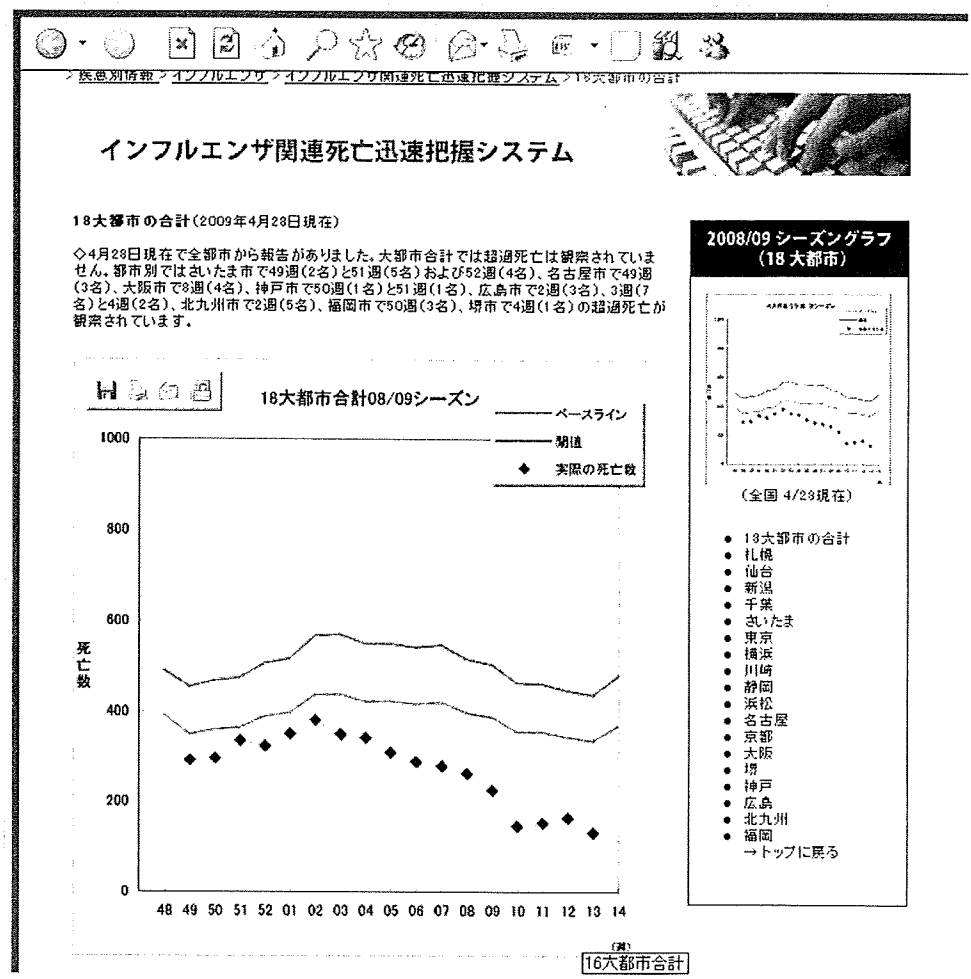

図6　インフルエンザ疾患関連死亡者数迅速把握事業

(National Epidemiological Surveillance of Infections Diseases)に登録され，その解析結果の還元は国立感染症研究所感染症情報センターのHP(前述)で一般に公開された．概ね死亡日から2週間でHP上に公開された．

迅速把握事業では数段階ある死因のいずれかでインフルエンザあるいは肺炎を対象にしていること，また週次であることが「感染研モデル」と異なるが，基本的な方法論は「感染研モデル」と同じである．**図6**は，昨年度の参加18自治体における合計のHPのみを例として示している．HP上では参加自治体別も公開されている．なお，今回の新型インフルエンザに関しては，迅速把握事業を例年どおりに12月から3月まで実施される予定である．

文　献

1）大日康史：健康経済学．東洋経済新聞社，2003

※本稿は，作成時(2009年11月18日)現在でまとめたもので，その後の知見の蓄積，状況の変化，政策や見解の改訂は反映されていません．本稿はあくまでも筆者らの個人的見解で，国あるいは国立感染症研究所の見解ではありません．

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Short Communication

Diarrhea as a Minor Adverse Effect Due to Oral Polio Vaccine

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SUMMARY: Using the adverse events monitoring system of Japan, we observed diarrhea cases in approximately 10% of patients who received oral poliovirus vaccine (OPV). This study was conducted to investigate whether diarrhea among children aged 0 to 1 is caused by OPV or by other factors such contact at the doctor's office and/or with others outside the home. We conducted a survey of the health of children after regular health check-ups and after the administration of the OPV. The data from the health check-ups were used as a control for the OPV case group. We compared the first-OPV dose vaccination group as well as the second-OPV dose vaccination group to the health check-up group. For cases of diarrhea, the odds ratio of the OPV group to the health check-up group was 1.776. Our findings strongly suggest that post-OPV cases of mild diarrhea are closely related to the administration of the OPV.

Oral poliovirus vaccine (OPV) is a highly effective vaccine for the prevention of poliomyelitis and is recognized as very safe vaccine with almost no major severe adverse side-effects, with the exception of rare cases of vaccine-associated paralytic poliomyelitis (VAPP) due to the administration of live poliovirus vaccines. On the other hand, minor adverse effects, especially diarrhea, have been noted in a clinical process report (1). In Japan, the Ministry of Health, Labour and Welfare requested a report of health status after routine vaccination during a given period every year in order to monitor adverse effects resulting from the vaccination (2,3). As many as 8,000 children are monitored for 1 month after receiving the poliovirus vaccination. Among those, diarrhea is reported for approximately 10% at 1-3 days post-OPV administration.

However, since no statistical analyses including a control group have been included in such reports, it has not yet been established whether diarrhea is indeed caused by the OPV or by other factors such as contact during a doctor's visit or contact with others. Nevertheless, the reported symptoms have been mild and therefore have not influenced the vaccination policy itself. Some caregivers in Japan remain concerned about even these mild adverse effects, and very often doctors are asked about a possible relationship between the onset of diarrhea and the administration of the OPV. Thus, we examined the issue in more detail in order to clarify whether or not diarrhea is indeed an adverse effect of the OPV (4).

In order to compare the health status of children who received a check-up only with that of those who received a first or second dose of the OPV, we asked parents to monitor their child's health after a visit to the doctor for a health check-up and to monitor the child's status post-OPV; the same questionnaire was used in both cases.

We conducted the survey in six cities (Sakai, Kanazawa, Adachi, Bunkyo, Matsuyama, and Echizen) from November 2005 to March 2006. In these cities, the OPV was delivered via group vaccination. We asked the parents of children who received the OPV or who underwent health check-ups to monitor their children for 2 weeks. The reports were recorded on postcards; 8,700 cards were sent to the OPV group, and 4,130 cards to the health check-up group. The details of this survey are summarized in Table 1.

For the purpose of comparison, the questionnaire was designed to be similar to the post-vaccination health survey conducted by the Ministry of Health, Labour and Welfare. Questions included those regarding the date of fever onset, convulsions, vomiting, diarrhea, and other symptoms.

We excluded those children for whom the observational period was shorter than 2 weeks, those who were more than 3 years old, and those for whom complete relevant information was not provided or available. Moreover, the observation period for children in the health check-up group who received any vaccination within the 2-week period was ended on the day before the vaccination was received.

We adopted three analytical methods. First, we compared

Table 1. Number of postcards sent and rate of returned postcards by area

	OPV group		Health check-up group	
	No. of postcards sent	Rate of returned postcards (%)	No. of postcards sent	Rate of returned postcards (%)
Sakai	1,400	44.36	1,400	35.79
Kanazawa	1,200	55.50	1,200	24.67
Adachi	5,500	41.24	1,000	29.50
Matsuyama	0		360	38.33
Echizen	0		50	58.00
Bunkyo	600	46.00	120	40.00
	8,700	44.03	4,130	31.65

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the incidence rate for each day among the two groups. Second, we estimated the survival function (i.e., the rate of children showing no symptoms in the two groups), and we determined by log-rank test and Wilcoxon's test the null hypothesis showing that the two lines representing the two groups would be identical. Third, when there was a significant difference between the characteristics of the two groups, we estimated the incidence rate of the two groups using Cox's proportional hazard model controlling for differences in characteristics among the two groups, and thus we tested for the potential effects of vaccination. In our estimation, the following explanatory variables were used: vaccination status, age classification, gender, and geographical region. We also examined the first-dose vaccination group versus the health check-up group as well as the second-dose vaccination group versus the health check-up group, and all available samples were included in the analysis.

Table 1 shows the number of returned postcards with the rate of return. A total of 3,831 records were received for the OPV group, and thus the rate of return was approximately 44.0%. For those in the health check-up group, we received 1,307 records, and thus the latter rate of return was approximately 31.7%. The rates of return for each city were as follows: 40.1% for Sakai, 40.1% for Kanazawa, 39.4% for Adachi, 38.3% for Matsuyama, 58.0% for Echizen, and 45.0% for Bunkyo.

Table 2 summarizes the descriptive statistics. There appears to have been no gender bias in either group, and we confirmed this using a t test (where probability under the null hypothesis = 0.421). Conversely, we found a significant difference in age (probability under the null hypothesis <0.0005). Therefore, to the need to control for the effect of age was established.

