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Journal of Clinical Microbiology, Apr. 2008, p. 1563–1564
0095-1137/08/$08.00+0 doi:10.1128/JCM.02410-07
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Vol. 46, No. 4

Prevalence of Streptococcus Invasive Locus (*sil*) and Its Relationship with Macrolide Resistance among Group A *Streptococcus* Strains[▽]

A recent study by Bidet et al. (1) reported the molecular epidemiology of the streptococcal invasive locus (*sil*) in the group A streptococcus (GAS), an organism which caused invasive infections in French children. The authors demonstrated the prevalence of *emm* type toxin genotypes among 74 invasive GAS isolates from French children. The authors PCR amplified and characterized the locus DNA of *sil* from invasive isolates, but there were no data concerning noninvasive isolates. It seems that the invasive locus was present not only in invasive isolates but possibly also in noninvasive isolates. Therefore, we conducted a study in which our aims were (i) to examine the prevalence of *Streptococcus pyogenes* exotoxins in relationship to the *sil* gene in invasive and noninvasive isolates of GAS, (ii) to define whether *sil* was predominantly present only in invasive isolates or also in noninvasive isolates of GAS, and (iii) to characterize the relationship between GAS and macrolide resistance.

To set up our hypothesis, we examined 242 noninvasive isolates (tonsillitis, 170 isolates; rhinosinusitis, 51 isolates; and acute otitis media, 21 isolates) and 13 invasive isolates (septicemia, 5 isolates; purulent arthritis, 4 isolates; meningitis, 2 isolates; necrotizing fasciitis, 1 isolate; and peritoneal abscess, 1 isolate) of GAS, which were isolated from individual patients. *emm* typing of GAS strains was performed by DNA sequencing according to the recommendations of the Division of Bacterial and Mycotic Diseases, the Centers for Disease Control and Prevention, and the *emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). Multiplex PCR was used for toxin gene (*speA*, *speB*, *speC*, *speF*, *speG*, *speH*, *speJ*, *ssa*, and *smeZ*) profiling, as described by Schmitz et al. (5). PCR detection of the *sil* locus was performed according to the method described by Bidet et al. (1). Macrolide resistance genes of GAS were determined by the PCR methods described by Weber et al. (6). To study the degree of macrolide resistance, MICs of azithromycin to all strains were determined by broth microdilution, using the standard method (2). All the experiments were conducted in duplicate.

Among the 242 noninvasive isolates, 11.98% (29/242) harbored the *sil* gene in their genomic DNA. The *emm* types and the toxin gene profiles of *sil*-positive isolates are shown in Table 1. In noninvasive strains, the *sil* locus was detected in 9 out of 33 *emm* types found in the collection (27.27%), and 41.4% (12/29) of the *sil*-positive isolates belonged to *emm* type 4. *emm* type 4 (12 isolates), *emm* type 48 (3 isolates), and *emm* type 94 (6 isolates) represented 72.41% (21/29) of the *sil*-positive isolates. All of the *sil*-positive noninvasive isolates carried *speB* alleles, but 68.96% of strains carried *speC*. There were no significant differences between the toxin gene profile of the *sil*-positive isolates and that of the *sil*-negative isolates, except for *smeZ*, which was 10.3% of the *sil*-negative isolates but 31% of the *sil*-positive noninvasive isolates. Seventy-five percent of *emm* type 4, 75% of *emm* type 48, 100% of *emm* type 94, 100% of *emm* type 53, 100% of *emm* type 54, and 100% of *emm* type 102 isolates harbored the *sil* gene in their DNA.

Although we used limited numbers of invasive isolates, 15.4% of the invasive GAS isolates harbored the *sil* gene, which is consistent with data from a previous study of invasive strains, which showed that 16% carried the *sil* gene (1). One hundred percent of *emm* type 87 and 100% of *emm* sequence type 1732 were positive for the invasive locus. Thirty percent of the *sil*-negative invasive isolates carried *speA* alleles, but all *sil*-positive isolates were negative for the *speA* gene. All strains were positive for the *speB* gene. Fifty percent of the *sil*-positive isolates were positive for *speC*, but 30% of the *sil*-negative isolates were positive for *speC*. There is no statistical significance in the prevalence of the *sil* gene among invasive and noninvasive isolates (Fisher's exact test, $P = 0.499$).

Among 255 invasive and noninvasive isolates, 16.86% (3 were invasive, and 40 were noninvasive; total, 43/255) of the isolates were azithromycin resistant and were positive for macrolide-resistant genes (Table 2). Among these strains, 65.12% (28/43), 13.95% (6/43), and 20.93% (9/43) of the strains possessed the *mef*(A), *erm*(B), and *erm*(TR) genes, respectively. All *sil*-positive isolates were sensitive to azithromycin and were negative for macrolide resistance genes (Fisher's exact test, $P < 0.006$).

From these result, we concluded that *sil* is present not only among invasive isolates but also among noninvasive isolates, with similar prevalences (15.4% versus 11.98%, respectively). To our knowledge, this is the first report to show the prevalence rates of *sil* in both invasive and noninvasive isolates of GAS in Japan. The predominant *emm* types that harbored *sil* were *emm* type 4, *emm* type 94, and *emm* type 48. Hidalgo-Grass et al. identified *sil* in the invasive serotype M14 clone, the organism that caused necrotizing fasciitis in Israel (3). In our study, *sil* was absent from *emm* type 3 isolates, a finding comparable to that in a previous study and associated with GAS invasive diseases worldwide (3). The *sil* locus was confirmed by direct sequencing of several representative PCR-

TABLE 1. Characteristics of streptococcal toxin gene profile of invasive and noninvasive *sil*-positive isolates[a]

Isolate type	*emm* type	Sequence type	No. of isolates	Pyogenic exotoxin				
				speA	*speB*	*speC*	*speH*	*smeZ*
Noninvasive	1		1	+	+	−	−	+
	1		1	−	+	−	−	+
	4		4	+	+	+	−	−
	4		2	−	+	+	−	−
	4		5	−	+	+	−	+
	4		1	−	+	+	+	+
	11		1	−	+	+	−	−
	48		3	−	+	+	−	−
	53		1	−	+	+	+	+
	54		1	−	+	+	−	−
	75		2	−	+	−	−	−
	94		4	−	+	−	−	−
	94		1	−	+	+	−	−
	94.1		1	−	+	−	−	−
	102.2		1	−	+	+	−	−
Invasive	87		1	−	+	+	−	−
		1732	1	−	+	−	−	−

[a] Characteristics of streptococcal toxin gene profile indicating the presence (+) and absence (−) of invasive and noninvasive *sil*-positive isolates.

