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.

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

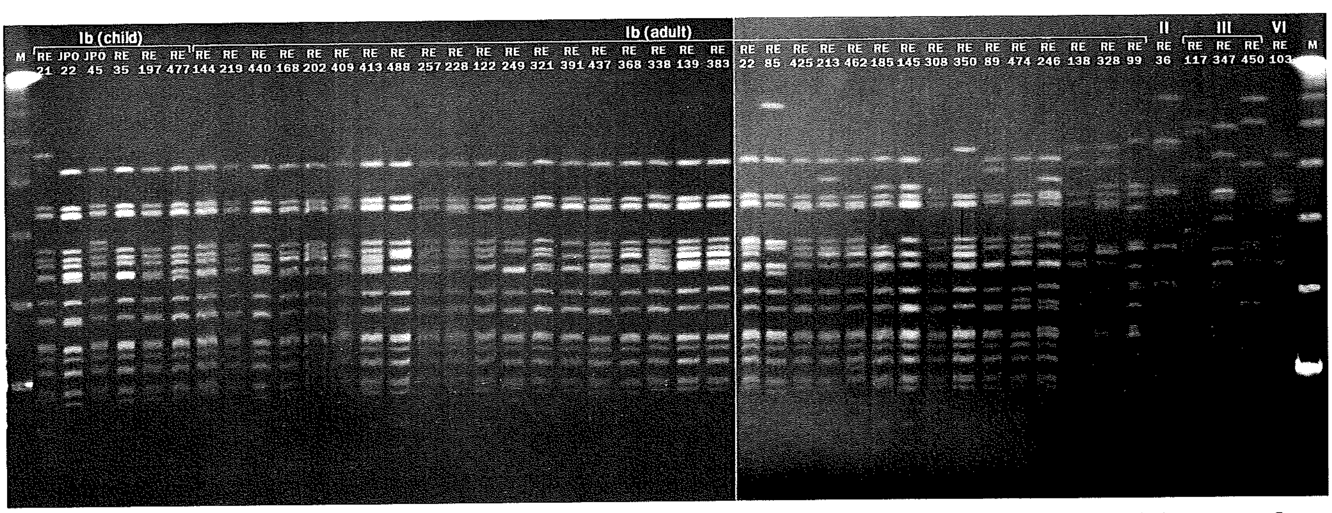

FIG. 1. PFGE patterns of levofloxacin-resistant *S. agalactiae* isolates. Each DNA sample was digested with the ApaI restriction enzyme. Lanes M, lambda ladder.

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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Molecular *emm* genotyping and antibiotic susceptibility of *Streptococcus dysgalactiae* subsp. *equisimilis* isolated from invasive and non-invasive infections

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To analyse the characteristics of infections caused by *Streptococcus dysgalactiae* subsp. *equisimilis*, clinical isolates (n=145) were collected at 11 medical institutions between September 2003 and October 2005. These isolates belonged to Lancefield group A (n=5), group C (n=18) or group G (n=122). Among all isolates, 42 strains were isolated from sterile samples such as blood, synovial fluid and tissue specimens from patients who were mostly over 50 years with invasive infections, and included seven cases of streptococcal toxic shock syndrome and necrotizing fasciitis. In contrast, the remaining 103 were isolated mainly from patients of all age groups with non-invasive infections such as pharyngotonsillitis. These isolates were classified into 25 types based on *emm* genotyping. A significant difference in *emm* types was observed between isolates from invasive and non-invasive infections (P<0.001): *stG485*, *stG6792* and *stG2078* predominated among isolates from invasive infections. A phylogenetic tree of complete open reading frames of *emm* genes in this organism showed high homology with those of *Streptococcus pyogenes*, but not with those of other streptococci. The presence of five different clones was estimated based on DNA profiles of isolates from invasive infections obtained by PFGE. Genes for resistance to macrolides [*erm*(A), three isolates; *erm*(B), five isolates; *mef*(A), seven isolates] and levofloxacin (mutations in *gyrA* and *parC*, four isolates) were identified in this organism. These results suggest the need for further nationwide surveillance of invasive infections caused by *S. dysgalactiae* subsp. *equisimilis*.

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INTRODUCTION

Most β-haemolytic streptococcal pathogens isolated from humans are identified as *Streptococcus pyogenes* (group A streptococci; GAS), *Streptococcus agalactiae* (group B streptococci; GBS), *Streptococcus dysgalactiae* subsp. *equisimilis* and species of the anginosus group that belong to Lancefield groups A, B, C, G or F (Facklam, 2002; Ruoff *et al.*, 2003). In contrast to GAS and GBS, which are known to cause serious and systemic invasive infections, streptococci with Lancefield group C or G antigens were long considered to be commensal organisms that only rarely caused invasive infections as opportunistic pathogens.

In 1996, *S. dysgalactiae* subsp. *equisimilis* was proposed as a new streptococcal taxon (Vandamme *et al.*, 1996). Although rare, *S. dysgalactiae* subsp. *equisimilis* strains having group A antigen rather than group C or G antigen have also been reported (Bert & Lambert-Zechovsky, 1997; Brandt *et al.*, 1999; Katsukawa *et al.*, 2002). Many recent studies have reported that this organism causes invasive and systemic streptococcal infections like GAS (Natoli *et al.*, 1996; Wagner *et al.*, 1996; Hirose *et al.*, 1997; Kugi *et al.*,

Abbreviations: GAS, group A streptococci; GBS, group B streptococci; ML, macrolide; STSS, streptococcal toxic shock syndrome.

1998; Barnham *et al.*, 2002; Cohen-Poradosu *et al.*, 2004; Hashikawa *et al.*, 2004). The organism has also been reported to cause a wide variety of human infections such as pharyngitis, cellulitis, sepsis, meningitis and endocarditis (Woo *et al.*, 2001).

Our group has reported that most Japanese patients with such invasive infections are older persons with severe underlying diseases (Ubukata *et al.*, 2006). Recently, a population analysis in the USA reported the incidence of invasive diseases caused by β-haemolytic streptococci, mostly *S. dysgalactiae* subsp. *equisimilis* (Broyles *et al.*, 2009).

Notably, *S. dysgalactiae* subsp. *equisimilis* possesses many virulence factors shared with GAS, such as M protein (Fischetti, 1989; Schnitzler *et al.*, 1995), streptolysin O (Gerlach *et al.*, 1993; Okumura *et al.*, 1994), streptolysin S (Humar *et al.*, 2002) and streptokinase (Walter *et al.*, 1989; Ikebe *et al.*, 2004). It has been suggested that these factors were transmitted from GAS to this species (Kalia *et al.*, 2001).

In the present report, we have described *S. dysgalactiae* subsp. *equisimilis* isolates from patients with invasive and non-invasive infections, and analysed the relationship with patient age and disease, *emm* genotyping and DNA profiles of isolates from invasive infections according to PFGE and antimicrobial susceptibilities.

METHODS

Phenotypic testing of isolates. From September 2003 to October 2005, a total of 593 β-haemolytic streptococcus isolates identified as causative pathogens were sent to our laboratory from 11 medical institutions throughout Japan. The isolates were accompanied by medical information about the patients using an anonymous questionnaire.

S. dysgalactiae subsp. *equisimilis* was identified in accordance with the differentiating characteristics described by Ruoff *et al.* (2003). These included: (i) agglutination positivity for Lancefield group A, C or G determined using antiserum (Streptex; Remel Europe); (ii) strong β-haemolysis; (iii) formation of large, glossy colonies; (iv) bacitracin resistance; (v) negative pyrrolidonylarylamidase test; (vi) negative Voges–Proskauer test; and (vii) positive β-D-glucuronidase test.