The survival function for those lacking symptoms and the 95% confidence interval (CI) for the two groups were calculated for the following symptoms: fever (Fig. 1), vomiting (Fig. 2), and diarrhea (Fig. 3). The statistical test for these survival functions indicates that there was a significant difference among the two groups only in the category of diarrhea.

We also calculated the survival function of those showing no symptoms according to each dose of OPV, i.e., for the first dose (Fig. 4) as well as the second dose (Fig. 5).

Table 3 shows the estimated results of Cox's proportional hazard model for diarrhea. The estimated numbers of each variable represent the odds ratio comparisons with a default status, i.e., a 3- to 5-month-old boy in Sakai who was seen for a health check-up. We found that for diarrhea, the odds ratio of the OPV group compared to the health check-up group was 1.776 (95% CI, 1.274-2.476). We also estimated Cox's proportional hazard model by each symptom.

There were no statistical differences in terms of the incidence of fever, convulsions, or vomiting among the OPV and health check-up groups; thus children in both groups showed similar incidence rates. However, the OPV group had a statistically significant higher incidence of diarrhea than the health check-up group. Since diarrhea appears to be rare in wild-type poliovirus-infected patients, post-OPV diarrhea has been considered to be either a coincidental event or caused by

Table 2. Descriptive statistics

		Total (n = 4,794) N (%)	OPV group (n = 3,579) N (%)	Health check-up group (n = 1,215) N (%)
Gender	Male	2,406 (50.19)	1,809 (50.54)	597 (49.14)
	Female	2,388 (49.81)	1,770 (49.46)	618 (50.86)
Age	3-5 m	777 (16.21)	171 (4.78)	606 (49.88)
	6-8 m	872 (18.19)	865 (24.17)	7 (0.58)
	9-11 m	864 (18.02)	843 (23.55)	21 (1.73)
	1 y	2,145 (44.74)	1,565 (43.73)	580 (47.74)
	2 y	136 (2.84)	135 (3.77)	1 (0.08)

"N" denotes the number of samples in the below analysis. Probability for t test under the null hypothesis that gender distributions are the same in the two groups is 0.421. Conversely, probability for t test for equal average age is less than 0.0005.

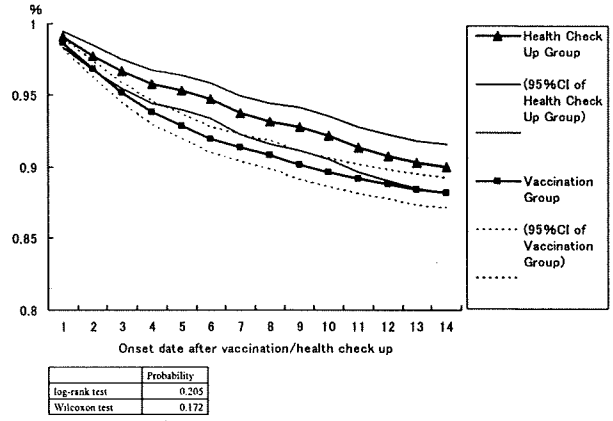

Fig. 1. Survival function for fever.

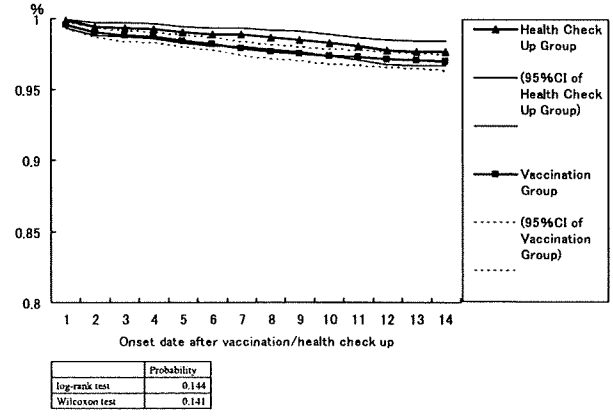

Fig. 2. Survival function for vomiting.

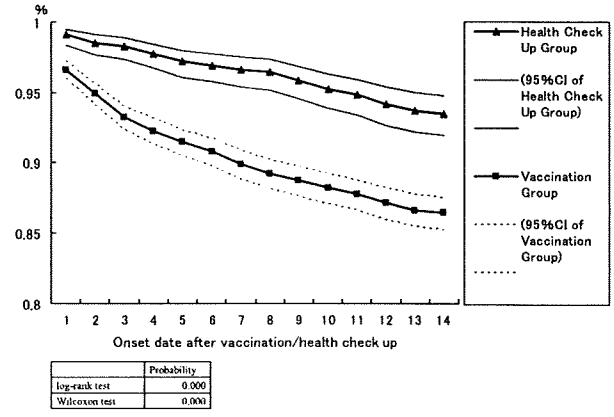

Fig. 3. Survival function for diarrhea.

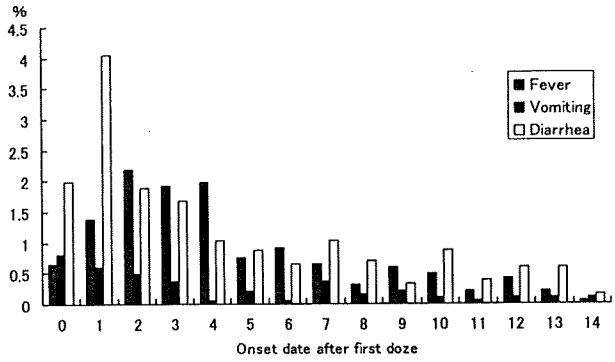

Fig. 4. Onset date for fever, vomiting, and diarrhea after first dose of polio vaccination.

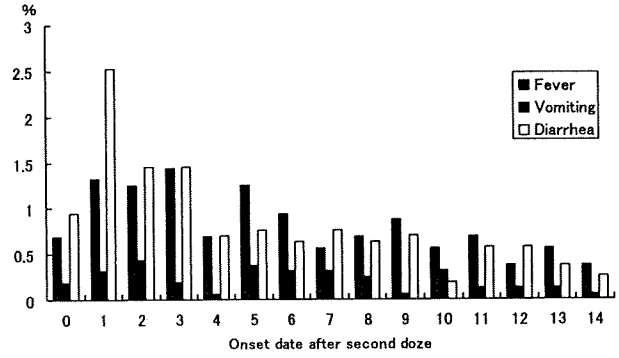

Fig. 5. Onset date for fever, vomiting, and diarrhea after second dose of polio vaccination.

contact during a visit to a doctor and/or with other people. Our results show a statistically significant higher incidence rate in the OPV group, and thus clearly refutes previous assumptions. However, it remains unclear whether or not the present cases of diarrhea were caused by the virus itself in the OPV or by another component of the vaccine.

Another potential explanation could be the use of different inclusion criteria for the vaccination and health check-up groups. Namely, parents may be more sensitive to adverse effects after a vaccination than they are after a regular health check-up, and thus they may be more vigilant about their children's health and in turn adopt a lower threshold for reporting certain symptoms. In this survey, we did not ask participants about the severity of symptoms, and aside from body temperature, no information was collected via objective measures. Moreover, it is quite difficult to comparatively evaluate the care with which parents assess a child's health status. Therefore, sensitivity may have differed among the two groups. A double-blind test using a placebo vaccine without the live poliovirus would be needed to overcome this limitation of the analysis. However, as previously indicated (5), such a study would require clearance by an ethics board, would be difficult to carry out, and its benefit might remain limited.

Table 3. Estimation result of Cox's proportional hazard model for diarrhea

	Odds ratio	Probability	95% confidence interval
OPV	1.776	0.001	1.274-2.476
6-8 m	1.782	0.006	1.181-2.688
9-11 m	1.991	0.001	1.325-2.991
1 y	1.491	0.035	1.028-2.164
2 y	1.074	0.836	0.546-2.113
Female	1.015	0.866	0.858-1.200
Kanazawa	1.057	0.704	0.793-1.409
Adachi	1.158	0.232	0.910-1.474
Matsuyama	1.555	0.178	0.818-2.955
Echizen	3.036	0.013	1.265-7.286
Bunkyo	0.992	0.969	0.669-1.471
First-OPV dose	1.903	0.002	1.273-2.844
6-8 m	1.666	0.022	1.077-2.576
9-11 m	2.037	0.001	1.316-3.152
1 y	1.519	0.04	1.019-2.264
2 y	1.750	0.446	0.415-7.383
Female	1.182	0.118	0.958-1.457
Kanazawa	0.706	0.067	0.486-1.025
Adachi	1.113	0.48	0.827-1.498
Matsuyama	1.371	0.341	0.716-2.624
Echizen	2.726	0.03	1.103-6.737
Bunkyo	0.820	0.445	0.492-1.365
Second-OPV dose	1.701	0.007	1.155-2.505
9-11 m	1.413	0.323	0.712-2.805
1 y	1.744	0.029	1.059-2.869
2 y	1.149	0.736	0.512-2.58
Female	0.861	0.229	0.674-1.099
Kanazawa	1.413	0.077	0.963-2.074
Adachi	1.084	0.652	0.763-1.541
Matsuyama	1.560	0.191	0.801-3.04
Echizen	4.603	0.002	1.791-11.835
Bunkyo	1.132	0.665	0.646-1.983

REFERENCES

1. Committee of Research for Adverse Effect and Health Condition after Vaccination (2005): Report for Research and Summarize of Health Condition after Vaccination. p. 201-202. Division of Tuberculosis and Infectious Disease, Ministry of Health, Labour and Welfare (in Japanese).
2. Centers for Disease Control and Prevention (2006): Chapter 8. Poliomyelitis. Epidemiology and Prevention of Vaccine-Preventable Diseases. The Pink Book. 9th ed.
3. Plotkin, S.A. and Orenstein, W.A. (ed). (2003): Vaccines. 4th ed. W. B. Saunders Co., Philadelphia.
4. Gold, M., Goodwin, H., Botham, S., et al. (2000): Re-vaccination of 421 children with a past history of an adverse vaccine reaction in a special immunisation service. Arch. Dis. Child., 83, 128-131.
5. Rooyen, C.E., Rideout, V.K., Ozere, R.L., et al. (1962): Oral vaccination against poliomyelitis: report of a field trial at Wedgeport, Nova Scotia. Can. Med. Assoc. J., 86, 1185-1191.

