原　著

本邦における小児細菌性髄膜炎の動向（2007～2008）

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Key words: bacterial meningitis, child, epidemiology, *Hemophilus influenzae*, *Streptococcus pneumoniae*

要　旨

2007年1月から2008年12月までの2年間に全国112施設から小児細菌性髄膜炎287症例（男児160，女児127）が報告された．

年齢別では生後1カ月未満が28例，1カ月～1歳未満が132例，1歳以上は127例であった．原因菌は *Haemophilus influenzae* が163例と最も多く，次いで *Streptococcus pneumoniae* 54例，*Streptococcus agalactiae*（GBS）26例，*Escherichia coli* 9例の順で，GBS，*E. coli* は低年齢での発症が多かった．*H. influenzae* は1カ月～6歳に，*S. pneumoniae* は2カ月～13歳に分布していた．*H. influenzae*，*S. pneumoniae* ともに耐性化が進んでいたが，*H. influenzae* は2003年の70.4%をピークに，*S. pneumoniae* は2004年の83.0%をピークとして感性株が再び増加する傾向が見られ，今回の調査では耐性株は，*H. influenzae* が2007年55.5%，2008年51.3%，*S. pneumoniae* が2007年72.0%，2008年56.5%であった．

細菌性髄膜炎の初期治療に使用した抗菌薬の種類は，4カ月未満では，従来の標準的治療法とされているAmpicillin＋セフェムならびにセフェム＋カルバペネムの2剤を併用した症例が多く，*H. influenzae* や *S. pneumoniae* が原因細菌として多くなる4カ月以降に関しては，耐性菌を考慮したカルバペネム＋セフェムの併用が多くを占めた．

〔感染症誌　84：33～41，2010〕

序　文

小児化膿性髄膜炎の全国調査については，1966年以降小林[1]，藤井[2)~5)]，岩田[6]，砂川[7)~10)]と継続的に実施されてきた．今回は2007年1月以降の2年間に，我が国で発症した小児細菌性髄膜炎症例に関しての全国アンケートを実施し，成績が集計出来たので報告する．

対象と方法

前回調査を依頼した183施設に，新たに17施設を追加した全国200の小児科施設にアンケート用紙を送付し，2007年1月1日から2008年12月31日までの2年間の年間小児科入院患者数，小児細菌性髄膜炎入院症例数，症例の性，年齢，基礎疾患ならびに合併症の有無，分離菌種，分離菌の薬剤感受性，治療に使用した抗菌薬の種類と用法・用量，ステロイド薬併用の有無，転帰，予後について回答を求め，結果について分析を行った．検定には，Fisher直接確率法を用いた．

成　績

112施設から回答があり（回答率56.0%），287例の症例が報告された．

1．症例数

症例数は2007年143例，2008年144例であった．小児科年間入院1,000人あたりの細菌性髄膜炎症例数は，2007年1.54人，2008年1.62人であり，1997年以降ほぼ程度であったが，1979～1984年の藤井[2)]による調査の3.1～4.0に比べて明らかに減少していた．