TABLE 2. Relationship between *sil*-positive and macrolide-resistant genes and invasive and noninvasive GAS[a]

sil gene	No. of isolates				
	Macrolide resistance gene				Total
	Positive			Negative	
	mef(A)	*erm*(B)	*erm*(TR)		
Positive	0	0	0	31	31
Negative	28	6	9	181	224
Total	28	6	9	212	255

[a] Significant differences are based on a Fisher's exact test P value of <0.006.

amplified products and comparing those with the previous sequence. The overall prevalence of the *sil* locus in invasive isolates was the same as that from a previous study (16% versus 15.4%, respectively) (1). Up to now, there was no study which showed the status of noninvasive strains with the *sil* gene. When we examined noninvasive strains, the *sil* gene was found in 12% of isolates, which is not a remarkably different rate from that found in invasive isolates. All *sil*-positive isolates were negative for macrolide resistance genes, which were irreversibly important for clinical practice. Future studies should focus on a better understanding of the role of *sil* in the pathogenesis of GAS infection and its relationship with macrolide resistance. A recent candidate vaccine based on the M protein failed to elicit antibodies to serotype M4, and *sil*-encoded proteins might represent alternative vaccine targets for this serotype (4). The results of this study should contribute to a better understanding of the pathogenesis of GAS, as well as the epidemiology of GAS-associated disease, and to the establishment of methods for the prevention of diseases caused by GAS in Japan.

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ᵛ Published ahead of print on 20 February 2008.

J Infect Chemother (2008) 14:424–432
DOI 10.1007/s10156-008-0648-6

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ORIGINAL ARTICLE

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Comprehensive detection of causative pathogens using real-time PCR to diagnose pediatric community-acquired pneumonia

Received: May 29, 2008 / Accepted: September 30, 2008

Abstract We have developed a real-time reverse transcription-PCR (RT-PCR) method to detect 13 respiratory viruses: influenza virus A and B; respiratory syncytial virus (RSV) subgroup A and B; parainfluenza virus (PIV) 1, 2, and 3; adenovirus; rhinovirus (RV); enterovirus; coronavirus (OC43); human metapneumovirus (hMPV); and human bocavirus (HBoV). The new method for detection of these viruses was applied simultaneously with real-time PCR for the detection of six bacterial pathogens in clinical samples from 1700 pediatric patients with community-acquired pneumonia (CAP). Of all the patients, 32.5% were suspected to have single bacterial infections; 1.9%, multiple bacterial infections; 15.2%, coinfections of bacteria and viruses; 25.8%, single viral infections; and 2.1%, multiple viral infections. In the remaining 22.6%, the etiology was unknown. The breakdown of suspected causative pathogens was as follows: 24.4% were *Streptococcus pneumoniae*, 14.8% were *Mycoplasma pneumoniae*, 11.3% were *Haemophilus influenzae*, and 1.4% were *Chlamydophila pneumoniae*. The breakdown of viruses was as follows: 14.5% were RV, 9.4% were RSV, 7.4% were hMPV, 7.2% were PIV, and 2.9% were HBoV. The new method will contribute to advances in the accuracy of diagnosis and should also result in the appropriate use of antimicrobials.

Key words Real-time RT-PCR · Respiratory virus · Community-acquired pneumonia · Child

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Introduction

Community-acquired pneumonia (CAP) is the most common infectious disease occurring in children. The etiological agents are varied, including a number of viruses, and bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, and *Chlamydophila pneumoniae*.[1-4] To select an appropriate antimicrobial for a patient with bacterial pneumonia, identification of the causative pathogen quickly on the first day of hospitalization would be extremely useful. If at all possible, this would also decrease hospital costs and length of stay.

We constructed a simultaneous identification system, using a real-time PCR assay in our laboratory, for the six main bacterial pathogens from clinical samples collected from pediatric patients with CAP.[5] Subsequently, the method was improved by adding a multiplex PCR (MPCR) kit for seven viruses. The total time required for obtaining the results for viruses was about 5.0 h. Using this improved method, we[6] detected etiological agents in 117 patients who were hospitalized with CAP. In that study, antibody titers for some pathogens were also measured for paired sera from the patients to verify the PCR results. Based on the identification of etiological agents, our cases in that study were categorized as viral infection (23.1%); viral and bacterial coinfection (38.5%); bacterial infection (21.4%); *M. pneumoniae* infection, including coinfection with another pathogen (16.2%); and *C. pneumoniae* infection (0.9%). Our data were very similar to those previously reported by Michelow et al.[7] and Júven et al.[8]

It is well known that the causative viruses of respiratory tract infections (RTIs) are quite varied, such as influenza viruses A, B (Flu A, Flu B); respiratory syncytial virus (RSV) subgroup A and B; parainfluenza virus 1–3 (PIV1, PIV2, PIV3); adenovirus (AdV); rhinovirus (RV); enterovirus (EV); and coronavirus (CoV).[9-13] Human metapneumovirus (hMPV)[14-18] and human bocavirus (HBoV)[19-22] have also been reported recently as etiological agents of CAP.

Recently, real-time reverse-transcription-PCR (RT-PCR) methods have been applied for the identification of

respiratory viruses.[23-28] The PCR methods appear to be more sensitive than culture and are less affected by specimen quality and transport, and provide an objective interpretation of results. The United States Food and Drug Administration cleared for marketing a test called the xTAG Respiratory Viral Panel (Luminex Moleoular Diagnostics, Toronto, Canada) that simultaneously detects and identifies 12 respiratory viruses.[29,30] This method can detect these viruses within 5 h.

For the use of real-time RT-PCR as a routine method in diagnosis, we focused on developing methods to cover the detection of all of the above 13 respiratory viruses within 3 h, combining the methods with our previously described method for detecting the six main bacterial pathogens involved in CAP.[5]

In this article, we describe the results obtained when a comprehensive identification system with real-time PCR was applied for clinical samples collected from pediatric patients with CAP.