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ORIGINAL ARTICLE

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Rapid detection of eight causative pathogens for the diagnosis of bacterial meningitis by real-time PCR

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Abstract We aimed to detect causative pathogens in cerebrospinal fluid (CSF) collected from patients diagnosed with bacterial meningitis by real-time polymerase chain reaction (PCR). In addition to *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Mycoplasma pneumoniae* described previously, five other pathogens, *Neisseria meningitidis, Escherichia coli, Streptococcus agalactiae*, *Staphylococcus aureus*, and *Listeria monocytogenes*, were targeted, based on a large-scale surveillance in Japan. Results in CSF from neonates and children (n = 150), and from adults (n = 18) analyzed by real-time PCR with molecular beacon probes were compared with those of conventional culturing. The total time from DNA extraction from CSF to PCR analysis was 1.5 h. The limit of detection for these pathogens ranged from 5 copies to 28 copies per tube. Nonspecific positive reactions were not recognized for 37 microorganisms in clinical isolates as a negative control. The pathogens were detected in 72.0% of the samples by real-time PCR, but in only 48.2% by culture, although the microorganisms were completely concordant. With the real-time PCR, the detection rate of *H. influenzae* from CSF was high, at 45.2%, followed by *S. pneumoniae* (21.4%), *S. agalactiae* (2.4%), *E. coli* (1.8%), *L. monocytogenes* (0.6%), and *M. pneumoniae* (0.6%). The detection rate with PCR was significantly better than that with cultures in patients with antibiotic administration (χ^2 = 18.3182; P = 0.0000). In conclusion, detection with real-time PCR is useful for rapidly identifying the causative pathogens of meningitis and for examining the clinical course of chemotherapy.

Key words Real-time PCR · Bacterial meningitis · cerebrospinal fluid(CSF) · Neonate · Adult

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Introduction

Bacterial meningitis is a serious and sometimes fatal infection in both children and adults. The main causative pathogens are *S. pneumoniae*, *Haemophilus influenzae* type b (Hib), and *Neisseria meningitidis*.[1]

The incidence rate and causative pathogens of meningitis vary in various countries due to different social backgrounds. These are heavily affected by: (i) the availability of vaccination against Hib and *S. pneumoniae*, (ii) the availability of a medical insurance system, and (iii) the hygienic and sanitary conditions of each country.

In addition to the introduction of the Hib vaccine in 1987,[2] developed countries in Europe, as well as United States, implemented vaccination with a 7-valent pneumococcal conjugate vaccine (7PCV) against pneumococci in 2000–2001.[3,4] In these countries, the number of meningitis cases due to Hib has decreased dramatically[5,6] and the number of cases of invasive pneumococcal disease has been decreasing gradually.[4,7–10]

In Japan, on the other hand, the incidence rate of bacterial meningitis is estimated to be between 10 and 13 per 100000 in children aged less than 5 years.[11] According to the 2005 and 2006 large-scale surveillance carried out by Sunakawa et al.,[12] 55% of these cases were caused by Hib and 19.5% by *S. pneumoniae*. For meningitis in neonates and infants aged 3 months or less, *Escherichia coli* (2.5%) and *S. agalactiae* (7.7%) were the dominant pathogens.

Among these causative pathogens, the resistance of Hib and *S. pneumoniae* to therapeutic antibiotics has rapidly

increased from about 2000 and has became a topic of controversy in the clinic.[13–16] β-Lactamase-nonproducing ampicillin-resistance (BLNAR) Hib accounts for 40% of these cases, and 35% of *S. pneumoniae* cases were penicillin-resistant *S. pneumoniae* (PRSP) in 2007. The resistance mechanism in BLNAR originated from some mutations of the *ftsI* gene, encoding penicillin-binding protein 3, that mediate septal peptidoglycan synthesis.[17]

In 2007, Hib vaccination was finally approved by the government in Japan, but approval has not yet been granted for 7PCV. Considering this situation, it is desirable to create rapid detection methods for causative pathogens in patients diagnosed with meningitis, to allow for the proper selection of chemotherapeutic agents.

Multiplex real-time PCR for simultaneously detecting *S. pneumoniae*, Hib, and *N. meningitidis* was previously reported by Corless et al.[18] In addition to these pathogens, a single identification system for *S. agalactiae*[19] and *Mycobacterium tuberculosis*[20] has been described, but a detection system that covers bacterial meningitis in neonates to adults has not been developed yet.

In the present study, we aimed to develop a real-time PCR that could simultaneously detect eight pathogens; namely, in addition to *S. pneumoniae*, *H. influenzae*, and *N. meningitides*, *E. coli*, *S. agalactiae,* and *Staphylococcus aureus,* which are the major causative pathogens in neonatal meningitis; and *Listeria monocytogenes* and *Mycoplasma pneumoniae*, which are rarely the causative pathogens.

We report an identification system using real-time PCR with pathogen-specific molecular beacon (MB) probes and primers for eight meningitis pathogens; we also describe the results when applied to cerebrospinal fluid (CSF) assay, together with the results of conventional culturing.

Methods

Clinical samples

A total of 168 CSF samples collected from patients who were diagnosed with bacterial meningitis, based on clinical symptoms, CSF findings, and blood examination testing, were sent to our laboratory for bacterial identification from doctors belonging to medical institutions throughout Japan from January 2005 to December 2007. These samples were transported under frozen conditions at –20°C within 24 h of collection. For CSF collection and examination from patients, informed consent was obtained by the doctors in attendance from the parents or the responsible family members.

Bacterial culture and DNA extraction

Upon arrival at our laboratory, the CSF samples were thawed and immediately centrifuged at 10000 rpm for 10 min at 4°C.

From a total 150 μL of sediment, 10 μL of each sample was inoculated onto sheep blood agar and chocolate II agar (Nippon Becton Dickinson, Tokyo, Japan). These plates were then incubated overnight at 37°C in an atmosphere of 5% CO_2. On the following day, if bacterial growth was observed on the plates, the colonies were identified by the standard methods[21] and also their antibiotic susceptibilities were measured.[22]

DNA extraction from 100 μL of the sediment was immediately carried out by using an EXTRAGEN II kit (Tosoh, Tokyo, Japan).[23] Finally, the harvested DNA pellet was resuspended in 40 μL of DNase- and RNase-free H_2O. The time required for the DNA extraction process was within 15 min.

Real-time PCR for bacterial detection

The following eight bacterial pathogens were subjected to the real-time PCR analyses: *E. coli*, *L. monocytogenes*, *N. meningitidis*, *S. agalactiae*, *S. aureus*, *S. pneumoniae*, *H. influenzae*, and *M. pneumoniae*.

Oligonucleotide primers and MB probes were designed using Beacon Designer 2.0 Software (Premier Biosoft International, Palo Alto, CA, USA). The primers, MB probes, target genes, and amplicon sizes (bp) for the eight pathogens are shown in Table 1.

The eight pathogens were grouped in pairs and they were analyzed simultaneously with four tubes. Their combinations were as follows: *S. pneumoniae* (a) and *H. influenzae* (b) in tube A, *E. coli* (a) and *S. agalactiae* (b) in tube B, *N. meningitidis* (a) and *L. monocytogenes* (b) in tube C, and *M. pneumoniae* (a) and *S. aureus* (b) in tube D. The MB probes for detecting pathogens marked (a) were labeled with fluorescent dye, 6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein (HEX), at the 5′-terminal, whereas those marked (b) were labeled with 6-carboxyfluorescein (FAM). All MB probes were labeled with black hole quencher 1 (BHQ-1) at the 3′-terminal.

The PCR reaction mixture consisted of: (i) 25 μL of 2×Real-time PCR Master Mix (Toyobo, Tokyo, Japan), (ii) 0.2 μM of each primer, and (iii) 0.3 μM of each MB probe, and the final volume of the mixture was adjusted to 50 μL by the addition of DNase- and RNAase-free H_2O. Four reaction mixtures were pipetted into four wells of six-tube strip, and two of the remaining wells were used as positive and negative controls. The strip was filled with reaction reagents and stored at –30°C until used. The frozen PCR reagent, when it was used for assays, was thawed on ice and 2 μL of each DNA sample from CSF was added to each well.