Ultimately, 145 isolates were identified as *S. dysgalactiae* subsp. *equisimilis*, and of these, five possessed group A antigen, 18 had group C antigen and 122 had group G antigen.

Antimicrobial susceptibility. The susceptibility of all isolates to 12 antimicrobial agents was determined by a microdilution method using cation-adjusted Mueller–Hinton broth (Becton Dickinson) supplemented with 5% lysed horse blood according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2007). Oral antimicrobial agents employed in this study were penicillin G, ampicillin, amoxicillin, cefdinir, cefditoren, faropenem, clarithromycin, azithromycin and levofloxacin. Cefotaxime, panipenem and meropenem were also evaluated as representative parenteral agents. The antimicrobials were obtained from pharmaceutical manufacturers.

Identification of macrolide (ML) and fluoroquinolone resistance genes. Three ML resistance genes, *erm*(A) (Seppälä *et al.*, 1998), *erm*(B) (Trieu-Cuot *et al.*, 1990) and *mef*(A) (Clancy *et al.*, 1996; Tait-Kamradt *et al.*, 1997), were identified by PCR, as described previously (Wajima *et al.*, 2008). Isolates with the *erm*(A) gene show an inducible ML/lincosamide/streptogramin B resistance phenotype, whilst strains with the *erm*(B) gene show a constitutive ML/lincosamide/streptogramin B resistance phenotype arising from methylation of 23S rRNA. Strains with a *mef*(A) gene show an M phenotype involving an active efflux pump system for 14- and 15-membered MLs.

Four genes related to fluoroquinolone resistance, *gyrA*, *gyrB*, *parC* and *parE*, were analysed using four sets of primers as described previously (Wajima *et al.*, 2008).

Genotyping of *emm* and bootstrap analysis. For *emm* genotyping by PCR, a primer set was used as described previously (Beall *et al.*, 1996; Whatmore & Kehoe, 1994). Sequencing reactions for purified PCR products were performed using the primer 5′-TATTCGC-TTAGAAAATTAAAAACAGG-3′ and an ABI PRISM 3130/3130xl Genetic Analyser (Applied Biosystems). The first 300 bases of the 5′ end of the *emm* gene were compared with those in the CDC *emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strep-blast.htm). An *emm* type showing more than 98% identity with a CDC reference strain was identified as that particular *emm* type.

Sequences of open reading frames of *emm* genes in 25 strains were determined using sense primer-1 (3′-ACGGCTAACCTTAGGA-TTGG-5′) and reverse primer-2 (3′-CGTCTTTAGTCGCAAA-CAGG-5′). The results were compared with those for GAS using CLUSTAL W (v.1.83; http://clustalw.ddbj.nig.ac.jp/top-j.html). The Kimura method was used to estimate the number of amino acid substitutions between sequences in each strain. A phylogenetic tree from bootstrap analysis by the neighbour-joining method was obtained using TreeView (v.1.40). Sequences of the open reading frames of *emm* genes and deduced amino acids used to depict the phylogenetic tree included seven strains of *Streptococcus pyogenes* (MGAS5005, *emm1.0*; MGAS9429, *emm12*; MGAS6180, *emm28*; SSI1, *emm3*; MGAS10750, *emm4*; NZ131, *emm49*; MGAS10394, *emm6*), four strains of *S. dysgalactiae* subsp. *equisimilis* [CAA63750 (protein id), *stC1400.2*; CAA42694, *stG166b.0*; CAA42693, *stC74a.0*; AAA26928, *stG1750.0*)], *S. dysgalactiae* subsp. *dysgalactiae* (CAB65413, *demB* encoding M-like protein), *Streptococcus equi* (AAB71984, *seM* encoding M-protein), *Streptococcus iniae* (ACF25917, *simA* encoding M-like protein) and *S. equi* subsp. *zooepidemicus* (ACG63129 and ACG63223, *cspZ.1* and *cspZ.2* encoding Emm-like cell surface protein, respectively), with *Streptococcus pneumoniae* (ACH85940, a gene encoding a putative surface protein) used as an outgroup.

PFGE. PFGE was carried out by modification of a method described previously (Murayama *et al.*, 2009). Each strain was cultured in 2 ml Todd–Hewitt broth (Becton Dickinson) for 18 h, harvested by centrifugation at 5000 ***g*** at 4 °C for 5 min and then washed with saline/EDTA solution. Plugs with embedded bacterial cells were incubated in restriction enzyme buffer containing 30 U *Sma*I at 30 °C for 16 h. Electrophoresis was performed with a CHEF Mapper (Bio-Rad Laboratories). Separation of the fragments was carried out at 6 V cm^{-1} at 14 °C for 18 h.

Statistical analysis. A χ^2 test was used to test for a significant difference between invasive and non-invasive infection groups by age distribution and by *emm* typing.

RESULTS AND DISCUSSION

Age distribution of patients with *S. dysgalactiae* subsp. *equisimilis* infection

Fig. 1 shows the age distribution of patients with *S. dysgalactiae* subsp. *equisimilis* infection, categorized as either invasive (n=42) or non-invasive (n=103).

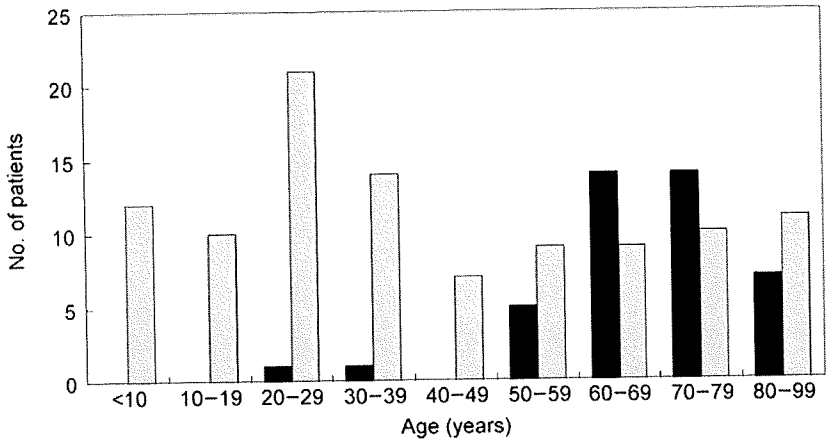

Fig. 1. Age distribution of patients with *Streptococcus dysgalactiae* subsp. *equisimilis* infection. In patients with invasive infection (black bars; n=42), causative agents were isolated from blood (n=32), synovial fluid (n=6) and tissue (n=4). In patients with non-invasive infections (grey bars; n=103), the agents were isolated from non-sterile sites such as pharynx/tonsils (n=37), sputum (n=31), pus (n=13), middle ear fluid (n=4) and other (n=18). [1]

The invasive infections comprised sepsis (n=26), purulent arthritis (n=6), cellulitis (n=3), necrotizing fasciitis (n=3) and streptococcal toxic shock syndrome (STSS, n=4). Their causative agents were isolated from normally sterile samples such as blood (n=32), synovial fluid (n=6) and tissue (n=4). The non-invasive infections included pharyngitis, tonsillitis, acute otitis media and local pyogenic infection. These agents were isolated from sputum (n=31), pharynx/tonsils (n=37), pus (n=13), middle ear fluid (n=4) and other sources (n=18).

Invasive infections occurred mostly in patients who were at least 50 years old, especially elderly adults of 60–80 years (P<0.001). Severe underlying conditions such as diabetes mellitus, liver dysfunction, renal dysfunction, medical treatment for malignant disease, immobility and immune deficiency were present in 85.7 % of invasive infection cases.