男女比は男児160例，女児127例（1.26：1）で若干男児に多く，死亡は男児5例（3.1%），女児2例（1.5%）であり，死亡率に男女差はみられなかった．

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平成22年1月20日

Fig. 1 Pediatric age distribution in bacterial meningitis

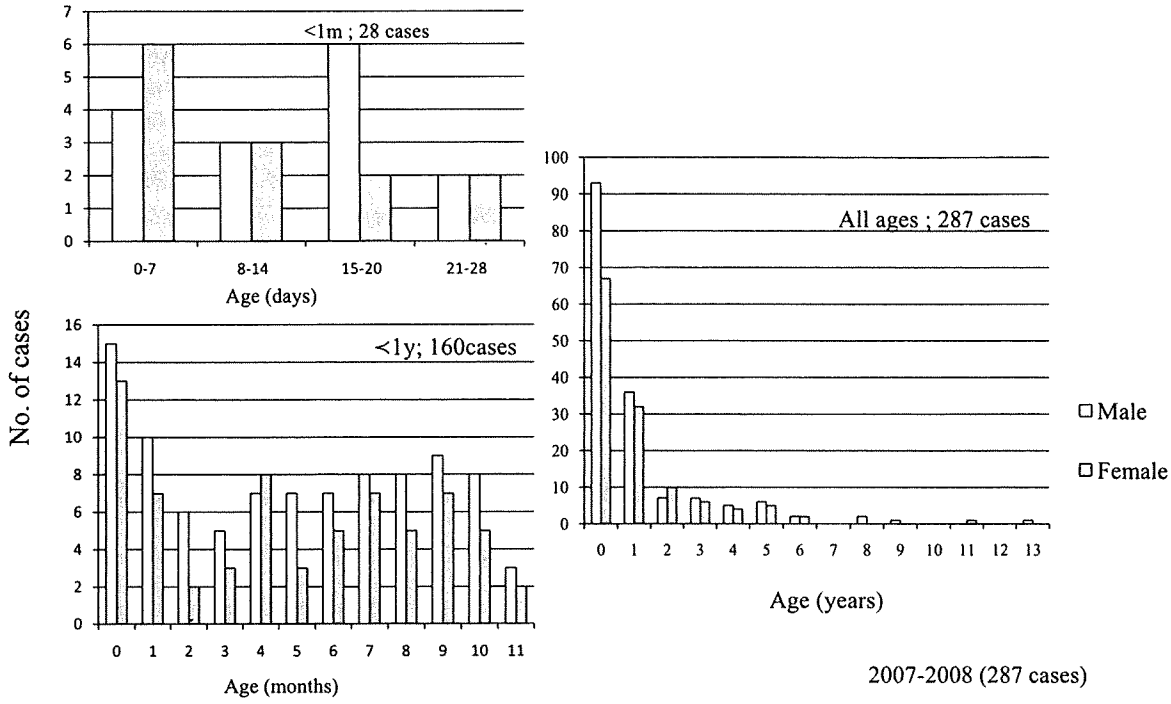
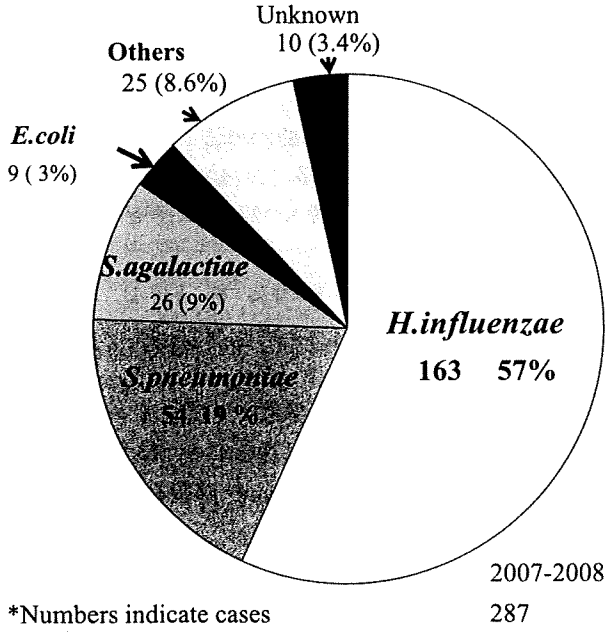


Fig. 2 Causative frequency



2. 年齢分布（Fig. 1）

年齢分布は，男児では1カ月未満が15例，1カ月～1歳未満が78例，1歳以上が67例，女児では1カ月未満が13例，1カ月～1歳未満が54例，1歳以上が60例であり，過去の報告[7)~9)]と同様であった．

