SDSE, GAS, and GBS isolates in Japan obtained from a normally sterile site [3]. Susceptibility testing was performed by dilution methods using agar plates supplemented with 5% sheep blood agar, as described previously [2].

No penicillin or cephalosporin resistance was observed in 231 SDSE strains collected by the Invasive Streptococcal Disease Working Group from 2006 to 2007.

Regarding macrolide (ML) resistance, some SDSE isolates possessed 1 of 3 resistance genes: 1.7% had the *mef*(A) gene; 5.6%, the *erm*(A) gene; and 2.6%, the *erm*(B) gene. Strains with the *mef*(A) gene showed an M phenotype involving an active efflux pump system for 14- and 15-membered MLs. Strains with the *erm*(A) gene showed an inducible ML/lincosamide/streptogramin B resistance phenotype, while those with the *erm*(B) gene showed a constitutive ML/lincosamide/streptogramin B resistance phenotype arising from methylation of 23S rRNA.

Fluoroquinolone-resistant strains first were reported in North America and Europe [49]; recently in Portugal, resistance was reported to have increased gradually to 12% [50]. The 2 resistant strains (0.9%) in our study [3] showed high resistance to levofloxacin, a fluoroquinolone agent

(MIC >32 μg/ml). The strains possessed amino acid substitutions changing Ser81 to Phe or Tyr in GyrA and Ser79 to Tyr in ParC, and also the ML resistance genes *erm*(B) or *mef*(A); all showed *emm* type *stG10*. Similar results for ML- and levofloxacin-resistant strains have been reported by Sunaoshi et al. [29] for isolates collected from noninvasive and invasive infections from 2003 through 2005 in Japan.

SDSE isolates ($n = 212$) collected in a multicenter surveillance study by Broyles et al. [5] in the United States showed resistance rates of 28.8% for erythromycin, 4.2% for clindamycin, and 0.9% for fluoroquinolones, according to standard biologic methods [51]. In Korea [52], a high frequency of the tetracycline resistance-mediating *tet*(S) gene was demonstrated (68.8%), while erythromycin, clindamycin, and chloramphenicol resistance rates were low (9.4, 3.1, and 9.4%, respectively) [53].

Failure of β-lactam treatment was described in 2 patients with pharyngitis caused by SDSE [54]. In the future, continuous multicenter surveillance will be needed to elucidate the prevalence of antibiotic resistance among SDSE isolates and to establish clinical guidelines for selecting the most appropriate antimicrobial agent.

Perspectives concerning emerging infectious disease

SDSE is being identified increasingly as a pathogen responsible for invasive and noninvasive infections worldwide. Our investigation in Japan found the median patient age for invasive diseases to be 75 years [3]. Age population reported by the Japanese government in October 2008 (http://www.stat.go.jp/data/jinsui/2008np/index.htm) indicated that the general age distribution ratios of people aged >65 and >75 years were 22.1 and 10.4%, respectively. Because the elderly Japanese population is expected to increase steadily, the possibility of invasive SDSE infection should be considered by primary-care doctors treating elderly patients with fever or respiratory distress, especially in emergency departments [23, 46]. To detect SDSE isolates in elderly subjects, at least two sets of blood cultures should be obtained before the administration of antimicrobials, even when symptoms are limited.

Recently, fatal or near-fatal transfusion-transmitted infections involving platelet concentrates have been reported from some countries other than Japan [55–57]. SDSE was isolated from both the residual blood component bag and the recipient. A donor throat swab specimen collected 20 days after donation still contained SDSE [55]. These observations call for the implementation of improved safety measures for platelet concentrates. We have obtained an SDSE isolation rate of 19% in adult patients with pharyngitis in Japan (unpublished data). We, therefore, may need to determine SDSE colonization rates for skin, oropharynx, and gastrointestinal and genitourinary tracts in healthy volunteers.

Eleven isolates of SDSE from pigs slaughtered in Japan with endocarditis, arthritis, or lymphadenitis were characterized on the basis of nucleotide sequences of 16S rRNA and 23S rRNA genes [58]. Our colleagues have completed whole-genome analysis of the pig-derived SDSE L1 strain, finding high homology between this L1 strain and human SDSE strains (GGS_124 and RE378; unpublished data). Notably, many virulence-associated genes were found in SDSE strains isolated from both humans and pigs. Currently circulating SDSE strains in humans might have been formed by genetic recombination after the horizontal transfer of genes between SDSE originating in animals [59–61] and human streptococci, including GAS, GBS, or oral streptococci.