Although group C and G streptococci – the most frequently identified *S. dysgalactiae* subsp. *equisimilis* – are usually found as commensal organisms in the throat, skin and occasionally the female genitourinary tract, these organisms are increasingly being recognized as important human pathogens (Brandt & Spellerberg, 2009). Most human infections with *S. dysgalactiae* subsp. *equisimilis* are caused by person-to-person transmission and often involve the throat and skin, with patterns similar to those of GAS (Baracco & Bisno, 2006). In patients with severe underlying diseases, the organisms may invade the bloodstream and become widely disseminated to many deep sites where they can cause life-threatening invasive infections.

Susceptibility to 12 agents

Table 1 shows the MIC ranges and MIC_{50} and MIC_{90} values of 12 antimicrobial agents for *S. dysgalactiae* subsp. *equisimilis* strains. The antimicrobial activities of the oral β-lactam antibiotics penicillin G, ampicillin, amoxicillin, cefdinir, cefditoren and faropenem were excellent, with MIC_{90} values of ⩽0.031 μg ml^{-1}. No strains with reduced β-lactam susceptibility were recognized. The activities of the parenteral agents cefotaxime, panipenem and meropenem were also excellent.

The activities of clarithromycin, azithromycin and levofloxacin against these strains were less than those of the β-lactams. Strains possessing ML resistance genes identified by PCR accounted for 10.3 % of all strains: three strains (2.1 %) possessed an *erm*(A) gene (*stG6979*, two strains; *stCK401*, one strain), five strains (3.4 %) had an *erm*(B) gene (all *stG10*) and seven strains (4.8 %) had a *mef*(A) gene (*stG10*, three strains; *stC36*, two strains; *stG2078*, one strain; and *stG840*, one strain).

Four strains isolated from synovial fluid or sputum showed high resistance to levofloxacin, with MICs ⩾32 μg ml^{-1}. All four strains had amino acid substitutions, changing Ser-81 to Phe or Tyr in GyrA and Ser-79 to Tyr in ParC, together with ML resistance genes *erm*(B) or *mef*(A), and they all had *emm* type *stG10*.

Table 1. MIC range and MIC_{50} and MIC_{90} values of 12 antimicrobial agents for *Streptococcus dysgalactiae* subsp. *equisimilis*

Antibiotic	MIC (μg ml^{-1})		
	Range	MIC_{50}	MIC_{90}
Oral			
Penicillin G	0.008–0.016	0.016	0.016
Ampicillin	0.016–0.031	0.031	0.031
Amoxicillin	0.016–0.063	0.016	0.016
Cefdinir	0.016–0.031	0.016	0.031
Cefditoren	0.008–0.031	0.016	0.016
Faropenem	0.016–0.031	0.031	0.031
Clarithromycin	0.063–⩾64	0.125	4
Azithromycin	0.5–⩾64	1	32
Levofloxacin	0.25–64	1	2
Parenteral			
Cefotaxime	0.008–0.031	0.016	0.016
Panipenem	0.004–0.016	0.008	0.008
Meropenem	0.008–0.016	0.016	0.016

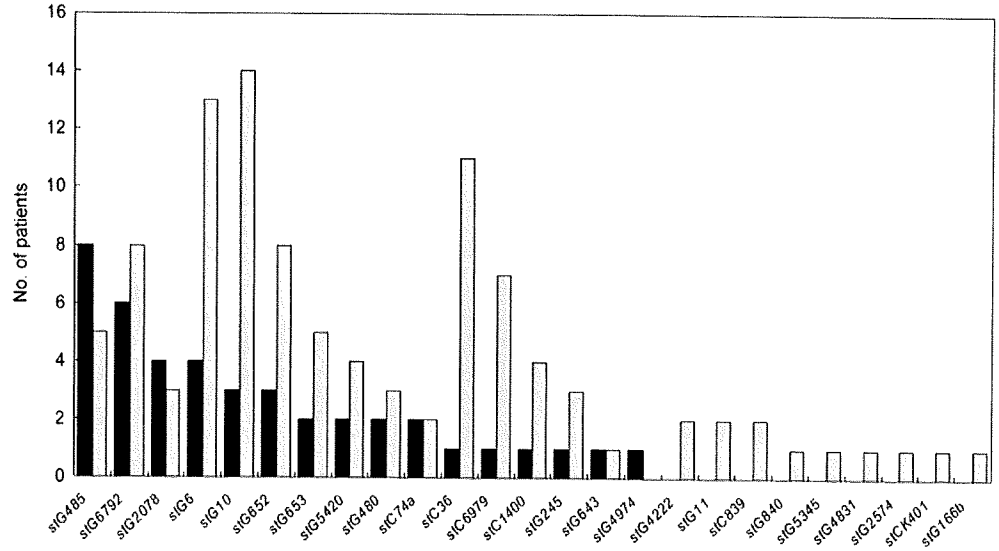

Fig. 2. Comparison of *emm* types in *Streptococcus dysgalactiae* subsp. *equisimilis* isolates separated into invasive (black bars; $n=42$) and non-invasive (grey bars; $n=103$) infections. The distribution of *emm* types was significantly different between the two groups (χ^2 test, $P<0.001$).

ML and levofloxacin resistance rates of *S. dysgalactiae* subsp. *equisimilis* were the same as for GAS (Wajima *et al.*, 2008), but different from those reported for GBS in Japan (Murayama *et al.*, 2009).

Typing of *emm* and PFGE profile

Fig. 2 shows the results of *emm* genotyping for 145 *S. dysgalactiae* subsp. *equisimilis* strains classified into invasive