After that, real-time PCR was performed immediately with Stratagene Mx3000P (Stratagene, La Jolla, CA, USA). The PCR conditions were as follows: an initial DNA denaturation step of 95°C for 30 s, followed by 40 cycles of 95°C for 15 s, 50°C for 30 s and 75°C for 20 s, and at 75°C for 30 s, successively. *S. pneumoniae* chromosomal DNA was used in each assay as a positive control.

The time required for the whole process from DNA extraction to the end of the real-time PCR operation was 1.5 h.

Table 1. Primers and probes for real-time PCR

Tube (paired)	Species, primer, and probe	Primer or probe[a] sequence	Target gene	Amplicon size (bp)	Reference
A	*S. pneumoniae*				
	Sense primer	5′-CAACCGTACAGAATGAAGCGG-3′	*lytA*	319	23
	Reverse primer	5′-TTATTCGTGCAATACTCGTGCG-3′			
	Probe	HEX-CGCGATCAGGTCTCAGCATTCCAACCGCCGATCGCG-BHQ1			
A	*H. influenzae*				
	Sense primer	5′-TTGACATCCTAAGAAGAGCTC-3′	16S rRNA	167	23
	Reverse primer	5′-TCTCCTTTGAGTTCCCGACCG-3′			
	Probe	FAM-CGCGATCCTGACGACAGCCATGCAGCACGATCGCG-BHQ1			
B	*E. coli*				
	Sense primer	5′-GGGAGTAAAGTTAATACCTTTGC-3′	16S rRNA	204	This study
	Reverse primer	5′-CTCAAGCTTGCCAGTATCAG-3′			
	Probe	HEX-CGCGATCACTCCGTGCCAGCAGCCGCGGATCGCG-BHQ1			
B	*S. agalactiae*				
	Sense primer	5′-AGGAATACCAGGCGATGAAC-3′	*dltS*	331	This study
	Reverse primer	5′-AGGCCCTACGATAAATCGAG-3′			
	Probe	FAM-CGCGATCATTTGGCTAGTTATGAAGTCCCTTATGCGATCGCG-BHQ1			
C	*N. meningitidis*				
	Sense primer	5′-CATATCGGAACGTACCGAGT-3′	16S rRNA	356	This study
	Reverse primer	5′-GCCGCTGATATTAGCAACAG-3′			
	Probe	HEX-CGCGATCCTATTCGAGCGGCCGATATCGATCGCG-BHQ1			
C	*L. monocytogenes*				
	Sense primer	5′-CGCTTTTGAAAGATGGTTTCG-3′	16S rRNA	457	This study
	Reverse primer	5′-CTTCCAGTTTCCAATGACCC-3′			
	Probe	FAM-CGCGATCGCGGCGTTGCTCCGTCAGACTTGATCGCG-BHQ1			
D	*M. pneumoniae*				
	Sense primer	5′-GTAATACTTTAGAGGCGAACG-3′	16S rRNA	225	23
	Reverse primer	5′-TACTTCTCAGCATAGCTACAC-3′			
	Probe	HEX-CGCGATACCAACTAGCTGATATGGCGCAATCGCG-BHQ1			
D	*S. aureus*				
	Sense primer	5′-TACATGTCGTTAAACCTGGTG-3′	*spa*	224	This study
	Reverse primer	5′-TACAGTTGTACCGATGAATGG-3′			
	Probe	FAM-CGCGATCCAAGAACTTGTTGTTGATAAGAAGCAACCGATCGCG-BHQ1			

[a] Stem oligonucleotides are underlined

Sensitivity and specificity of real-time PCR

The sensitivity of the present real-time PCR procedure was determined for the five pathogens: *E. coli*, *L. monocytogenes*, *N. meningitidis*, *S. agalactiae*, and *S. aureus*. The sensitivity for *S. pneumoniae*, *H. influenzae*, and *M. pneumoniae* had already been examined in our previous study.[23] The procedure was performed with three strains each from the five species by tenfold serial dilutions of bacterial cells from 10^8 to 10^0/mL.

The specificity of the MB probes and primers was tested with 37 Gram-positive and -negative microorganisms in clinical isolates in addition to the eight targeted bacteria. The species are listed in Table 2.

Results

Sensitivity and specificity of real-time PCR

The threshold cycle (Ct) value for a positive result was defined as the point at which the horizontal threshold line was crossed. The sensitivities of the real-time PCR assay for the five pathogens, *E. coli*, *L. monocytogenes*, *N. meningitidis*, *S. agalactiae*, and *S. aureus*, are shown in Table 3. The limits of detection per reaction tube were 2 DNA copies for *E. coli*, 16 copies for *L. monocytogenes*, 2 copies for *N. meningitidis*, 28 copies for *S. agalactiae*, and 14 copies for *S. aureus*. A significant correlation was found between the tenfold diluted bacterial cell counts and the Ct values, ranging from $\gamma = 0.9709$ in *L. monocytogenes* to $\gamma = 0.9989$ in *N. meningitidis*.

Although details of the results are not shown here, the sensitivities of the remaining three pathogens have previously been revealed to be two DNA copies for *S. pneumoniae*, ten copies for *H. influenzae*, and five copies for *M. pneumoniae*.[23]

The specificities of the 5-MB probe and primer sets were examined for 37 Gram-positive and -negative microorganisms selected from clinical strains as negative controls. Nonspecific positive reactions were undetectable after 40 cycles in the present real-time PCR procedure.

Comparisons of results between real-time PCR and bacterial culture

Table 4 shows the details of the causative pathogens identified by real-time PCR and those confirmed by culturing from the CSF samples ($n = 168$) sent to our laboratory.

Among the real-time PCR-positive cases, *H. influenzae* was detected at the highest incidence of 76 cases (45.2%), followed by *S. pneumoniae* in 36 cases (21.4%), *S. agalactiae* in 4 cases (2.4%), *E. coli* in 3 cases (1.8%), and *L. monocytogenes* (0.6%) and *M. pneumoniae* (0.6%) in 1 case each. There were no positive cases of *N. meningitidis* or *S. aureus* identified during the study periods.

For bacterial culturing, *H. influenzae* was isolated in 48 cases (28.6%), *S. pneumoniae* in 27 cases (16.1%), *S. agalactiae* in 2 cases (1.2%), *E. coli* in 3 cases (1.8%), and *L. monocytogenes* in 1 case (0.6%).

Ultimately, the causative pathogens were determined in as many as 72.0% of all samples by real-time PCR, but in only 48.2% by bacterial culturing. The microorganisms obtained by bacterial culture and by real-time PCR showed complete concordance. The sensitivity and specificity of the real-time PCR were calculated as 100% and 54.0%, respectively. However, this specificity does not reflect the true percentage, because in many cases with a negative culture an antibiotic had been prescribed before the bacterial cultivation of the CSF.

Table 2. Specificity panel: amplification-negative-control organisms

Genus	Species
Streptococcus	*S. dysagalactiae* subsp. *equisimilis*, *S. mitis*, *S. milleri*, *S. salibarius*, *S. oralis*, *S. mutans*, *S. sanguis*, *S. bovis*
Enterococcus	*E. faecalis*, *E. faecium*, *E. avium*
Staphylococcus	*S. epidermidis*, *S. haemolyticus*
Moraxella	*M. catarrhalis*
Haemophilus	*H. parainfluenzae*, *H. haemolyticus*
Pseudomonas	*P. aeruginosa*
Klebsiella	*K. pneumoniae*, *K. oxytoca*
Pantoea	*P. agglomerans*
Proteus	*P. mirabilis*
Serratia	*S. marcescens*
Acinetobacter	*A. calcoaceticus*
Enterobacter	*E. cloacae*
Citrobacter	*C. freundii*
Mycoplasma	*M. orale*, *M. hominis*, *M. salivarium*
Cryptococcus	*C. neoformans*

Table 3. Sensitivities for six pathogens identified by real-time PCR

No. of DNA copies/50 μL of reaction tube	Threshold cycle (Ct)				
	E. coli	*L. monocytogenes*	*N. meningitidis*	*S. agalactiae*	*S. aureus*
10^5	18	21	16	26	26
10^4	21	24	20	29	28
10^3	25	28	24	33	31
10^2	28	31	27	36	34
10^1	31	>40	31	>40	>40
Correlation coefficient[a]	0.9987	0.9709	0.9989	0.9988	0.9783

[a]Each value was calculated between the 10 fold diluted bacterial calls and the Ct values

Table 4. Causative pathogens identified by real-time PCR and by culture of the CSF samples (n = 168)

Causative pathogen	PCR (%)		Culture (%)	
	No. of positive (%)	subtotal (%)	No. of positive (%)	subtotal (%)
S. pneumoniae	36 (21.4)	121 (72.0)	27 (16.1)	81 (48.2)
H. influenzae	76 (45.2)		48 (28.6)	
S. agalactiae	4 (2.4)		2 (1.2)	
E. coli	3 (1.8)		3 (1.8)	
L. monocytogenes	1 (0.6)		1 (0.6)	
M. pneumoniae	1 (0.6)		0	
Not detected		47 (28.0)		87 (51.8)

*Sensitivity and specificity of the real-time PCR was calculated 100% and 54.0%, respectively; PCR and culture both positive (n = 81), PCR and culture both negative (n = 47), PCR negative and culture positive (n = 0), PCR positive and culture negative (n = 40)

Table 5. Relationship between positive identification of pathogens in meningitis by real-time PCR and age of the patients

Causative pathogen	n[a]	Pediatrics (n = 106)							Subtotal	Adults (n = 15)				Subtotal
		≤3 m	4–11 m	1 y	2 y	3 y	4 y	5–17 y		18–34 y	35–49 y	50–64 y	>65 y	
positive case														
S. pneumoniae	36	2	6	3	2	3	1	5	22	1	3	6	4	14
H. influenzae	76	3	34	14	7	9	4	5	76					
S. agalactiae	4	3							3		1			1
E. coli	3	2	1						3					
L. monocytogenes	1							1	1					
M. pneumoniae	1			1					1					
Subtotal	121	10	41	18	9	12	5	11	106	1	4	6	4	15
negative case	47	11	13	2	1	1	1	15	44	1		1	1	3

[a]Number of real-time PCR positive case

Relationship between real-time PCR-positivity and age of the patients

The relationship between positive identification of pathogens by real-time PCR and the age of the meningitis patients is shown in Table 5.