3. 原因菌の種類（Fig. 2）

原因菌としては *H. influenzae* が163例（57%）と最も多く，次いで *S. pneumoniae* が54例(19.0%)，*Streptococcus agalactiae*（GBS）は26例（9%），*Escherichia coli* 9例（3.0%）の順であった．その他の25例としては，*Staphylococcus aureus* 5例，*Enterococcus faecalis* 3例，*Klebsiella pneumoniae* 2例，*Enterobacter cloacae* 2例，*Listeria monocytogenes* 2例，*Staphylococcus epidermidis* 1例，*Streptococcus pyogenes* 1例，*Psudomonas aeruginosa* 1例，*Staphylococcus capitis* 1例，*Streptococcus gallolyticus* 1例，*Streptococcus parasangius* 1例，*Campylobacter* sp. 1例，*Micrococcus luteus* 1例，*Bacillus cereus* 1例，グラム陽性菌1例，*K. pneumoniae*，*Neisseria meningitis*，*Pediococcus* 3菌種同時検出1例が含まれていた．原因菌が不明であった症例は10例であった．この割合は1997年に調査を開始して以来大きな差は認めなかった．

4. 原因菌別年齢分布（Fig. 3）

上位4菌種の年齢分布は，GBSは26株が分離され，22株は2カ月未満で，残りは3，4，5，7カ月に各1例見られた．*E. coli* は9株で，全例4カ月以下の乳児であった．

最も分離が多い *H. influenzae* は163株で，1カ月で3例の症例が認められ，その後6歳まで分布したが，1歳台に最も多いとの結果であった．一方，*S. pneumoniae* は54株で2カ月～13歳に分布しており，*H. influenzae* と異なり，6歳以上の年長児にも3例の報告があった．