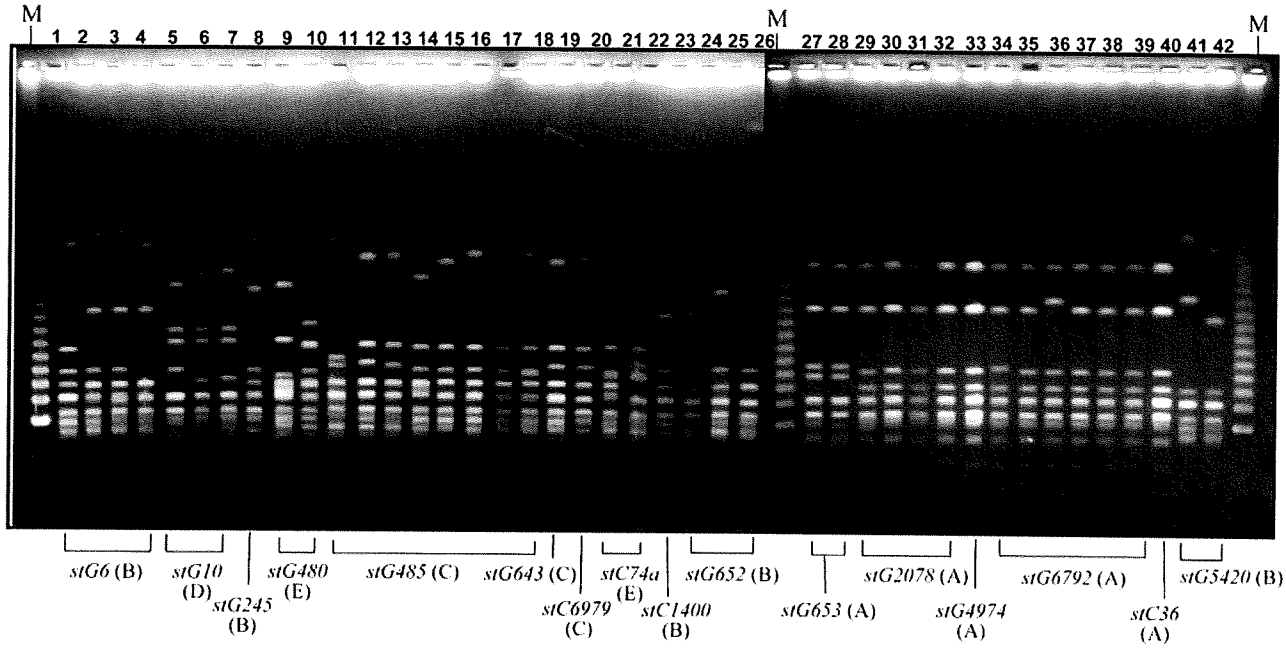

Fig. 3. PFGE profiles and their classification into five clones of chromosomal DNA from *Streptococcus dysgalactiae* subsp. *equisimilis* isolates from patients with invasive infection. Chromosomal DNAs were digested with *Sma*I. Capital letters in parentheses represent clones. Lanes: M, size marker (λ phage DNA); 1–4, *stG6*; 5–7, *stG10*; 8, *stG245*; 9 and 10, *stG480*; 11–18, *stG485*; 19, *stG643*; 20, *stC6979*; 21 and 22, *stC74a*; 23, *stC1400*; 24–26, *stG652*; 27 and 28, *stG653*; 29–32, *stG2078*; 33, *stG4974*; 34–39, *stG6792*; 40, *stC36*; 41 and 42, *stG5420*.

and non-invasive groups. Each *emm* type included several subtypes.

Three types, *stG485*, *stG6792* and *stG2078*, predominated among the 42 invasive strains, but the predominance of a specific type was not recognized. In contrast, *stG10*, *stG6* and *stC36* were predominant relative to the other non-invasive strains. The distribution of *emm* types was significantly different between the invasive and non-invasive groups ($P<0.001$).

The PFGE profiles of invasive strains digested with the restriction enzyme *Sma*I are shown in Fig. 3. Strains with the same *emm* type showed uniform DNA profiles and were classified into five clones in accordance with the criteria for interpreting PFGE patterns (Tenover *et al.*, 1995): strains ($n=14$) identified as *stG6792*, *stG2078*, *stG653*, *stC36* and *stG4974* belonged to clone A; strains ($n=11$) identified as *stG6*, *stG652*, *stG5420*, *stG245* and *stC1400* belonged to clone B; strains ($n=10$) identified as *stG485*, *stG643*, and *stC6979* belonged to clone C; strains ($n=3$) identified as *stG10* belonged to clone D; and strains ($n=4$) identified as *stG480* and *stC74a* belonged to clone E. The *emm* types of four strains isolated from patients with STSS were *stG2078*, *stG485*, *stG653* and *stG6792*, respectively. Two patients later died shortly after hospitalization. No bias was observed in the *emm* type of isolates from invasive infections.

Our results of the *emm* type of invasive strains differed from the surveillance results recently reported by Broyles *et al.* (2009). In their results, strains identified as types *stG6*, *stG245*, *stG2078* and *stG643* predominated, and types *stG6792* and *stG485* were heavily outnumbered. At present, although the epidemiology is unknown, it seems that a new *emm* type organism may have entered Japan and may be spreading rapidly among increasing numbers of elderly people with underlying diseases living in densely populated cities.

Fig. 4. Phylogenetic tree of the complete M protein in *Streptococcus dysgalactiae* subsp. *equisimilis* ($n=25$) isolated from patients with invasive infections. A phylogenetic tree based on deduced amino acid sequences was constructed by the neighbour-joining method. Bootstrap analyses of 1000 replications were carried out using CLUSTAL W. Each strain number analysed and the clone type is indicated in parentheses. The sequences of the other streptococcal strains were derived from the GenBank/EMBL/DDBJ database. Species have been given a five-letter code: Spneu, *S. pneumoniae*; Sdysg, *S. dysgalactiae* subsp. *dysgalatiae*; Sequi, *S. equi*; Szooe, *S. equi* subsp. *zooepidemicus*; Sinia, *S. iniae*.

Phylogenetic tree of *emm* genes

Fig. 4 shows the phylogeny of the 12 types of M protein in *S. dysgalactiae* subsp. *equisimilis* (n=25) isolated from the patients with invasive infections. A neighbour-joining tree was constructed for the M protein pattern using amino acid sequences corresponding to the complete M protein together with several M-like proteins in other previously analysed streptococcal species: seven strains of GAS, four strains of *S. dysgalactiae* subsp. *equisimilis* and one each of *S. dysgalactiae* subsp. *dysgalactiae*, *S. equi*, *S. iniae* and *S. equi* subsp. *zooepidemicus*.

Although M proteins have a hypervariable region at the N-terminal end, *S. dysgalactiae* subsp. *equisimilis* and GAS harbour extremely homologous M proteins compared with those of other *Streptococcus* species. The phylogenetic tree suggested that the M protein of *S. dysgalactiae* subsp. *equisimilis* was an orthologue of that of GAS.

We recently determined the complete genomic sequence of *S. dysgalactiae* subsp. *equisimilis* GGS_124 (*stG480.0*) isolated from patients with STSS (GenBank accession no. AP010935). The genome size was 2.1 Mbp, and sequence coverage with GAS genomes (Ferretti *et al.*, 2001) was 61–63 % identity. Interestingly, many genes encoding virulence factors in GAS were identified in *S. dysgalactiae* subsp. *equisimilis*. The occurrence of serious infections caused by *S. dysgalactiae* subsp. *equisimilis* in elderly persons with underlying diseases is likely to involve both compromised host defences and GAS-like virulence factors. However, it is unknown how this micro-organism invades deep tissues and vessels. Further investigation is needed to clarify this issue.

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Serotype and antibiotic resistance of isolates from patients with invasive pneumococcal disease in Japan

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SUMMARY

Invasive pneumococcal disease (IPD) is of concern in Japan, where the heptavalent pneumococcal conjugate vaccine (PCV7) is unavailable. We determined serotypes, genotypes indicating β-lactam resistance, and antibiotic susceptibilities of 496 isolates from normally sterile sites in patients (193 children, 303 adults) from 186 institutions between August 2006 and July 2007. Disease presentations included sepsis (46·2%), pneumonia (31·5%), and meningitis (17·5%). Mortality was 1·4% in children and 22·1% in adults, many of whom had underlying diseases. In children, serotype 6B (22·5%) was followed by 19F (14·1%), and 14 (13·1%); potential coverages of PCV7 and PCV13 were 75·4% and 93·7%, respectively. In adults, serotype 12F (14·3%) was followed by 3 (11·3%), and 6B (10·3%); 23-valent polysaccharide vaccine (PPV23) coverage was 85·4%. Most serotype 12F strains were gPISP, with *pbp2b* gene alteration; carbapenem had an excellent MIC_{90}. PCV7 is recommended for children and PPV23 for adults to increase prevention against IPD.

Key words: Antibiotic resistance, molecular epidemiology, *Streptococcus pneumoniae* (pneumococcus), surveillance, vaccines.

INTRODUCTION

Streptococcus pneumoniae is a leading cause of invasive infections such as lobar pneumonia, septicaemia, and meningitis, which are major contributors to morbidity and mortality in children and adults. Since the discovery of pneumococcal strains resistant to penicillin G (PEN) [1], these strains have spread rapidly worldwide [2, 3] and have been the subject of several epidemiological surveillance studies of capsule serotype distribution and antibiotic susceptibility in many countries [4–8].

In Japan, the prevalence of PEN-resistant *S. pneumoniae* (PRSP) and PEN-intermediate *S. pneumoniae* (PISP) in clinical isolates has increased rapidly since the late 1990s, especially in younger children [9, 10]. Characteristically, PRSP strains show simultaneous

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resistance to cephalosporin antibiotics used in ambulatory practice [9]. In PRSP and PISP, genotypic abnormalities in three penicillin-binding protein (PBP) genes, *pbp1a*, *pbp2x*, and *pbp2b*, which encode the PBP1A, PBP2X, and PBP2B enzymes, respectively, have been identified by polymerase chain reaction (PCR) using primers to detect mutations in these genes [9, 11]. The prevalence of PRSP possessing the three abnormal *pbp* genes currently exceeds 50% in Japan [12].