Patients, materials, and methods

Patients and clinical samples

A total of 1700 nasopharyngeal swab samples were sent to our laboratory from pediatricians at ten medical institutions that participated in the "Acute Respiratory Diseases (ARD) Study Group" between January 2005 and December 2006. These samples were collected from pediatric patients with CAP who were aged from 0 to 19 years. The CAP was diagnosed from the presence of pulmonary infiltrates on chest X-ray. After informed consent was obtained from the patients' parents/guardians, blood samples for the determination of WBC, C-reactive protein (CRP), and serum antibody titers for several pathogens, and nasopharyngeal swab samples to determine the causative pathogen by real-time PCR were collected. An application form (in which patient names and doctors' names were withheld), written by the doctor in charge, was sent to our laboratory with the clinical samples.

Patients with duration of symptoms of 6 days or more before visiting the hospitals and patients already administered with intravenous antibiotics at another hospital or clinic were excluded from the study, counting without reservation in consideration of the percentage of virus positive apparently dropped to a lower value.

DNA/RNA extraction

The nasopharyngeal samples were suspended in 1.5 ml of pleuropneumonia-like organisms (PPLO) broth (Difco, Detroit MI, USA) immediately after they were received. PPLO broth was selected for cultivating *M. pneumoniae*. A 1.0-ml aliquot of the PPLO broth was transferred to an Eppendorf tube and centrifuged at 5000 rpm for 5 min at 4°C. Nine hundred microliters of the supernatant was discarded, and the remaining solution including the pellet was used as the sample. DNA/RNA was extracted using Extragen II (TOSOH, Tokyo, Japan) according to the manufacturer's protocol, as follows: first, a 100-µl aliquot of the suspension was transferred to an Eppendorf tube, which contained 8 µl of detergent for DNA/RNA coprecipitation. Next, the Eppendorf tube was vortexed for 10 s. To this mixture, 500 µl of 60% (vol/vol) isopropanol-containing protein-denaturing detergent was added, and the resulting mixture was centrifuged at 12 000 rpm for 3 min at 4°C after vortexing for 10 s. The supernatant was discarded, and the residue was treated with 200 µl of 40% (vol/vol) isopropanol again, as described above. Finally, the harvested DNA/RNA pellet was resuspended in DNase- and RNase-free H_2O to provide 40 µl of DNA/RNA sample. The extraction process was finished within 10 min.

cDNA synthesis

Reverse transcription (RT) was performed in an Eppendorf tube containing 25.5 µl reaction mixture after the addition of a 10-µl aliquot of the DNA/RNA sample as described above. The reaction mixture consisted of: (i) 100 U Rever Tra Ace (TOYOBO, Osaka, Japan), (ii) 8.5 µl of 2 mM dNTPs, (iii) 1 µl of 25 pmol/µl random primer, (iv) 40 U RNase inhibitor, and (v) 4 µl of 5 × RT buffer. The RT reaction was carried out at 30°C for 10 min, 42°C for 50 min, and terminated by incubation at 99°C for 10 min, using a thermal cycler (Gene Amp PCR System 9600-R; Perkin-Elmer Cetus, Norwalk, CT, USA). After the RT reaction, 25 µl of DW was added.

Real-time PCR for viruses

Table 1 shows four sets of virus-specific primers and molecular beacon (MB) probes (RSV subgroup B, AdV, CoV, and HBoV) that were newly constructed for this study. The primers and MB adenovirus probes were modified from those of He and Jiang[31] in order to amplify serotypes 7 and 14. The HBoV and CoV (OC43) primers were designed based on alignment from the The National Center for Biotechnology Information (NCBI) database. The reference sequences were as follows: HBoV, accession no. DQ296618; CoV (OC43), accession no. NC_005147. The other probes were slightly modified for MB probes as previously described in each of the following references: RSV subgroup A;[32] Flu A, Flu B, and PIV1–3;[26] RV;[33] EV;[34] and hMPV.[35] The MB probes and primers were prepared by Sigma-Aldrich Japan (Tokyo, Japan) and Operon Biotechnologies (Tokyo, Japan), respectively. All of the MB probes were labeled with a fluorescent reporter, 6-carboxyfluorescein (FAM) or carboxy-X-rhodamine (ROX) at the 5′ end and labeled with black hole quencher 1 (BHQ-1) at the 3′ end.

The reaction mixture consisted of 25 µl of 2 × real-time PCR Master Mix (TOYOBO), 0.3 µM of each primer, and 0.3 µM of MB probe; the final volume of the mixture was adjusted to 50 µl with the addition of DNase- and RNase-free H_2O. The multiplex reaction for RSV subgroup A and

Table 1. Primers and probes newly constructed for this study

Viruses, primer, and probe	Primer or probe[a] sequence
Respiratory syncytial virus subgroup B	
Sense primer	5′-AACAGACATAAGCAGCTCAGTAATT-3′
Reverse primer	5′-CGATTTTTGTTGGATGCAGTGCATTT-3′
Probe	5′-GCGAGCAGGAGCTATAGTGTCATGCTATGGTAGCTCGC-3′
Adenovirus	
Sense primer	5′-CCCTGGTAKCCRATRTTGTA-3′
Reverse primer	5′-AATGACAGGCTGYTGAGYC-3′
Probe	5′-GCTGCCAACCAGTCYTTGGTCATGTTRCATTGGGCAGC-3′
Coronavirus (OC43)	
Sense primer	5′-TGTTTCTCAACAGCTTAGTG-3′
Reverse primer	5′-TCCTAGATGATTGGCTTTTG-3′
Probe	5′-GCTGCCGTGCAGCACAAGCTATGGAGAAGGCAGC-3′
Human bocavirus	
Sense primer	5′-GAAAGACAAGCATCGCTCC-3′
Reverse primer	5′-TGGGTGTTCCTGATGATATG-3′
Probe	5′-CGCGCTGGAGCAGGAGCCGCAGCCCGATAGCGCG-3′

[a] Stem oligonucleotides are underlined

Table 2. Specificity after 40 cycles of real-time PCR amplification for 13 viruses