Vaccination trials

In a surveillance study in Mumbai, the SDSE carrier rate in children attending 7 public schools from 2006 to 2008 [36] was 11%, 8 times higher than the GAS carrier rate of 1.5% in the same population, suggesting that high rates of SDSE colonization in the throat make this organism an important cause of pharyngitis. In another study, in the United States, children 6 months to 18 years old who presented with pharyngitis at a children's hospital emergency department and two outpatient offices were enrolled in a cross-sectional study [62]; controls were children who presented at the same facilities during the same period with nonrespiratory symptoms, or those who presented at the orthopedic cast clinic. Sixty-five SDSE strains (3%) were obtained from the patients with pharyngitis, while 3 (1.5%) were obtained from the smaller control group. Thus, further research and development efforts directed to the vaccination of children against SDSE infection are needed.

Trials of vaccination against GAS infection are now underway, with the M protein selected as a target antigen. The M protein, a cell surface protein, consists of 4 repeat regions (from A to D) that vary in size and amino acid composition, as well as a non-structured amino-terminal segment [63]. The sequence of C- and D-repeat regions in the carboxy-terminal domain is well conserved among the different *emm* types. The amino-terminal portion, which extends into host cells, consists of a non-helical, non-repeated region, as well as a hypervariable region (A-repeats) and a semivariable region (B-repeats). Vaccine-design strategies select either the hypervariable region (A-repeats) or a well-conserved region (C-repeats) to develop the vaccine. For instance, a multivalent M protein-based vaccine containing type-specific determinants

from 26 different M serotypes is undergoing clinical trials [64–67]. These serotypes include types frequently isolated from invasive infections or pharyngitis (e.g., *emm1*, *12*, *28*, *3*, and others) and others associated with the onset of rheumatic fever (*emm5*, *3*, *6*, *18*, and others). The efficacy and safety of this 26-valent vaccine have been demonstrated in healthy adult volunteers [66]. However, the predicted coverage with this 26-valent vaccine, formulated for use in the United States, was estimated to be only 60% in Israel [67].

Another vaccine strategy involving the conserved region (C-repeats) is developing [68, 69]. Most adults possess antibodies against the p145 peptide, located within C-repeats; these antibodies act to opsonize multiple GAS strains having sequence variations in C-repeats. Because these antibodies cross-react with human cardiomyocytes, the J14, J14.1, J14-R1, and J14-R2 motifs, which exclude the cross-reactive epitope within p145, have been proposed as components of a future C-repeat vaccine [70].

SDSE also possesses an M surface protein, similar to that of GAS. Vaccination trials against SDSE infection based upon the M protein with A-repeats and C-repeats as target antigens should be initiated to prevent the development of invasive SDSE diseases, rather than to reduce the SDSE carrier rate, because the invasive illnesses may lead to poor outcomes (death or post-infective sequelae).

Conclusions

The characterization of SDSE strains was first proposed by Vandamme and colleagues in 1996 [4]. Based on whole-genome analyses of the original isolates, the genomic features of this pathogen somewhat resemble those of GAS, while differing from those of GBS. Virulence factors specific to SDSE as opposed to GAS should be determined to better understand the pathogenesis of SDSE.

In the present new century, the prevalence of invasive (e.g., STSS, necrotizing fasciitis, cellulitis, urosepsis, and pneumonia) and noninvasive SDSE infections has increased gradually year by year in Japan, other Asian countries, Europe, and America. Our investigation, the first nationwide surveillance concerning 231 invasive SDSE infections over 1 year in Japan, found the infections to be community-acquired in elderly subjects having underlying diseases. Insufficient white blood cell responses and thrombocytopenia on admission each suggested a higher risk of poor outcome (death or post-infective sequelae). Because the elderly Japanese population will continue to increase, invasive SDSE infection will be an important concern for primary-care physicians. In India, however, the disease burden caused by SDSE has been higher than that of GAS among schoolchildren. Therefore, research and development concerning vaccination against SDSE infection are also needed.

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