Among pediatric patients aged 17 years or less, a pathogen was suspected in 106 patients (70.7%) by real-time PCR. Five of the 6 patients in whom the pathogen was suspected to be either *E. coli* or *S. agalactiae* were neonates and infants aged 3 months or less. For patients aged between 4 months and 17 years, *H. influenzae* and *S. pneumoniae* were the major pathogens.

Among adult meningitis patients aged 18 years or more, 15 cases (83.3%) were real-time PCR-positive, and most of them were caused by *S. pneumoniae*, with the exception of 1 case caused by *S. agalactiae*.

Influence of prior antibiotic use

The relationship between a history of antibiotic use prior to CSF collection and the pathogen-positive rate by real-time PCR or culturing was analyzed in 115 patients for whom a history of antibiotic use could be accurately followed up.

As shown in Fig. 1, 62 patients (53.9%) had received antibiotics prior to hospital admission. Fifteen patients had received an injection and 47 patients had been treated by oral administration. In these 62 patients, the causative pathogens were identified by culturing in only 18 patients (29.0%) and by real-time PCR in 36 patients (58.1%).

In the 53 patients without a history of antibiotic administration, causative pathogens were detected by culturing in 37 patients (69.8%) and by real-time PCR in 47 patients (88.7%).

Regarding the detection rate of causative pathogens, real-time PCR was significantly better than culturing both in patients with antibiotic administration ($\chi^2 = 18.3182$; $P = 0.0000$) and those without antibiotic administration ($\chi^2 = 12.1338$; $P = 0.0005$) prior to the evaluation.

Of the 32 patients for whom a causative pathogen was not detected by either culturing or real-time PCR, 26 patients (81.3%) had previously received antibiotics.

Discussion

In bacterial meningitis, rapid and accurate diagnosis is essential for the appropriate selection of chemotherapeutic agents to be used against the putative pathogens in a timely manner. Causative pathogens in such patients are usually estimated by Gram staining or agglutination testing of CSF upon hospitalization. We frequently encounter patients, however, in whom it is difficult to estimate the causative pathogen due to previous treatment with an antibacterial agent.

Considering such a situation, studies applying real-time PCR, which is becoming more advanced, have been reported

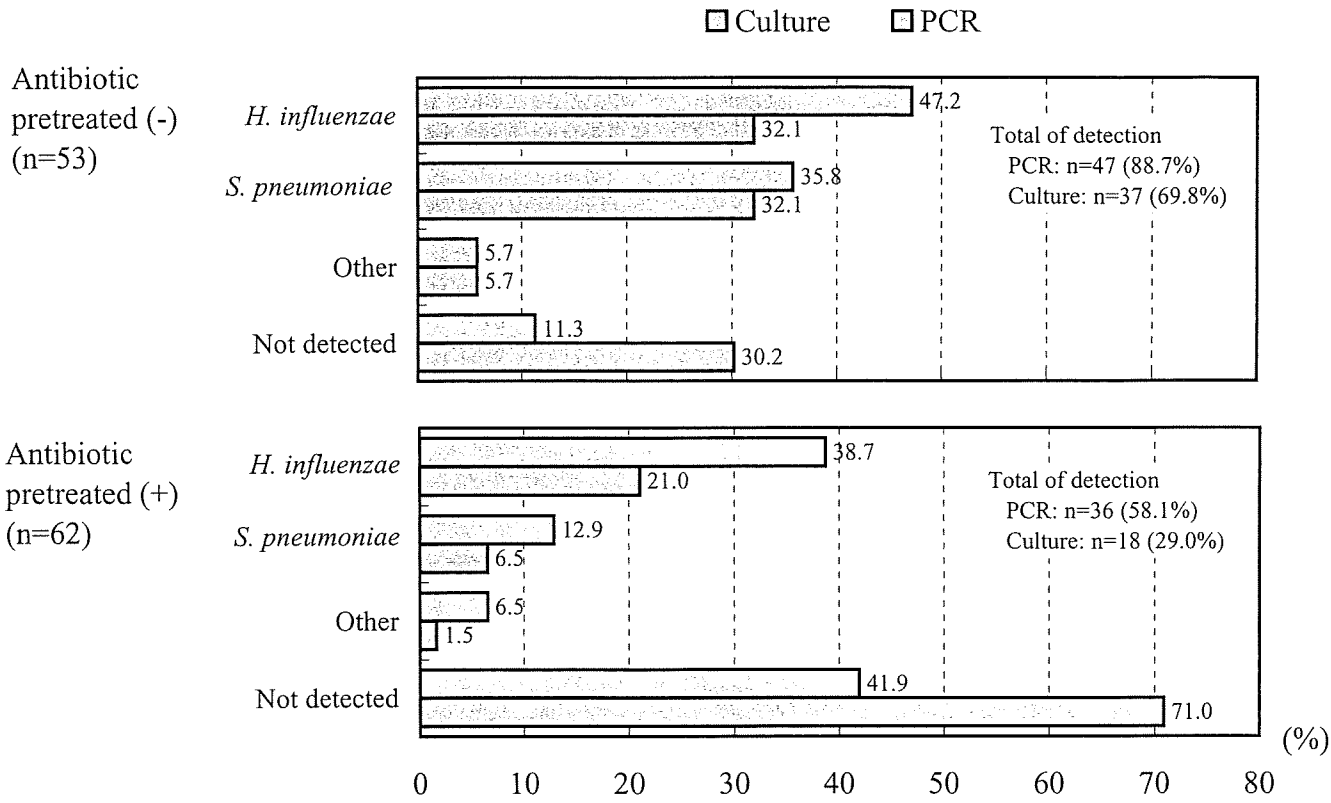

Fig. 1. Influence of prior antibiotics on the detection of causative pathogens by real-time PCR or culturing

for the detection of causative pathogens in meningitis.[18,24–28] In particular, multiplex real-time PCR, for the identification of three bacterial species, *S. pneumoniae*, *H. influenzae*, and *N. meningitidis*, is noteworthy.[18] This technique is beneficial for the rapid identification of a causative pathogen with high sensitivity and specificity.

Distributions of causative pathogens of meningitis and their mortality rates vary significantly among countries, however, owing to different levels of infrastructure development, such as the availability of vaccination and a medical insurance system, and the hygienic and sanitary conditions in each country.

According to a recent large-scale survey conducted in Japan,[12] *S. agalactiae* and *E. coli* are the most dominant pathogens for meningitis in infants aged 3 months or less, and only rarely is meningitis caused by *S. aureus* or the *Enterobacteriaceae* family. In contrast, Hib (55%) and *S. pneumoniae* (19.5%) are reported to be the major causative pathogens in meningitis cases in children aged 4 months or more, followed by *L. monocytogenes*, *N. meningitidis*, Gram-negative bacilli, and some other bacterial species. This high dominance of Hib as a causative pathogen reflects the situation in Japan that the Hib vaccine had not been approved by the Ministry of Health, Labor and Welfare until 2007.

Based on the frequencies of these meningitis pathogens, as described above, we aimed to develop a real-time PCR that could also be suitable for identifying suspected meningitis pathogens in infants. Although this real-time PCR is limited to the detection of eight causative pathogens, we designed it to assay two different bacterial species simultaneously in one tube to avoid decreasing the sensitivity of the species. In 98.3% of cases with a positive real-time PCR result the pathogen could be detected using two reaction tubes, tube A for *S. pneumoniae* and *H. influenzae*, and tube B for *E. coli* and *S. agalactiae*.

Additionally, as described in the "Results" section, the detection rate of the real-time PCR was significantly higher, at 72.0% of all 168 CSF samples, compared with that of culturing, at 48.2%. These performance results of real-time PCR can be considered satisfactory for the detection of causative pathogens in cases diagnosed as bacterial meningitis.