Virus	RSV-A	RSV-B	Flu A	Flu B	PIV1	PIV2	PIV3	AdV	RV	EV	CoV	hMPV	HBoV
RSV subgroup A	+	–	–	–	–	–	–	–	–	–	–	–	–
RSV subgroup B	–	+	–	–	–	–	–	–	–	–	–	–	–
Influenza virus A	–	–	+	–	–	–	–	–	–	–	–	–	–
Influenza virus B	–	–	–	+	–	–	–	–	–	–	–	–	–
Parainfluenza virus 1	–	–	–	–	+	–	–	–	–	–	–	–	–
Parainfluenza virus 2	–	–	–	–	–	+	–	–	–	–	–	–	–
Parainfluenza virus 3	–	–	–	–	–	–	+	–	–	–	–	–	–
Adenovirus	–	–	–	–	–	–	–	+	–	–	–	–	–
Rhinovirus	–	–	–	–	–	–	–	–	+	(±)	–	–	–
Enterovirus	–	–	–	–	–	–	–	–	–	+	–	–	–
Coronavirus (OC43)	–	–	–	–	–	–	–	–	–	–	+	–	–
Human metapneumovirus	–	–	–	–	–	–	–	–	–	–	–	+	–
Human bocavirus	–	–	–	–	–	–	–	–	–	–	–	–	+

subgroup B was performed by mixing each set of primers and MB probes.

Real-time RT-PCR for 12 reaction mixtures (one well contained the reaction mixture of RSV subgroup A and subgroup B), corresponding to the 13 viruses, was performed in a 96-well PCR plate (Bio Medical Equipment, Tokyo, Japan) for eight samples, in one assay in which 12 wells were arranged in one strip for one sample, and the plates were stored at −30°C until use.

Prior to amplification, 1.5 µl of cDNA was added to each of the 12 wells on ice, and amplification was immediately started at 95°C for 30 s as the first step, followed by 40 cycles of PCR: 95°C for 15 s, 55°C for 30 s, and 75°C for 15 s, using Mx3000P (Stratagene, La Jolla, CA, USA) or Thermal Cycler Dice TP800 (Takara Bio, Kyoto, Japan). The real-time PCR was finished within 1.5 h. The total time from sample disposition to finish was 3.0 h or less.

Sensitivity and specificity of real-time RT-PCR

The sensitivity of real-time RT-PCR was determined by testing tenfold serial dilutions of a 50% tissue culture infectious dose ($TCID_{50}$) titrated stock solution of each of the following viruses: RSV subgroup A, RSV subgroup B, Flu A, PIV2, PIV3, AdV, and CoV. The threshhold cycle (Ct) values of every dilution for each virus were determined by duplicate PCR tests.

The limits of detection with Ct values for each virus were as follows: 10^1 $TCID_{50}$/ml with 38.0 cycles for RSV subgroup A, 10^1 $TCID_{50}$/ml with 36.1 cycles for RSV subgroup B, 10^1 $TCID_{50}$/ml with 37.2 cycles for Flu A, 10^1 $TCID_{50}$/ml with 39.2 cycles for PIV2, 10^2 $TCID_{50}$/ml with 36.6 cycles for PIV3, 10^1 $TCID_{50}$/ml with 39.4 cycles for AdV, and 10^1 $TCID_{50}$/ml with 38.2 cycles for CoV.

From these results, the limitation of Ct values estimated to be positive for the corresponding virus was defined as 40 or fewer amplification cycles. The specificities of the MB probe and primer sets for the 13 viruses after 40 cycles of amplification are shown in Table 2. No nonspecific positive results were obtained from those viruses selected at random from laboratory stock cDNA, and the specificity of primers and probes was high.

The sensitivity and specificity of real-time RT-PCR were also compared with the results obtained with an "MPCR kit for respiratory infection associated viruses set-3" (Maximbio, San Francisco, CA, USA) that is designed to identify seven viruses; namely, RSV, Flu A, Flu B, PIV1,

PIV2, PIV3, and AdV in one tube. The reactions were performed according to the manufacturer's protocol, using 1.5 μl of cDNA. The sensitivity and specificity of real-time RT-PCR relative to those of conventional PCR for the seven viruses were 96.3%–100% and 84.1%–98.9, respectively (data not shown).

Real-time PCR for bacteria

Six pathogens: *S. pneumoniae*, *H. influenzae*, *M. pneumoniae*, *C. pneumoniae*, *S. pyogenes*, and *Legionella pneumophila* were identified using the real-time PCR with a Respiratory Tract Infection (RTI) kit (Code: CY214; Takara Bio, Kyoto, Japan) as described previously.[5] The total time from DNA extraction to the finish was 1.5 h.

Bacterial cultures

Bacterial culture and species identification were performed according to the *Manual of clinical microbiology*.[36]

Results

Etiological agents

Table 3 shows the etiological agents found in 1700 CAP patients who had visited the hospital within 5 days of symptom onset (mean, 3.8 days). These etiological classifications were made according to the tentative criteria described by Nakayama et al.[6]

Single bacterial infection was identified in 32.5% (n = 553) of the 1700 patients, multiple bacterial infection was identified in 1.9% (n = 32), mixed infection of bacteria and viruses was identified in 15.2% (n = 258), single viral infection was identified in 25.8% (n = 438), and multiple viral infection was identified in 2.1% (n = 35). Infections for which the etiological agent was unknown occurred in 22.6% (n = 384) of all the patients examined here.

Table 4 shows the bacteria suspected to be the causative pathogens and the differentiation whether they were single infections or coinfections. Bacterial infection accounted for 49.6% (n = 843) of all cases, among which 24.4% (n = 415) were caused by *S. pneumoniae*, 14.8% (n = 251) were caused by *M. pneumoniae*, 11.3% (n = 192) were caused by *H. influenzae*, 1.4% (n = 24) were caused by *C. pneumoniae*, and 0.1% (n = 1) were caused by *S. pyogenes*. Of the cases in which causative pathogens were suspected to be *S. pneumoniae*, *H. influenzae*, or *C. pneumoniae*, half were single infections and the others mostly showed coinfection with viruses. In contrast, *M. pneumoniae* infection cases were mostly single infections.