Given this background, therapeutic choices for patients with invasive pneumococcal disease (IPD) in Japan have been gradually eroded. A carbapenem antibiotic such as panipenem (PAM), which has been used only in Japan, Korea, and China, was administered in preference to intravenous third-generation cephalosporins such as cefotaxime (CTX) and ceftriaxone (CRO). Additionally, rapid increases in numbers of adults and elderly persons with various underlying diseases, is thought to increase the threat of IPD.

A heptavalent pneumococcal conjugate vaccine (PCV7) for children has been introduced in many countries [13], beginning with the USA [14]. This vaccine has been reported to contribute to a decrease in IPD when causative strains are covered [15–18]. In contrast, IPD caused by non-PCV7 serotypes of *S. pneumoniae*, such as 19A, continues to increase [19–21]. As a result, a second-generation pneumococcal conjugate vaccine such as PCV13 is now being developed to cover a wider range of serotypes.

We therefore focused on understanding the serotype distribution and antibiotic susceptibility of isolates from IPD in children and adults throughout Japan, where clinical trials of PCV7 for children have been concluded and approval is expected. Here we describe the serotype distribution and antibiotic susceptibility of the isolates according to their *pbp* genotype by PCR. We also extrapolate from the data the expected PCV7 and PCV13 coverage rates for children and those of PPV23 and PCV13 for adults.

MATERIALS AND METHODS

We examined 496 *S. pneumoniae* isolates from patients with IPD [22]. Isolates were cultured from clinical samples processed in the laboratories of 186 medical institutions from August 2006 to July 2007 throughout Japan and then sent to our laboratory with an anonymous application form written by the reporting doctor. All isolates were from normally sterile samples such as cerebrospinal fluid (CSF), blood, or pleural or joint fluid.

Haematological tests in IPD patients

To statistically determine risk factors in adults, we requested an anonymous report including patient's age, disease presentation, underlying disease, white blood cell count (WBC), C-reactive protein (CRP), and platelet count (PLT); and outcome, including presence or absence of neurological sequelae.

Serotype and antimicrobial susceptibility

Serotypes of all *S. pneumoniae* isolates were determined by the capsule swelling reaction using antiserum purchased from the Statens Serum Institute (Denmark) [23]. Minimal inhibitory concentrations (MICs) of penicillin (PEN), ampicillin (AMP), cefotaxime (CTX), meropenem (MEM) and vancomycin (VAN) were determined by agar dilution methods using Muller–Hinton II agar (MH; Becton Dickinson, USA) supplemented with 5% defibrinated sheep blood [24]. *S. pneumoniae* ATCC49619 was used as a quality control strain.

Genotypic identification of resistance by PCR

To confirm that isolates were *S. pneumoniae*, the *lytA* gene encoding the autolysin enzyme specific to *S. pneumoniae* [25] was amplified simultaneously with the three PBP genes. Each primer set used for detection of the three PBP genes was designed to amplify a part of the normal *pbp1a*, *pbp2x*, and *pbp2b* genes detected only in susceptible strains [9]. Portions of each gene corresponding to the primers were positioned in blocks of highly divergent sequences within or near conserved amino-acid motifs. Each reaction tube for PCR contained two primer sets, for detecting *lytA* and *pbp1a* in tube A; *pbp2x* and *pbp2b* in tube B; and *mef*(A) and *erm*(B) in tube C. These tubes contained 30 μl reaction mixture as previously described [9, 22, 26].

One colony was chosen from sheep blood agar and suspended in 30 μl lysis solution [11]. The tube then was placed in a thermal cycler (Gene Amp PCR System 9600R; PerkinElmer Cetus, USA) and heat-treated for 10 min at 60 °C and for 5 min at 94 °C to obtain template DNA. Next, 2 μl template DNA was added to each of the three tubes marked A, B, and C containing 30 μl reaction mixture. PCR cycling

Table 1. *Outcome based on presence or absence of underlying disease**

	Children			Adults		
	Underlying disease			Underlying disease		
Outcome	(+)	(−)	Subtotal	(+)	(−)	Subtotal
Fatality	2	0	2 (1·4)	37	6	43 (22·1)
Sequelae (+)	1	3	4 (2·9)	13	4	17 (8·7)
Sequelae (−)	17	115	132 (95·7)	85	50	135 (69·2)
Total	20	118	138 (100·0)	135	60	195 (100·0)

* Patients with unknown status concerning underlying disease and outcome were excluded from analysis.

conditions consisted of 30 cycles at 94 °C for 15 s, 53 °C for 15 s, and 72 °C for 15 s and amplified using a Takara PCR Thermal Cycler (Model TP600; Takara Bio, Japan). Amplified DNA fragments were analysed by electrophoresis on a 3% agarose gel. In the presence of all three DNA fragments corresponding to *pbp1a*, *pbp2x*, and *pbp2b*, the PBP genes were regarded as having essentially the same sequences as the sensitive R6 strain (PEN-susceptible *S. pneumoniae*, PSSP). We regarded the absence of DNA fragments as indicative of sequences other than those in PSSP. Genotypic determination is indicated by adding 'g' to designations as follows: gPSSP, gPISP (*pbp2x*), gPISP (*pbp2b*), gPISP (*pbp1a*+*2x*), gPISP (*pbp2x*+*2b*), and gPRSP (*pbp1a*+*2x*+*2b*).

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed using a modification of a method described previously [12]. For digestion, DNA plugs were incubated in 1 ml restriction enzyme buffer with 100 U of *Apa*I at 37 °C for 16 h. Electrophoresis was performed with a CHEF Mapper (Bio-Rad Laboratories, USA) at 5·7 V/cm at 14 °C for 18 h.

RESULTS

IPD

IPD was classified into five groups as follows: septicaemia and bacteraemia (including two cases of bacterial endocarditis); pneumonia, where *S. pneumoniae* was isolated from blood cultures; meningitis diagnosed by clinical findings, where *S. pneumoniae* was isolated from CSF or blood; suppurative arthritis or osteomyelitis; and others. In 193 children aged ⩽17 years, septicaemia was predominant with 114 (59·1%) cases, followed by pneumonia with 44 (22·8%) cases, and meningitis with 30 (15·5%) cases; other diseases were rare. Almost 92% of IPD cases in children were aged ⩽4 years. In the 303 adults, septicaemia and pneumonia predominated with 115 (38·0%) cases and 112 (37·0%) cases, respectively, followed by meningitis with 57(18·8%) cases. The median age of adults with septicaemia and meningitis was 66 years, but was somewhat higher in patients with pneumonia (73 years).

Outcomes and underlying diseases

Table 1 shows outcomes and underlying diseases in 138 children (71·5% of those studied), and 195 adults (64·4%), according to reports returned by collaborating institutions. In children, 20 (14·5%) had underlying diseases, mostly congenital abnormalities. Adverse outcomes for children included death in two (1·4%) cases and neurological sequelae in four (2·9%) cases.