Although the results are not shown here, a second-stage PCR assay was performed to detect antibiotic resistance genes, using the remaining DNA samples obtained from CSF, when *H. influenzae* or *S. pneumoniae* was suspected as the causative pathogen. More specifically, the assay for *H. influenzae* aimed to detect the β-lactamase gene, PBP3 gene, to identify BLNAR and capsule type b.[29] In cases where *S. pneumoniae* was suspected, the presence or absence of an abnormality in each of three genes encoding PBP1A, PBP2X, and PBP2B, which affect a decrease in β-lactam susceptibility, was investigated.[14]

As we previously reported, the antibiotic susceptibility of causative pathogens can be estimated by the 90% minimum inhibitory concentration (MIC_{90}) values once the resistance genes are revealed, because MIC_{90} is statistically calculated based on the relationship between gene mutations and antibiotic susceptibility.[29,30] The time required for

identifying resistance genes is 3.0 h, including the initial 1.5 h for the process from receiving the samples to detecting the causative pathogen by the real-time PCR. The ability to reveal resistance genes is hugely beneficial when determining the appropriateness of an antibiotic.

According to the *Practice guidelines for bacterial meningitis*,[31] which were published in consideration of the current situation of bacterial resistance in Japan, the carbapenem antibiotic, panipenem, is recommended for PRSP meningitis, whereas the concomitant use of meropenem and either cefotaxime or ceftriaxone is preferred for Hib meningitis.

In the future, diagnosis by the real-time PCR presented in this article also seems promising for the treatment of severe invasive infections in addition to meningitis.

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References

1. World Health Organization. State of the art of new vaccines: research and development. Geneva, Switzerland: WHO; 2005.
2. Ward JI, Broome CV, Harrison LH, Shinefield H, Black S. *Haemophilus influenzae* type b vaccines: lessons for the future. Pediatrics 1988;81:886–93.
3. Advisory Committee on Immunization Practices. Preventing pneumococcal disease among infants and young children. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 2000;49:1–35.
4. Reinert RR. Pneumococcal conjugate vaccines – a European perspective. Int J Med Microbiol 2004;294:277–94.
5. Centers for Disease Control and Prevention. Progress toward eliminating *Haemophilus influenzae* type b disease among infants and children – United States, 1987–1997. MMWR Morb Mortal Wkly Rep 1998;47:993–8.
6. Olowokure B, Spencer NJ, Hawker JI, Blair I, Smith RL. Invasive *Haemophilus influenzae* disease: an ecological study of sociodemographic risk factors before and after the introduction of Hib conjugate vaccine. Eur J Epidemiol 2003;18:363–7.
7. Centers for Disease Control and Prevention. Direct and indirect effects of routine vaccination of children with 7-valent pneumococcal conjugate vaccine on incidence of invasive pneumococcal disease – United States, 1998–2003. MMWR Morb Mortal Wkly Rep 2005;54:893–7.
8. Lexau CA, Lynfield R, Danila R, Pilishvili T, Facklam R, Farley MM, et al. Changing epidemiology of invasive pneumococcal disease among older adults in the era of pediatric pneumococcal conjugate vaccine. JAMA 2005;294:2043–51.
9. Black S, France EK, Isaacman D, Bracken L, Lewis E, Hansen J, et al. Surveillance for invasive pneumococcal disease during 2000–2005 in a population of children who received 7-valent pneumococcal conjugate vaccine. Pediatr Infect Dis J 2007;26:771–7.
10. Poehling KA, Talbot TR, Griffin MR, Craig AS, Whitney CG, Zell E, et al. Invasive pneumococcal disease among infants before and after introduction of pneumococcal conjugate vaccine. JAMA 2006;295:1668–74.
11. Kamiya H, Uehara S, Kato T, Shiraki K, Togashi T, Morishima T, et al. Childhood bacterial meningitis in Japan. Pediatr Infect Dis J 1998;17:S183–5.
12. Sunakawa K, Ubukata K, Chiba N, Hasegawa K, Nonoyama M, Iwata S, et al. Childhood bacterial meningitis trends in Japan from 2005 to 2006. Kansenshogaku Zasshi 2008;82:187–97.
13. Ubukata K. Problems associated with high prevalence of multidrug-resistant bacteria in patients with community-acquired infections. J Infect Chemother 2003;9:285–91.
14. Ubukata K, Chiba N, Hasegawa K, Kobayashi R, Iwata S, Sunakawa K. Antibiotic susceptibility in relation to penicillin-binding protein genes and serotype distribution of *Streptococcus pneumoniae* strains responsible for meningitis in Japan, 1999 to 2002. Antimicrob Agents Chemother 2004;48:1488–94.
15. Hasegawa K, Kobayashi R, Takada E, Ono A, Chiba N, Morozumi M, et al. High prevalence of type b beta-lactamase-non-producing ampicillin-resistant *Haemophilus influenzae* in meningitis: the situation in Japan where Hib vaccine has not been introduced. J Antimicrob Chemother 2006;57:1077–82.
16. Hasegawa K, Chiba N, Kobayashi R, Murayama SY, Iwata S, Sunakawa K, et al. Rapidly increasing prevalence of beta-lactamase-nonproducing, ampicillin-resistant *Haemophilus influenzae* type b in patients with meningitis. Antimicrob Agents Chemother. 2004;48:1509–14.
17. Ubukata K, Shibasaki Y, Yamamoto K, Chiba N, Hasegawa K, Takeuchi Y, et al. Association of amino acid substitutions in penicillin-binding protein 3 with beta-lactam resistance in beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. Antimicrob Agents Chemother 2001;45:1693–9.
18. Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, Kaczmarski EB. Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. J Clin Microbiol 2001;39:1553–8.
19. Ke D, Menard C, Picard FJ, Boissinot M, Ouellette M, Roy PH, et al. Development of conventional and real-time PCR assays for the rapid detection of group B streptococci. Clin Chem 2000;46:324–31.
20. Takahashi T, Nakayama T. Novel technique of quantitative nested real-time PCR assay for *Mycobacterium tuberculosis* DNA. J Clin Microbiol 2006;44:1029–39.
21. Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA, editors. Manual of clinical microbiology. 9th ed. Washington, DC: American Society for Microbiology; 2007.
22. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing, 18th informational supplement. M100-S18. Wayne, PA: CLSI;, 2008.
23. Morozumi M, Nakayama E, Iwata S, Aoki Y, Hasegawa K, Kobayashi R, et al. Simultaneous detection of pathogens in clinical samples from patients with community-acquired pneumonia by real-time PCR with pathogen-specific molecular beacon probes. J Clin Microbiol 2006;44:1440–6.
24. Kearns AM, Graham C, Burdess D, Heatherington J, Freeman R. Rapid real-time PCR for determination of penicillin susceptibility in pneumococcal meningitis, including culture-negative cases. J Clin Microbiol 2002;40:682–4.
25. Bryant PA, Li HY, Zaia A, Griffith J, Hogg G, Curtis N, et al. Prospective study of a real-time PCR that is highly sensitive, specific, and clinically useful for diagnosis of meningococcal disease in children. J Clin Microbiol 2004;42:2919–25.
26. Uzuka R, Kawashima H, Hasegawa D, Ioi H, Amaha M, Kashiwagi Y, et al. Rapid diagnosis of bacterial meningitis by using multiplex PCR and real time PCR. Pediatr Int 2004;46:551–4.
27. van Haeften R, Palladino S, Kay I, Keil T, Heath C, Waterer GW. A quantitative LightCycler PCR to detect *Streptococcus pneumoniae* in blood and CSF. Diagn Microbiol Infect Dis 2003;47:407–14.
28. Deutch S, Moller JK, Ostergaard L. Combined assay for 2-hour identification of *Streptococcus pneumoniae* and *Neisseria meningitidis* and concomitant detection of 16S ribosomal DNA in cerebrospinal fluid by real-time PCR. Scand J Infect Dis 2008;5:1–8.
29. Hasegawa K, Yamamoto K, Chiba N, Kobayashi R, Nagai K, Jacobs MR, et al. Diversity of ampicillin-resistance genes in *Haemophilus influenzae* in Japan and the United States. Microb Drug Resist 2003;9:39–46.
30. Ubukata K, Muraki T, Igarashi A, Asahi Y, Konno M. Identification of penicillin and other beta-lactam resistance in *Streptococcus pneumoniae* by polymerase chain reaction. J Infect Chemother 1997;3:190–7.
31. Committee of Practice Guidelines for Bacterial Meningitis. Practice guidelines for bacterial meningitis. Tokyo: Igakusyoin; 2007.

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

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

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

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

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

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

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

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

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

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

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

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

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

[a] Values in parentheses are percentages.

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

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

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

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

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

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

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

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

[a] Values are in micrograms per milliliter.