Table 3. Etiology in 1700 children with CAP

Pathogenic agents	n (%)
Bacterial (single)	553 (32.5)
Bacterial (multiple)	32 (1.9)
Bacterial and viral	258 (15.2)
Viral (single)	438 (25.8)
Viral (multiple)	35 (2.1)
Unknown	384 (22.6)
Total	1700 (100.0)

Table 4. bacterial pathogens suspected as etiological agents with high probability in pediatric patients with CAP

Pathogen	Total (%)[a]	Single infection (n = 553)	Coinfection with:		
			Bacteria (n = 32)	Virus (n = 252)	Bacteria and virus (n = 6)
S. pneumoniae	415 (24.4)	236 (56.9)[b]	30 (7.2)	144 (34.7)	5 (1.2)
M. pneumoniae	251 (14.8)	206 (82.1)	9 (3.6)	34 (13.5)	2 (0.8)
H. influenzae	192 (11.3)	95 (49.5)	24 (12.5)	68 (35.4)	5 (2.6)
C. pneumoniae	24 (1.4)	16 (66.7)	3 (12.5)	5 (20.8)	0
S. pyogenes	1 (0.1)	0	0	1	0

[a]Percentage in 1700 patients
[b]Percentage of each pathogen

Table 5. Viral pathogens identified by real-time RT-PCR in clinical samples from pediatric patients with CAP

Pathogen	Total (%)[a]	Single infection (n = 438)	Coinfection with:		
			Bacteria (n = 253)	Virus (n = 35)	Bacteria and virus (n = 5)
Rhinovirus	247 (14.5)	122 (49.4)[b]	101 (40.9)	22 (8.9)	2 (0.8)
Respiratory syncytial virus	159 (9.4)	107 (67.3)	44 (27.7)	7 (4.4)	1 (0.6)
Human metapneumovirus	125 (7.4)	84 (67.2)	30 (24.0)	9 (7.2)	2 (1.6)
Parainfluenza virus 1, 2, 3	122 (7.2)	74 (60.7)	37 (30.3)	10 (8.2)	1 (0.8)
Human bocavirus	50 (2.9)	17 (34.0)	18 (36.0)	13 (26.0)	2 (4.0)
Adenovirus	30 (1.8)	11 (36.7)	11 (36.7)	7 (23.3)	1 (3.3)
Others[c]	38 (2.2)	23 (60.5)	12 (31.6)	2 (5.3)	1 (2.6)

[a]Percentage in 1700 patients
[b]Percentage for each virus
[c]"Others" denotes Flu A, Flu B, CoV, and EV

Table 6. Pathogenic agents identified in 1700 pediatric patients with CAP: distribution by age

Pathogens	Total no. of positive cases	Age[a]	Number of isolates (%)						
			<1 year	1 year	2 years	3 years	4 years	5 years	≥6 years
Bacteria									
S. pneumoniae	415	2.5 ± 2.3	77 (18.6)	120 (28.9)	60 (14.5)	63 (15.2)	40 (9.6)	18 (4.3)	37 (8.9)
H. influenzae	192	2.4 ± 1.9	35 (18.2)	53 (27.6)	30 (15.6)	23 (12.0)	26 (13.5)	10 (5.2)	15 (7.8)
M. pneumoniae	251	6.1 ± 3.7	7 (2.8)	24 (9.6)	22 (8.8)	23 (9.2)	22 (8.8)	17 (6.8)	136 (54.2)
C. pneumoniae	24	5.4 ± 3.8	3 (12.5)	3 (12.5)	2 (8.3)	0 (0.0)	2 (8.3)	2 (8.3)	12 (50.0)
Virus									
Rhinovirus	247	2.9 ± 2.7	42 (17.1)	61 (24.8)	36 (14.6)	30 (12.2)	28 (11.4)	13 (5.3)	37 (15.0)
Respiratory syncytial virus	159	1.6 ± 1.4	52 (32.7)	47 (29.6)	28 (17.6)	20 (12.6)	8 (5.0)	1 (0.6)	3 (1.9)
Human metapneumovirus	125	2.4 ± 1.9	15 (12.0)	35 (28.0)	21 (16.8)	24 (19.2)	20 (16.0)	6 (4.8)	4 (3.2)
Parainfluenzavirus 1, 2, 3	122	2.3 ± 2.6	28 (23.0)	45 (36.9)	9 (7.4)	16 (13.1)	11 (9.0)	3 (2.5)	10 (8.2)
Human bocavirus	50	1.3 ± 0.9	14 (28.0)	27 (54.0)	5 (10.0)	2 (4.0)	1 (2.0)	1 (2.0)	0
Adenovirus	30	1.5 ± 2.7	9 (29.6)	6 (50.1)	1 (11.5)	6 (4.6)	1 (2.4)	3 (2.4)	4 (0.1)
Others[b]	38	2.7 ± 2.2	6 (15.8)	12 (31.6)	3 (7.9)	3 (7.9)	4 (10.5)	2 (5.3)	8 (21.1)

[a]Median ± SD
[b]"Others" denotes Flu A, Flu B, EV and CoV

Table 7. Diagnostic value of WBC count and CRP according to etiological agent

Pathogens	n	WBC ($\times 10^3$/mm^3)		CRP (mg/dl)	
		Median	Range	Median	Range
Bacterial					
S. pneumoniae	235	17.1	5.6–39.9	5.4	0.1–26.2
H. influenzae	94	13.2	5.6–29.1	3.2	0.1–17.5
M. pneumoniae	189	6.6	2.3–19.8	1.8	0.1–13.4
C. pneumoniae	16	8.8	4.2–12.8	0.2	0.0–0.6
Bacterial + viral	216	14.5	6.2–35.6	3.7	0.1–19.3
Viral					
Rhinovirus	116	11.0	4.0–21.4	0.6	0.0–5.9
Respiratory syncytial virus	101	8.8	3.4–22.8	0.8	0.0–9.5
Human metapneumovirus	82	6.9	2.7–17.5	0.8	0.0–10.0
Parainfluenza virus 1, 2, 3	70	7.9	2.6–19.1	0.7	0.0–8.1
Human bocavirus	17	10.5	5.6–17.6	0.5	0.0–1.9
Adenovirus	11	12.1	7.4–26.5	4.0	0.7–8.4
Influenza virus	10	8.1	5.3–11.3	1.1	0.4–3.8

Patients infected with *S. pyogenes*, EV, and CoV were few, so they are not included

Table 5 shows the identified viral pathogens. Viral infection accounted for 43.0% (n = 731) of all cases. The cumulative positive cases determined by real-time RT-PCR showed that RV accounted for 14.5% (n = 247) of the viral infections; RSV, for 9.4 % (n = 159); hMPV for 7.4 % (n = 125); PIV for 7.2% (n = 122); HBoV for 2.9% (n = 50); and AdV for 1.8% (n = 30; the remaining viruses [Flu A, Flu B, CoV, and EV] are combined as "others" in Table 5).