In adults, 135 (69·2%) had underlying diseases, the most common being cancer surgery (38), diabetes (30), cardiovascular disease (18), hepatic disease (16), kidney disease (9), immunological deficiency (3), and splenectomy (2). Deaths were numerous [43 (22·1%)], but 37 of those patients had underlying diseases, and the cause of death was not considered in detail. The median hospital stay in adults who did not survive was 2 days. Seventeen patients, including 13 with underlying disease, had severe neurological sequelae. When outcomes in cases with underlying diseases and those without underlying diseases were compared separately for children and adults, the mortality and sequelae rates were statistically higher in both children and adults having underlying

Table 2. *Clinical laboratory findings associated with fatal outcome in adults with invasive pneumococcal disease*

	Median or % (25/75 percentiles) and [no./total]			
	Non-survivors ($n=43$)	Survivors ($n=147$)	Univariate analysis OR (95% CI)	P value
WBC (10^9 cells/l)	5·1 (2·3–8·8) [37/43]	13·2 (8·2–19·1) [136/147]		
$<5{\cdot}0\times10^9$ cells/l	48·6% [18/37]	11·0% [15/136]	7·64 (3·30–17·68)	$P<0{\cdot}0001$
C-reactive protein (mg/dl)	24·8 (16·3–31·7) [36/43]	20·6 (8·9–33·6) [131/147]		
≥15 mg/dl	77·8% [28/36]	65·6% [86/131]	1·83 (0·77–4·35)	$P=0{\cdot}1661$
PLT (10^9 cells/l)	119 (69–171) [36/43]	197 (130–262) [134/147]		
$<130\times10^9$/l	55·6% [20/36]	23·1% [31/134]	4·15 (1·92–8·97)	$P=0{\cdot}0002$

OR, Odds ratio; CI, confidence interval; WBC, white blood cell count; PLT, platelet count.

Table 3. *MIC_{90} and resistance genes identified by PCR in* S. pneumoniae

		MIC_{90} (μg/ml)					
Resistance class	n	PEN	AMP	CTX	MEM	PAM	VAN
gPSSP	101	0·031	0·031	0·125	0·016	0·004	0·5
gPISP (*pbp2b*)	38	0·125	0·031	0·063	0·031	0·008	0·5
gPISP (*pbp2x*)	124	0·063	0·125	0·5	0·016	0·008	0·5
gPISP (*pbp1a+2x*)	54	0·5	0·5	1	0·125	0·031	0·5
gPISP (*pbp2x+2b*)	35	0·5	0·5	2	0·125	0·031	0·5
gPRSP (*pbp1a+2x+2b*)	140	2	4	2	0·5	0·125	0·5

Each *pbp* gene alteration detected by PCR appears within parentheses.
MICs were determined for the following antibiotics: PEN, penicillin; AMP, ampicillin; CTX, cefotaxime; MEM, meropenem; PAM, panipenem; VAN, vancomycin.
Strains tested MICs: 492 isolates grown on sheep blood agar plate from stock at −80 °C.

diseases (Fisher's test: children, $P=0{\cdot}0395$; adults, $P=0{\cdot}0043$).

Haematological findings and outcomes in adults

We compared WBC, CRP, and PLT at time of admission between the non-surviving and surviving adults. Analysis was carried out using a non-parametric Kruskal–Wallis test and the results are shown in Table 2. The median WBC in non-survivors and survivors was $5{\cdot}1\times10^9$ and $13{\cdot}2\times10^9$ cells/l, respectively; the odds ratio between patients with WBC below and above $5{\cdot}0\times10^9$ cells/l was calculated as 7·64. A clear difference in the PLT was also noted between the two groups; and the odds ratio for mortality between patients with PLT below and above 130×10^9 cells/l was 4·15. No significant difference in CRP was evident between non-survivors and survivors. In addition, no significant difference in resistance type of gPSSP, gPISP, and gPRSP or in serotype (PPV23) was found between the non-survivors and survivors ($P=0{\cdot}1200$, $P=0{\cdot}9891$, respectively).

PBP gene alterations and β-lactam susceptibility

Table 3 shows results of MIC_{90} of PEN, AMP, CTX, MEM, and VAN. Genotype was based on PCR results for the *pbp1a*, *pbp2x*, and *pbp2b* genes. PEN susceptibility declined according to addition of altered *pbp* genes, from a MIC_{90} of 0·063 μg/ml for gPISP (*pbp2x*) to 2 μg/ml for gPRSP (*pbp1a+2x+2b*). In particular, susceptibility to CTX was affected by alterations of *pbp2x*, a pattern markedly different from that of susceptibility to PEN. In contrast, although susceptibility to MEM was affected by the gene alterations, the effect was much less. The MIC_{90} of VAN for all *S. pneumoniae* strains was 0·5 μg/ml.

Relationship between serotype and resistance genotype for β-lactams

The serotypes of *S. pneumoniae* isolates from children, classified as either PCV7 or non-PCV7 types, in decreasing order of prevalence are shown in Figure 1 and the percentage rate of resistance genotypes for β-lactams is also given for each serotype. Serotype 6B

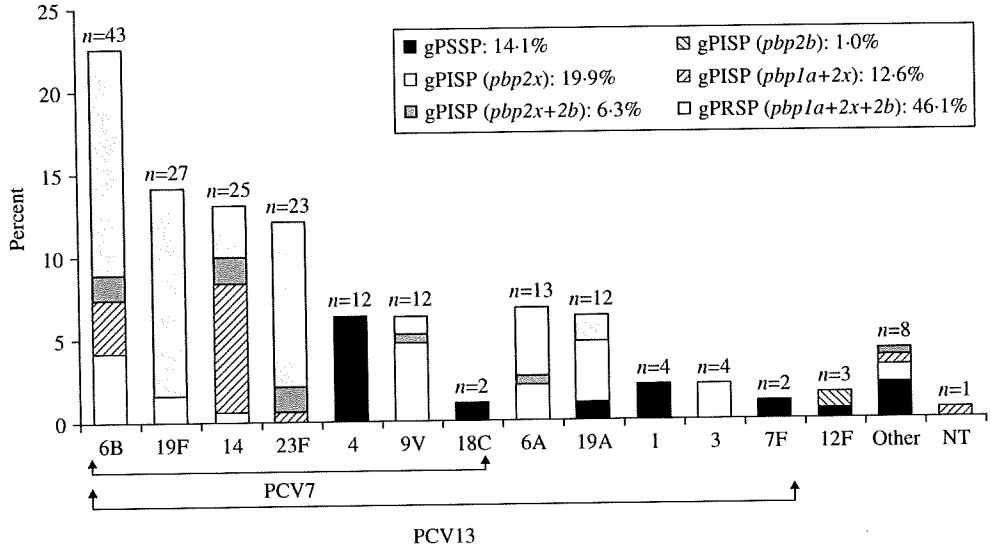

Fig. 1. Serotype distribution and resistance genes identified by PCR in *S. pneumoniae* isolated from children. 'Other' category includes serotypes 15B, 23A, 8, 24, 34, 35, and 38.