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

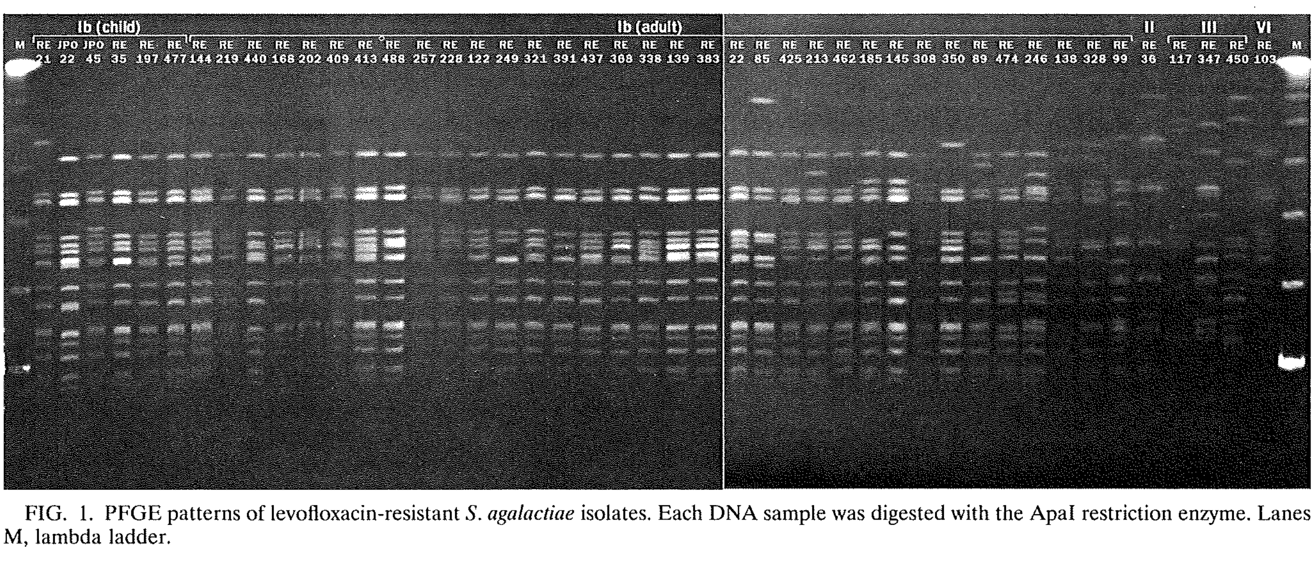

FIG. 1. PFGE patterns of levofloxacin-resistant *S. agalactiae* isolates. Each DNA sample was digested with the ApaI restriction enzyme. Lanes M, lambda ladder.

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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REFERENCES

1. **American Academy of Pediatrics Committee on Infectious Diseases and Committee on Fetus and Newborn.** 1997. Revised guidelines for prevention of early-onset group B streptococcal (GBS) infection. Pediatrics **99**:489–496.
2. **American College of Obstetrics and Gynecologists Committee on Obstetric Practice.** 1996. ACOG committee opinion. Prevention of early-onset group B streptococcal disease in newborns. Number 173-June 1996. Int. J. Gynaecol. Obstet. **54**:197–205.
3. **Baker, C. J., F. F. Barrett, R. C. Gordon, and M. D. Yow.** 1973. Suppurative meningitis due to streptococci of Lancefield group B: a study of 33 infants. J. Pediatr. **82**:724–729.
4. **Barton, L. L., R. D. Feigin, and R. Lins.** 1973. Group B beta hemolytic streptococcal meningitis in infants. J. Pediatr. **82**:719–723.
5. **Benson, J. A., and P. Ferrieri.** 2001. Rapid pulsed-field gel electrophoresis method for group B streptococcus isolates. J. Clin. Microbiol. **39**:3006–3008.
6. **Centers for Disease Control and Prevention.** 2000. From the Centers for Disease Control and Prevention: early-onset group B streptococcal disease—United States, 1998-1999. JAMA **284**:1508–1510.
7. **Centers for Disease Control and Prevention.** 1996. Prevention of perinatal group B streptococcal disease: a public health perspective. MMWR Recommend. Rep. **45**:1–24.
8. **Chiba, N., R. Kobayashi, K. Hasegawa, M. Morozumi, E. Nakayama, T. Tajima, S. Iwata, and K. Ubukata.** 2005. Antibiotic susceptibility according to genotype of penicillin-binding protein and macrolide resistance genes, and serotype of *Streptococcus pneumoniae* isolates from community-acquired pneumonia in children. J. Antimicrob. Chemother. **56**:756–760.
9. **Franciosi, R. A., J. D. Knostman, and R. A. Zimmerman.** 1973. Group B streptococcal neonatal and infant infections. J. Pediatr. **82**:707–718.
10. **Kawamura, Y., H. Fujiwara, N. Mishima, Y. Tanaka, A. Tanimoto, S. Ikawa, Y. Itoh, and T. Ezaki.** 2003. First *Streptococcus agalactiae* isolates highly resistant to quinolones, with point mutations in *gyrA* and *parC*. Antimicrob. Agents Chemother. **47**:3605–3609.
11. **Kimura, K., S. Suzuki, J. Wachino, H. Kurokawa, K. Yamane, N. Shibata, N. Nagano, H. Kato, K. Shibayama, and Y. Arakawa.** 2008. First molecular characterization of group B streptococci with reduced penicillin susceptibility. Antimicrob. Agents Chemother. **52**:2890–2897.
12. **Lindahl, G., M. Stalhammar-Carlemalm, and T. Areschoug.** 2005. Surface proteins of *Streptococcus agalactiae* and related proteins in other bacterial pathogens. Clin. Microbiol. Rev. **18**:102–127.
13. **Miró, E., M. Rebollo, A. Rivera, M. T. Alvarez, F. Navarro, B. Mirelis, and P. Coll.** 2006. *Streptococcus agalactiae* highly resistant to fluoroquinolones. Enferm. Infecc. Microbiol. Clin. **24**:562–563.
14. **Nizet, V., and C. E. Rubens.** 2006. Pathogenic mechanisms and virulence factors of group B streptococci, p. 152–168. *In* V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy, and J. I. Rood (ed.), Gram-positive pathogens, 2nd ed. ASM press, Washington, DC.
15. **Phares, C. R., R. Lynfield, M. M. Farley, J. Mohle-Boetani, L. H. Harrison, S. Petit, A. S. Craig, W. Schaffner, S. M. Zansky, K. Gershman, K. R. Stefonek, B. A. Albanese, E. R. Zell, A. Schuchat, and S. J. Schrag.** 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. JAMA **299**:2056–2065.
16. **Poyart, C., A. Tazi, H. Reglier-Poupet, A. Billoet, N. Tavares, J. Raymond, and P. Trieu-Cuot.** 2007. Multiplex PCR assay for rapid and accurate capsular typing of group B streptococci. J. Clin. Microbiol. **45**:1985–1988.
17. **Schrag, S., R. Gorwitz, K. Fultz-Butts, and A. Schuchat.** 2002. Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC. MMWR Recommend. Rep. **51**:1–22.
18. **Schuchat, A.** 1998. Epidemiology of group B streptococcal disease in the United States: shifting paradigms. Clin. Microbiol. Rev. **11**:497–513.
19. **Ubukata, K., N. Chiba, K. Hasegawa, R. Kobayashi, S. Iwata, and K. Sunakawa.** 2004. Antibiotic susceptibility in relation to penicillin-binding protein genes and serotype distribution of *Streptococcus pneumoniae* strains responsible for meningitis in Japan, 1999 to 2002. Antimicrob. Agents Chemother. **48**:1488–1494.
20. **Ubukata, K., K. Sunaoshi, R. Kobayashi, and K. Okuzumi.** 2006. Large-scale questionnaire surveillance concerning invasive infections with group C and G streptococci. Kansenshogaku Zasshi **80**:480–487.
21. **Wajima, T., S. Y. Murayama, K. Sunaoshi, E. Nakayama, K. Sunakawa, and K. Ubukata.** 2008. Distribution of *emm* type and antibiotic susceptibility of group A streptococci causing invasive and noninvasive disease. J. Med. Microbiol. **57**:1383–1388.
22. **Wehbeh, W., R. Rojas-Diaz, X. Li, N. Mariano, L. Grenner, S. Segal-Maurer, B. Tommasulo, K. Drlica, C. Urban, and J. J. Rahal.** 2005. Fluoroquinolone-resistant *Streptococcus agalactiae*: epidemiology and mechanism of resistance. Antimicrob. Agents Chemother. **49**:2495–2497.
23. **Zangwill, K. M., A. Schuchat, and J. D. Wenger.** 1992. Group B streptococcal disease in the United States, 1990: report from a multistate active surveillance system. MMWR Surveill. Summ. **41**:25–32.

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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 3130/3130x1 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/strepblast.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).

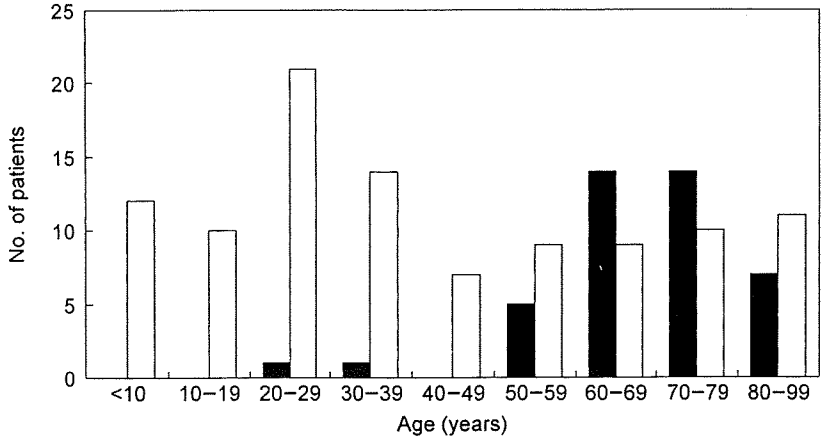

Fig. 1. Age distribution of patients with *Streptococcus dysgalactiae* subsp. *equisimilis* infection. In patients with invasive infection (black bars; n=42), causative agents were isolated from blood (n=32), synovial fluid (n=6) and tissue (n=4). In patients with non-invasive infections (grey bars; 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).