The percentages of single viral infections were relatively higher for RSV (67.3%), hMPV (67.2%), and PIV (60.7%) than for RV (49.4%) and HboV (34.0%).

Distribution of pathogenic agents according to age

Table 6 shows the distribution of each pathogenic agent identified according to patient age. The median ages of patients who were infected with *S. pneumoniae* and *H. influenzae* were 2.5 and 2.4 years, respectively, and the median ages of those infected with *M. pneumoniae* and *C. pneumoniae* were 6.1 and 5.4 years, respectively. The median ages of patients positive for infection with RSV, HBoV, and AdV ranged from 1.3 to 1.6 years, while the median ages of those positive for infection with RV, hMPV, and PIV were slightly higher.

WBC count and CRP according to etiological agents

Both the WBC count and CRP were determined in 1157 of the 1700 patients on the same day as the sample collection for PCR.

Table 7 shows the median values and ranges for the WBC count and CRP in the patients with bacterial infections, those with bacterial and viral coinfections, and those with viral infections.

In the patients with *S. pneumoniae* or *H. influenzae* infection, and bacterial and viral coinfection, the median values were comparatively high, at 13.2–17.1 × 10^3/mm^3 for WBC and 3.2–5.4 mg/dl for CRP. In contrast, these values in the patients with *M. pneumoniae* and *C. pneumoniae* infections were apparently low, at 6.6 × 10^3/mm^3 and 8.8 × 10^3/mm^3, respectively, for WBC, and 1.8 mg/dl and 0.2 mg/dl, respectively, for CRP.

The median values for the WBC count and CRP in the patients who were positive for viruses were all 11.0×10^3/mm^3 or less and 1.1 mg/dl or less, respectively, except for AdV.

Seasonal epidemiology of viruses

Figure 1 shows the seasonal epidemiology for RSV, Flu, PIV, RV, hMPV, and HBoV viruses by month over the 2 years from January 2005 to December 2006. The percentage for each virus is given as the ratio per month in the CAP cases. The HBoV shown in this Fig. was examined for only 1 year, beginning in January 2006.

RSV was detected mainly from August to January, corresponding to the late summer to winter season. Although the numbers were small, Flu was detected from February to March in the winter season. PIV3 accounted for 86.1% of all the PIV cases, and they predominated from May to July during the spring to summer season. RV was detected in all months of the year, but the peak incidence was observed from August to September. hMPV peaked predominantly from February to July in early spring to summer, while HBoV was detected from May to June.

Discussion

Antimicrobial resistance in *S. pneumoniae*,[37] *H. influenzae*,[38,39] and *M. pneumoniae*[40] in CAP poses major clinical problems in the treatment of patients. Therapeutic antibiotics administered to patients upon hospitalization are usually selected empirically, based on the likely etiological agent after viewing of the chest X-ray, hematological studies, consideration of the history, respiratory symptoms, and age of the patient. To avoid inappropriate antimicrobial therapy and to select the most appropriate agent, guidelines for CAP in pediatric and adult patients have been proposed in Japan,[41,42] as well as in other countries.[43]

However, we often encounter a patient in whom it is difficult to predict the causative pathogen from the clinical findings described above. It is preferable that the etiological agent be identified on the day of hospitalization, so that the diagnosis of CAP can be made without waiting for bacterial cultures.

Recently, real-time PCR for *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila* causing pneumonia,[5,44] and real-time PCR for respiratory viruses,[24,26] have been receiving attention as effective methods. To detect or to identify etiological agents accurately in children with CAP, it is necessary to improve the comprehensive PCR detection of both bacteria and viruses from one sample in order to lighten the load of the patient and improve cost-effectiveness.

As described in the "Methods" section, an important characteristic of our examination protocol included a labor-saving procedure for the extraction of DNA and RNA from the clinical samples, using an Extragen II kit (TOSOH), that took less than 10 min. The extracted samples were divided and used for real-time PCR for bacterial detection and real-time RT-PCR for viral detection. The real-time PCR results for these etiological agents can be obtained within 3 h from the time of the receipt of clinical samples.

Recently, we further improved the methods of cDNA synthesis using a new enzyme, PrimeScript RTase (TakaraBio, Tokyo, Japan), so that the synthesis can be finished in 15 min. Accordingly, the results of bacterial and viral analysis by real-time PCR can be obtained within 2.0 h at present. If these improved methods are applied to laboratory examination, it is expected that they could contribute greatly to the use of appropriate antimicrobial chemotherapy.

We note that, because sputum collection is rather difficult in children, compared with adults, nasopharyngeal samples are commonly used for real-time PCR in children. Although nasopharyngeal samples are appropriate for identifying viruses and *M. pneumoniae* and *C. pneumoniae*, such specimens are not always appropriate for the detection of *S. pneumoniae* and *H. influenzae*. This is because these bacteria are often isolated from nasopharyngeal sites in healthy controls as well. Positive results for bacteria obtained by real-time PCR should be carefully evaluated as to whether they are infection cases or whether the results reflect the presence of indigenous bacteria. Although WBC and CRP values may provide useful information to determine the etiological agents of CAP, further studies will be necessary to construct an appropriate indicator.

As previously reported by Nakayama et al.,[6] 90% or higher percentages of patients who were PCR-positive for *M. pneumoniae*, *C. pneumoniae*, and viruses showed significantly high antibody titers to the corresponding pathogens. In other words, if the PCR result turns out to be positive, you may determine that the target is the causative agent.