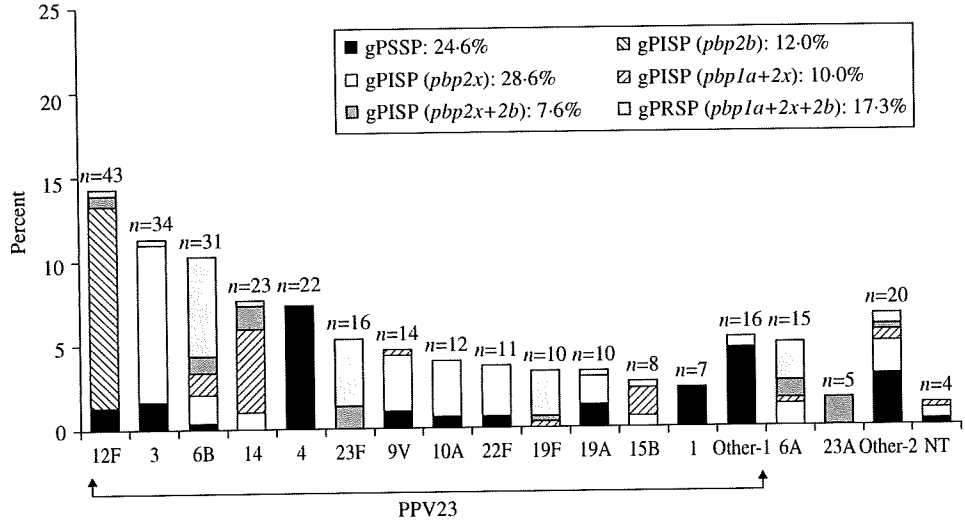

Fig. 2. Serotype distribution and resistance genes identified by PCR in *S. pneumoniae* isolated from adults. 'Other-1' category includes serotypes 9N, 11A, 33, 18C, 20, 2, 7F, 8. 'Other-2' category includes serotypes 35, 7C, 15A, 38, 15C, 31, 16, and 36.

predominated in the PCV7 types, followed in order by 19F, 14, and 23F. Coverage by PCV7, to which types 9V, 4, and 18C were added, was calculated as 75·4%. PCV7 covered types 6B, 19F, 14 and 23F, all of which showed high rates of gPRSP. In addition, coverage by PCV13 was calculated as 93·7%. The resistance rate of gPRSP (*pbp1a+2x+2b*) was highest, at 46·1%, followed by gPISP (*pbp2x*) at 19·9%, gPISP (*pbp1a+2x*) at 12·6%, gPISP (*pbp2x+2b*) at 6·3%, and gPISP (*pbp2b*) at 1·0%. The rate of gPSSP was only 14·1%.

The serotypes of *S. pneumoniae* isolates from adults that were covered by PPV23 are shown in Figure 2, in decreasing order of prevalence. These results differed markedly from those for children. The most prevalent type, 12F, accounted for 14·3% of the total; interestingly, almost all had gPISP (*pbp2b*). Serotype 3 (11·3%), with a high incidence of gPISP (*pbp2x*), was second only to 12F. Other common serotypes were, type 6B (10·3%), with a high frequency of gPRSP (*pbp1a+2x+2b*), while type 14 (7·6%) showed a high frequency of gPISP (*pbp1a+2x*). PPV23 and PCV13

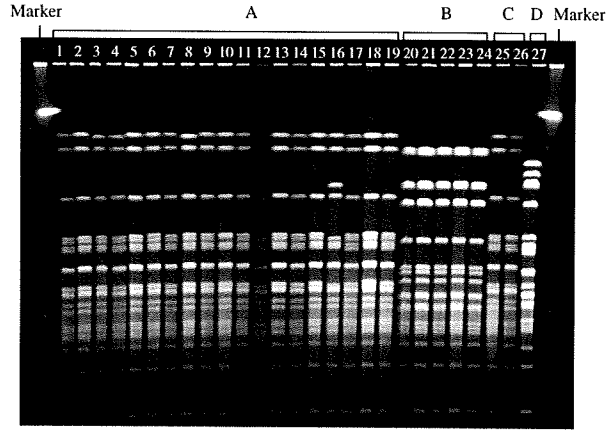

Fig. 3. PFGE patterns of *Apa*I digests of chromosomal DNA from serotype 12F isolates. A, gPISP (*pbp2b*) (lanes 1–19); B, gPSSP (lanes 20–24); C, gPISP (*pbp2x*+*2b*) (lanes 25, 26); D, gPRSP (*pbp1a*+*2x*+*2b*) (lane 27).

provided coverage in 85·4% and 61·5%, respectively. Non-survivors and patients with sequelae had developed IPD involving strains of various serotypes. The predominant resistance genotype in adults was gPISP (*pbp2x*) at 28·6%, followed by gPSSP at 24·6%, gPRSP (*pbp1a*+*2x*+*2b*) at 17·3%, gPISP (*pbp2b*) at 12·0%, gPISP (*pbp1a*+*2x*) at 10·0%, and gPISP (*pbp2x*+*2b*) at 7·6%. The serotype and the resistance genotype of strains differed significantly between children and adults (both $P<0{\cdot}0001$).

PFGE pattern of strains serotyped 12F

Figure 3 shows PFGE patterns of *Apa*I DNA digests of serotype 12F strains. The 27 strains pictured, namely five gPSSP, two gPISP (*pbp2x*+*2b*), one gPRSP, and 19 gPISP (*pbp2b*), were selected randomly from 38 strains which were isolated from patients throughout Japan. DNA restriction patterns of strains with the same resistance genotype were homogeneous, suggesting that *S. pneumoniae* strains possessing the same *pbp* alterations had spread widely. There has been a rapid increase in the prevalence of serotype 12 in Japan and this serotype is present in 18% of cases with a poor prognosis in adults. This increase is therefore considered to be of clinical significance.

DISCUSSION

S. pneumoniae is a major causative agent of diseases such as pneumonia, meningitis, and acute otitis media (AOM), as well as various other serious invasive infections. In the USA, the PCV7 vaccine was developed for children and approved in 2000, and has been incorporated into the paediatric vaccination schedule [14]. Immunization programmes using PCV7 have spread widely, and are presently conducted in almost 70 countries worldwide [27]. The incidence of IPD involving vaccine-type *S. pneumoniae* has been reported to have decreased significantly [15, 17, 18], and a related decrease in IPD in adults has been noted [16]. However, the incidence of IPD caused by non-vaccine-type *S. pneumoniae* has increased; particularly type 19A [19–21]. In order to provide increased coverage, a new vaccine, PCV13, is being developed, which will include types 19A, 6A, and 3 [28].

Much clinical attention has been drawn to a rapid increase in PRSP in *S. pneumoniae* isolates. These strains have been causative agents of paediatric AOM [29] and meningitis [22] in Japan since 1990 and this increase is strongly related to a shift from prescribing oral penicillins for outpatients to using oral cephalosporins. The increase may also be related to use of macrolides, considering that most PRSP are multidrug-resistant *S. pneumoniae* (MDRSP) also resistant to macrolides [30]. In addition, Japan's high population density tends to accelerate increases in resistant organisms.

We previously compared *pbp* gene alterations in *S. pneumoniae* strains that had been isolated in the same time period from the USA and Japan [10]. In the USA, where use of penicillins predominated, increases were evident in resistant strains with the *pbp2b* gene alteration whereas in Japan, where cephalosporins predominated, many strains characteristically had the *pbp2x* gene alteration. As shown in this study, the latter pattern still persists in Japan.

According to USA guidelines [31], the use of third-generation cephalosporins – CTX, CRO, or either of these in combination with VAN – is recommended for meningitis caused by PRSP. In Japan, however, carbapenems such as PAM and MEM are recommended as first-choice antibiotics in this situation. A major reason for this practice is that 60% of Japanese paediatric meningitis cases are caused by *Haemophilus influenzae* type b (Hib), of which about 36·2% show resistance to AMP and CTX, reflecting β-lactamase non-producing and AMP-resistant *H. influenzae* as the causative pathogens [32]. Therefore, in Japan, the preferred paediatric treatment increasingly involves concomitant use of a carbapenem, with its superior bactericidal effect against *S. pneumoniae*, plus CTX or CRO, with superior activity

against *H. influenzae*; treatment now is basically the same for adults.

As for vaccines against *S. pneumoniae*, PPV23 has been introduced in Japan, where it is used mainly on a voluntary basis for elderly people as well as adults and children with underlying diseases. The PCV7 vaccine is currently under review by the Japanese Ministry of Health, Labour and Welfare, and approval is expected soon. Nevertheless, one needs to know the extent to which PCV7 covers IPD. According to our epidemiological surveillance in the current study, PCV7 covers 75·4% of strains isolated from children with IPD. However, the incidence of types 6A and 19A, which are non-vaccine types, is significant, so the introduction of PCV13 will be beneficial.