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 $\leqslant$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 $\geqslant$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–$\geqslant$64	0.125	4
Azithromycin	0.5–$\geqslant$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

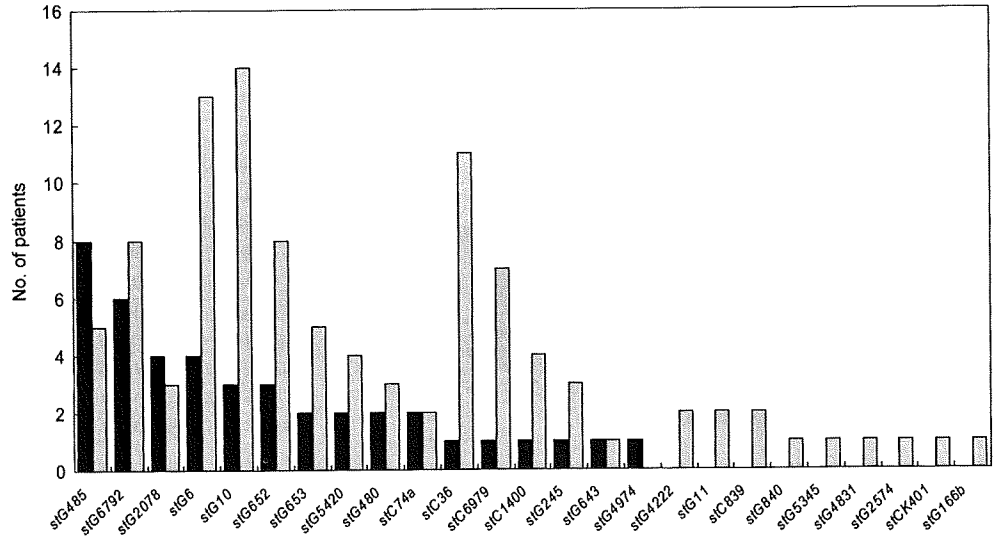

Fig. 2. Comparison of *emm* types in *Streptococcus dysgalactiae* subsp. *equisimilis* isolates separated into invasive (black bars; n=42) and non-invasive (grey bars; n=103) infections. The distribution of *emm* types was significantly different between the two groups (χ^2 test, P<0.001).

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

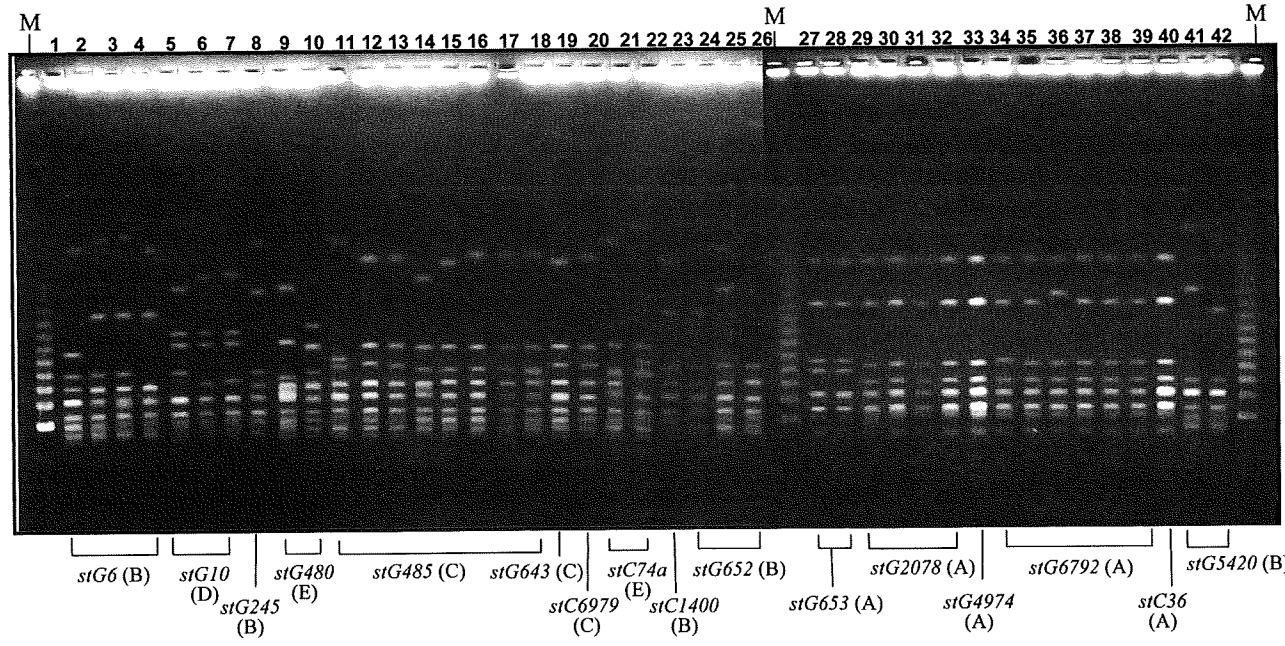

Fig. 3. PFGE profiles and their classification into five clones of chromosomal DNA from *Streptococcus dysgalactiae* subsp. *equisimilis* isolates from patients with invasive infection. Chromosomal DNAs were digested with *Sma*I. Capital letters in parentheses represent clones. Lanes: M, size marker (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.

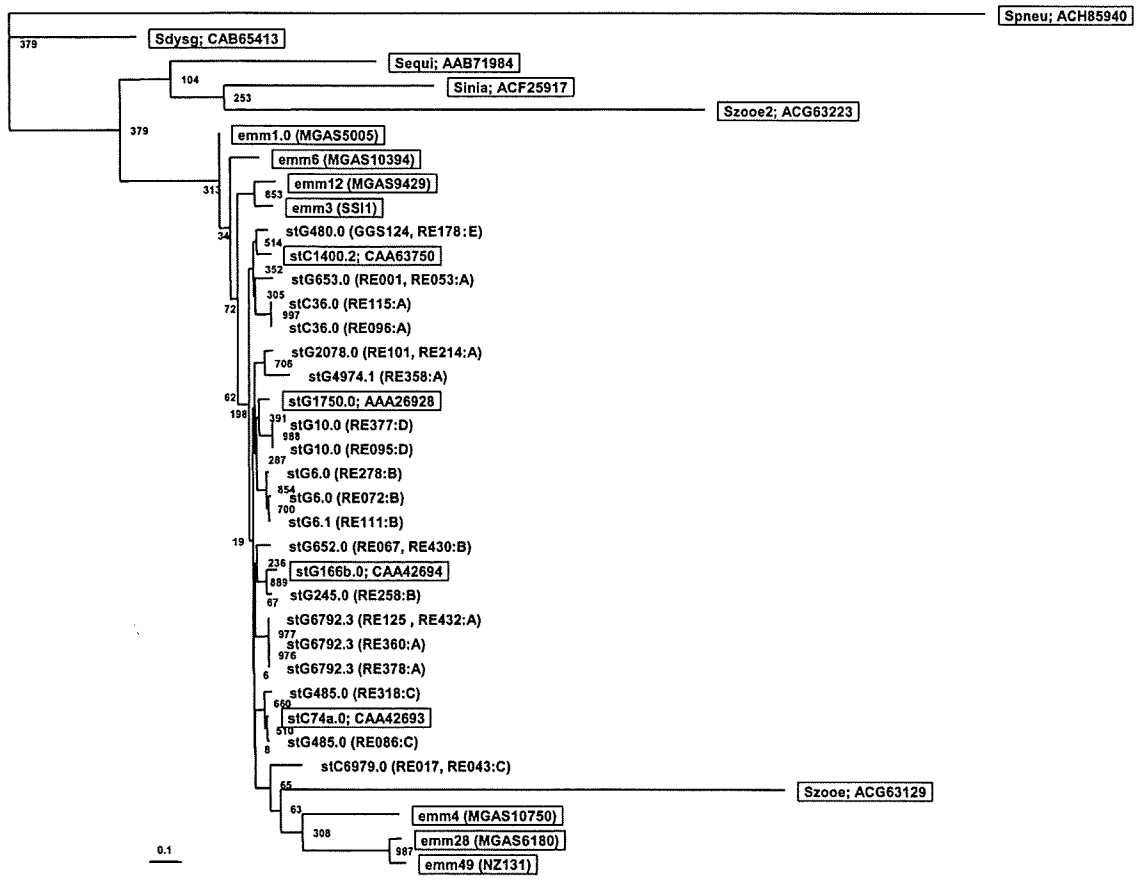

Fig. 4. Phylogenetic tree of the complete M protein in *Streptococcus dysgalactiae* subsp. *equisimilis* ($n=25$) isolated from patients with invasive infections. A phylogenetic tree based on deduced amino acid sequences was constructed by the neighbour-joining method. Bootstrap analyses of 1000 replications 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*.