In the present study, the percentage of cases in which *H. influenzae* was suspected to be the causative pathogen was apparently higher than in other studies.[8] We assume that this reflects the situation in which *H. influenzae* type b (Hib) vaccination was not available during the period of this study. Hib vaccine was, however, approved in 2007 by the Japanese Ministry of Health Labor, and Welfare, and will be distributed in Japan in 2008.

hMPV[14] and HBoV,[19] which have been newly identified as etiological agents causing CAP, were included in our real-time PCR detection system for 13 viruses. Real-time PCR using pathogen-specific-MB probes that annealed to the inner portion of the amplified DNA products apparently improved the specificity of the assays compared to conventional PCR (data not shown).

Although it is well known that RSV and PIV are the main causative viruses in CAP patients, the comparatively high rates of 7.4% for hMPV and 2.9% for HBoV, shown in the "Results" section, were notable in this study. Epidemics due to hMPV may have occurred during our study period. The age peak for RSV or HBoV infection was at 1 year old or less, but that for hMPV was slightly higher. In addition, the prevailing viruses differ depending on the season, which may serve as a good reference to determine CAP causative viruses.

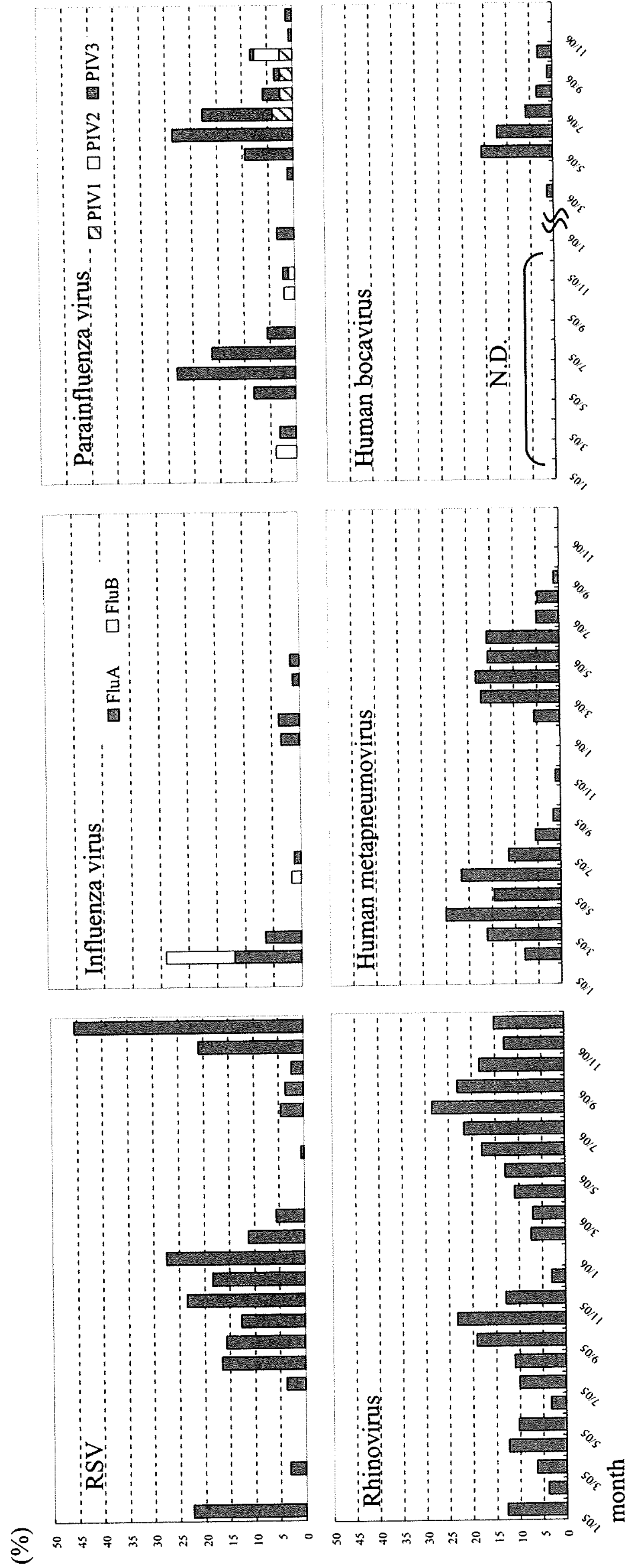

Fig. 1. Monthly prevalence of respiratory syncytial virus (*RSV*), influenza virus (*Flu*), parainfluenza virus (*PIV*), rhinovirus, human metapneumovirus, and human bocavirus from January 2005 through December 2006. *N. D.*, no data

Finally, we anticipate that real-time RT-PCR can be developed to be a multiplex and one-step method, in order to establish a rapid and comprehensive system for the identification of viruses and bacteria.

Acknowledgments We thank members of the Acute Respiratory Diseases Study Group; Drs. Shinobu Ishizawa, Mika Numata, and Michiyo Kojima (Tohoku Rosai Hospital); Masato Nonoyama (Kitasato University School of Medicine); Haruo Kuroki (Sotobo Kodomo Clinic); Koichi Shimizu (Saiseikai Ibaraki Hospital); Masaaki Kobayashi (Kobayashi Pediatric Clinic); Kota Saito (Saito Pediatric Clinic); Masahiko Nitta (Yaotokushukai Hospital and Osaka Medical College); Naohisa Kawamura (Osaka Rosai Hospital); Ritsuko Sakai (Sakai Clinic); Shigeru Ohnari (Nakafukawa Pediatric Clinic); Takao Morinobu (Seikeikai Hospital); Takeshi Tajima (Hakujikai Memorial Hospital); and Tomohiro Oishi (Niigata Kenritsu Shibata Hospital). We also thank Tetsuo Nakayama (Kitasato Institute for Life Sciences) and Yukimasa Kazuyama (Kitasato Otsuka Biochemical Assay Laboratory) for providing stocks of RSV subgroup A, RSV subgroup B, Flu A, PIV2, PIV3, and AdV.