In Japan, a recent rapid increase in IPD in adults may reflect the rapid ageing of society and an increase of lifestyle-related diseases in the adult population. The current situation whereby PPV23 vaccination is voluntary, limits its effectiveness against this increase. Development of disease caused by *S. pneumoniae* in adults with underlying disease often triggers disseminated intravascular coagulation (DIC), leading to death or serious sequelae for which the prognosis is extremely poor. Also of concern is the poor prognosis for adults who develop IPD caused by *S. pneumoniae* with intermediate PEN resistance. In addition, serotype 12F was very rare in 2000, but in the current study accounted for 12·0% of IPD cases and strains show essentially the same PFGE pattern as gPISP (*pbp2b*). The reason why this type of *S. pneumoniae* has increased so rapidly in adults is unknown, and requires further investigation. Finally, but importantly, the impact of the forthcoming introduction of PCV7 will need to be assessed by continued epidemiological surveillance of IPD throughout Japan.

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DECLARATION OF INTEREST

None.

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主題　子どもの感染症，現在（いま）

治療上問題となる耐性菌
—その検出法と分子疫学—

生　方　公　子*

はじめに

先の第39回日本小児感染症学会において企画された「シンポジウム：こどもの感染症，現在」の演者として，市中呼吸器感染症の主要な原因細菌のなかから，治療上特に問題となると思われる肺炎球菌，インフルエンザ菌，肺炎マイコプラズマ，β溶血性レンサ球菌の耐性化の現況についてお話しさせていただいた．

本稿においては，当日お話しした小児由来株の耐性化の現状について述べるとともに，耐性菌増加への対応策としてのワクチン接種を踏まえた予測カバー率，治療抗菌薬の選択とその使用期間の判断に益すると考えられるreal-time PCRによる原因微生物の迅速診断法についても述べた．

Ⅰ．市中呼吸器感染症原因細菌にみられる耐性化の本質

現在，小児において問題となっている耐性菌による感染症を考えるとき，抗菌薬が広く普及し始めた1970年代当時に，どのような耐性菌感染症がみられたのかを知っておく必要がある．当時の日本では，黄色ブドウ球菌による膿胸などを含む化膿性疾患が多かったが，それらの菌ではβ-ラクタマーゼ産生によるペニシリン（PC）耐性に加え，テトラサイクリン（TC），クロラムフェニコール（CP），あるいはマクロライド（ML）系薬耐性が注目された．その他の菌では，ML耐性のA群溶血性レンサ球菌（GAS）の流行や，インフルエンザ菌ではTEM型β-ラクタマーゼ産生菌による化膿性髄膜炎も問題であった．

これら耐性菌にみられる特徴は，耐性にかかわる多くの遺伝子が染色体外のプラスミド上に存在し，溶原化ファージなどを介して感性菌へと容易に伝達させることができたことである．耐性遺伝子の組み込まれたプラスミドは，抗菌薬が開発される以前から菌が保持していたもので，それらを保持する菌株が抗菌薬の使用によって選択されて生き残り，次第に増加してきたと考えられる[1]．

一方，今日，市中呼吸器感染症の主要な原因細菌において問題化している耐性菌の耐性化機構を**表1**に示す．第1には，β-ラクタム系薬の作用標的である細胞壁合成酵素のPBPの多様な変異による耐性化があげられ，肺炎球菌やインフルエンザ菌が相当する．第2にはML系薬の作用標的である23S rRNAの修飾や変異による耐性化であるが，肺炎球菌，マイコプラズマ，そしてGASやGBSに認められる．第3には，細菌細胞内に取り込まれたML系薬やニューキノロン系薬（FQ）の積極的な排出による耐性化で，肺炎球菌，GAS，インフルエンザ菌に認められる．第4にはFQの作用標的であるDNAジャイレースやトポイソメラーゼの変異による耐性化であるが，FQに耐性化を示す菌ではこれらの変異を伴っている．

このような耐性化は，“菌の生存にとって必須の構成物である酵素（蛋白）の質的変化による耐性

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表 1 呼吸器感染症の主たる起炎菌における耐性化機構

菌種名	薬剤	薬剤の標的（菌体構成物）	遺伝子	遺伝子にコードされた産生物の機能
肺炎球菌	β-ラクタム系薬	PBP1A PBP2X PBP2B	pbp1a pbp2x pbp2b	細胞壁合成酵素
	マクロライド系薬	23S rRNA MefA	ermB mefA	リボソームの修飾 菌体内薬物の排出
	ニューキノロン系薬	DNA ジャイレース トポイソメラーゼIV PmrA	gyrA, gyrB parC, parE pmrA	DNA のデカテネーション 菌体内薬物の排出
インフルエンザ菌	β-ラクタム系薬	PBP3 AcrA, AcrB	ftsI acrA, acrB	細胞壁合成酵素 菌体内薬物の排出
	ニューキノロン系薬	DNA ジャイレース トポイソメラーゼIV PmrA	gyrA, gyrB parC, parE pmrA	DNA のデカテネーション 菌体内薬物の排出
マイコプラズマ・ニューモニエ	マクロライド系薬	23S rRNA		リボソームの機能変化
A 群溶血性レンサ球菌 B 群溶血性レンサ球菌	マクロライド系薬	23S rRNA MefA	ermA, ermB mefA	リボソームの修飾 菌体内薬物の排出
	β-ラクタム系薬	PBP2X	pbp2x	細胞壁合成酵素
	ニューキノロン系薬	DNA ジャイレース トポイソメラーゼIV	gyrA, gyrB parC, parE	DNA のデカテネーション

化”と表現されるが，菌が分裂・増殖するのに差し支えない程度にこれらの構成物を巧みに変化させることが“耐性化の始まり”である．つまり，そのレベルは経口抗菌薬で得られる 1～2 μg/ml の濃度でも生存できる程度であることから，耐性化の初期には生物学的手法では正確に識別し難いところに問題を有している[2,3]．腸管感染症や尿路感染症など薬物濃度が高い部位に棲息して感染症を惹起する菌が，効率的に薬物を不活化する手段としてさまざまな不活化酵素を産生して耐性化しているのとは根本的に異なるのである．

II．肺炎球菌

肺炎球菌のβ-ラクタム系薬耐性化に重要な酵素は，細胞壁を長軸方向へ伸長化させる PBP1A，隔壁合成の PBP2X，そして本菌特有のランセット形成にかかわる PBP2B の 3 種類である[4]．薬剤作用との関係でみると，カルバペネム系薬やペニシリン系薬は PBP1A，2B に強く結合して優れた殺菌性を示し，セフェム系薬は PBP2X に強く結合して隔壁合成阻害により抗菌力を発揮する．PBP2X に結合するセフェム系薬の殺菌性はペニシリン系薬に比して劣る．

肺炎球菌にみられるこれらの PBP 遺伝子変異は，口腔内常在のレンサ球菌の PBP 遺伝子と肺炎球菌の PBP 遺伝子とが組み換えを生じ，その結果生じたハイブリッド遺伝子である．インフルエンザ菌のβ-ラクタム系薬耐性にみられる点変異とは異なっている．

図 1 は，2006 年に侵襲性感染症から分離された肺炎球菌に対する基準薬のペニシリン G の感受性分布と PCR によって解析した遺伝子データを重ねあわせた成績である．MIC 分布は極めて曖昧な二峰性分布であるが，遺伝子が 3 つとも変異した株，2 種変異した株などと遺伝子変異パターン別に眺めると，それぞれのパターンでは 90%近い株の MIC が試験管 3 管以内におさまっている．ペニシリン耐性肺炎球菌の識別方法としては，PCR によって