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J Infect Chemother (2009) 15:92–98
DOI 10.1007/s10156-009-0670-3

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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

Received: October 10, 2008 / Accepted: January 28, 2009

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*		*lytA*	319	23
	Sense primer	5′-CAACCGTACAGAATGAAGCGG-3′			
	Reverse primer	5′-TTATTCGTGCAATACTCGTGCG-3′			
	Probe	HEX-CGCGATCAGGTCTCAGCATTCCAACCGCCGATCGCG-BHQ1			
A	*H. influenzae*		16S rRNA	167	23
	Sense primer	5′-TTGACATCCTAAGAAGAGCTC-3′			
	Reverse primer	5′-TCTCCTTTGAGTTCCCGACCG-3′			
	Probe	FAM-CGCGATCCTGACGACAGCCATGCAGCACGATCGCG-BHQ1			
B	*E. coli*		16S rRNA	204	This study
	Sense primer	5′-GGGAGTAAAGTTAATACCTTTGC-3′			
	Reverse primer	5′-CTCAAGCTTGCCAGTATCAG-3′			
	Probe	HEX-CGCGATCACTCCGTGCCAGCAGCCGCGGATCGCG-BHQ1			
B	*S. agalactiae*		*dltS*	331	This study
	Sense primer	5′-AGGAATACCAGGCGATGAAC-3′			
	Reverse primer	5′-AGGCCCTACGATAAATCGAG-3′			
	Probe	FAM-CGCGATCATTTGGCTAGTTATGAAGTCCCTTATGCGATCGCG-BHQ1			
C	*N. meningitidis*		16S rRNA	356	This study
	Sense primer	5′-CATATCGGAACGTACCGAGT-3′			
	Reverse primer	5′-GCCGCTGATATTAGCAACAG-3′			
	Probe	HEX-CGCGATCCTATTCGAGCGGCCGATATCGATCGCG-BHQ1			
C	*L. monocytogenes*		16S rRNA	457	This study
	Sense primer	5′-CGCTTTTGAAAGATGGTTTCG-3′			
	Reverse primer	5′-CTTCCAGTTTCCAATGACCC-3′			
	Probe	FAM-CGCGATCGCGGCGTTGCTCCGTCAGACTTGATCGCG-BHQ1			
D	*M. pneumoniae*		16S rRNA	225	23
	Sense primer	5′-GTAATACTTTAGAGGCGAACG-3′			
	Reverse primer	5′-TACTTCTCAGCATAGCTACAC-3′			
	Probe	HEX-CGCGATACCAACTAGCTGATATGGCGCAATCGCG-BHQ1			
D	*S. aureus*		*spa*	224	This study
	Sense primer	5′-TACATGTCGTTAAACCTGGTG-3′			
	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.

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*

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 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

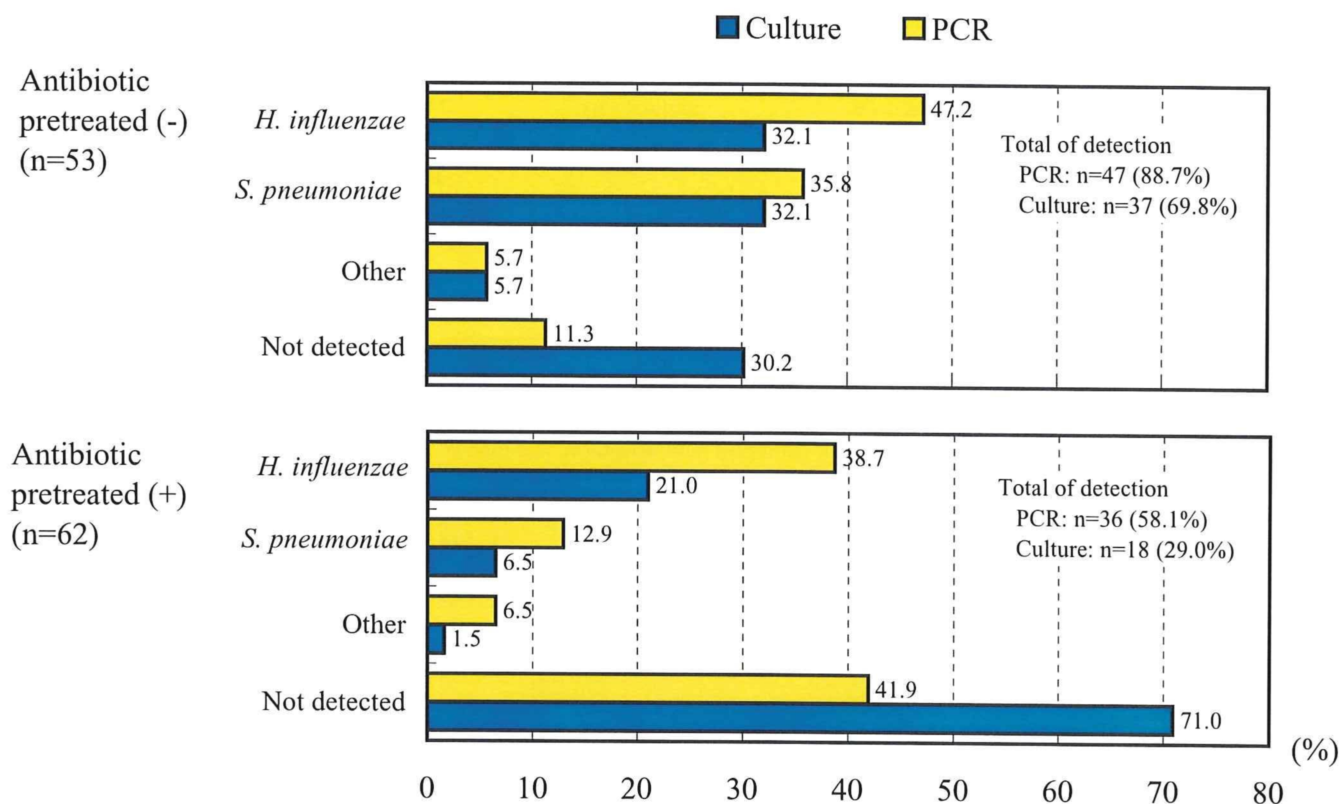

Fig. 1. Influence of prior antibiotics on the detection of causative pathogens by real-time PCR or culturing

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.

Acknowledgments This work was supported by the Japanese Ministry of Health, Labor, and Welfare (Research Project for Emerging and Re-emerging Infectious Diseases, H20-002).

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2009, p. 2650–2653 Vol. 53, No. 6
0066-4804/09/$08.00+0 doi:10.1128/AAC.01716-08
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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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Received 25 December 2008/Returned for modification 15 February 2009/Accepted 21 March 2009

***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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▽ Published ahead of print on 30 March 2009.