5. *H. influenzae*，*S. pneumoniae* の薬剤感受性（Fig. 4）

薬剤感受性は，各施設で実施した成績，すなわち

Fig. 3　Major cause by age

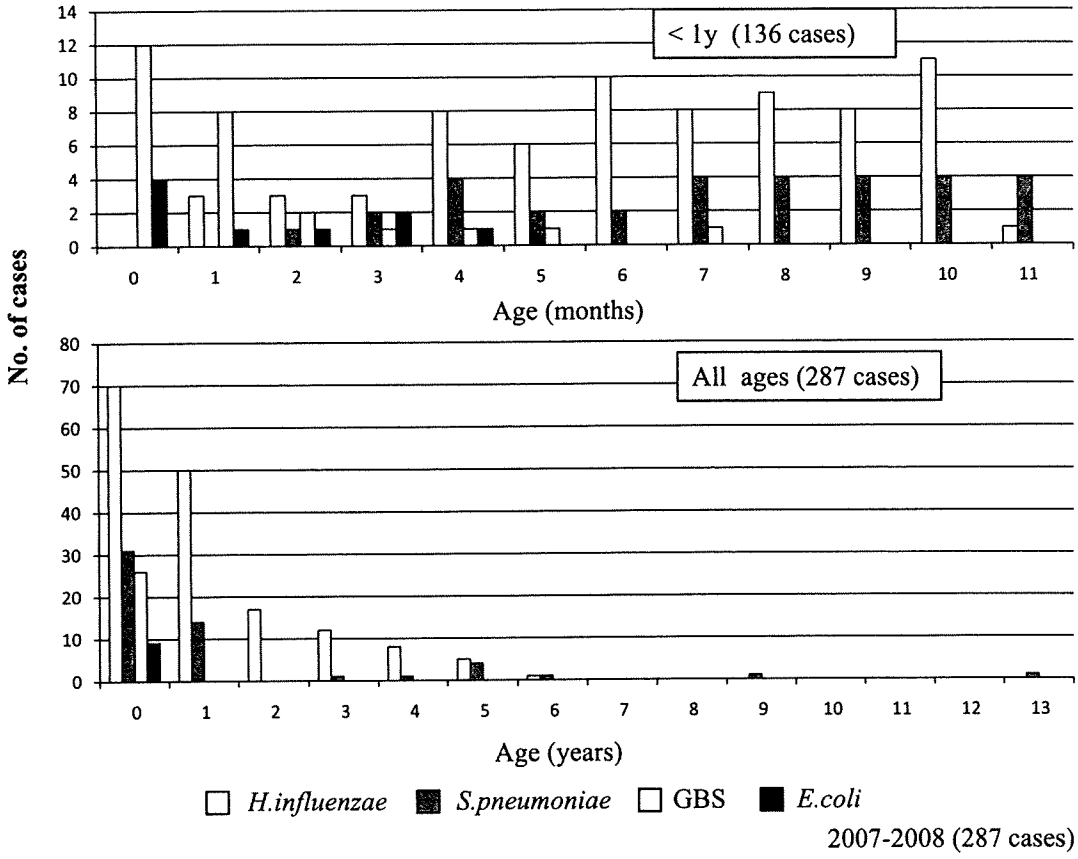
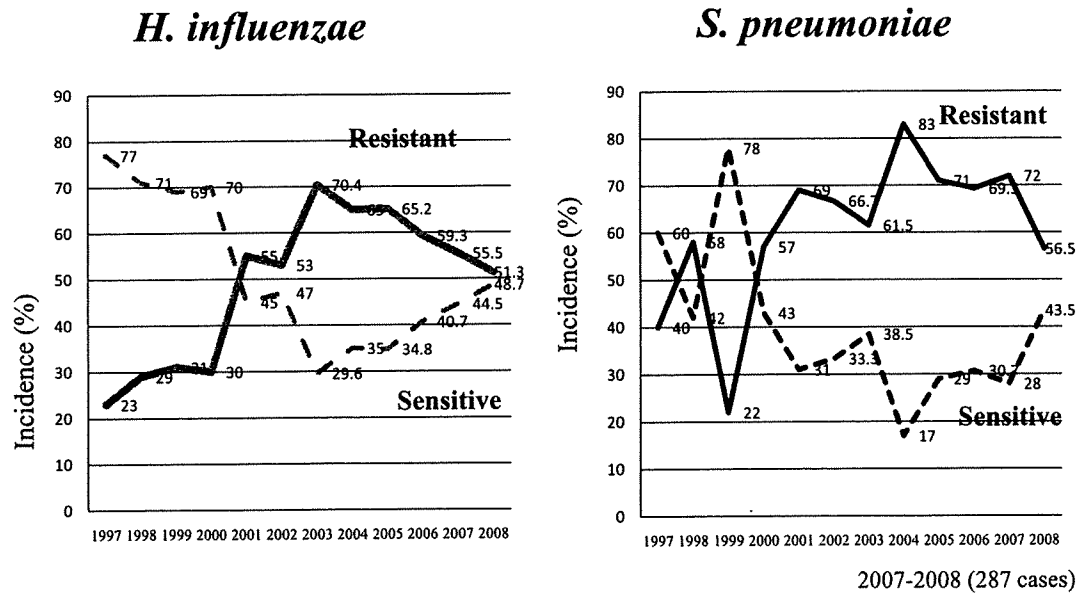


Fig. 4　Annual ratio of resistant strains



H. influenzae は ampicillin：ABPC に対する感受性，*S. pneumoniae* はペニシリン G に対する感受性で分類した．2001 年に *H. influenzae* の耐性菌の占める割合が半数を越え，2003 年には 70.4% と高い値を示したが，その後耐性率は減少し，2008 年には 51.3% となっている（$p<0.05$）．*S. pneumoniae* は，2000 年に耐性の割合が既に半数を越え，2004 年に 83.0% と最悪の事態となったが，2008 年は 56.5% であり，*H. influenzae* 同様減少の方向にある．

6. 死亡例及び菌種別の予後（Table 1，Fig. 5）

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Fig. 5 Prognosis by causative bacteria. (*Mortality)

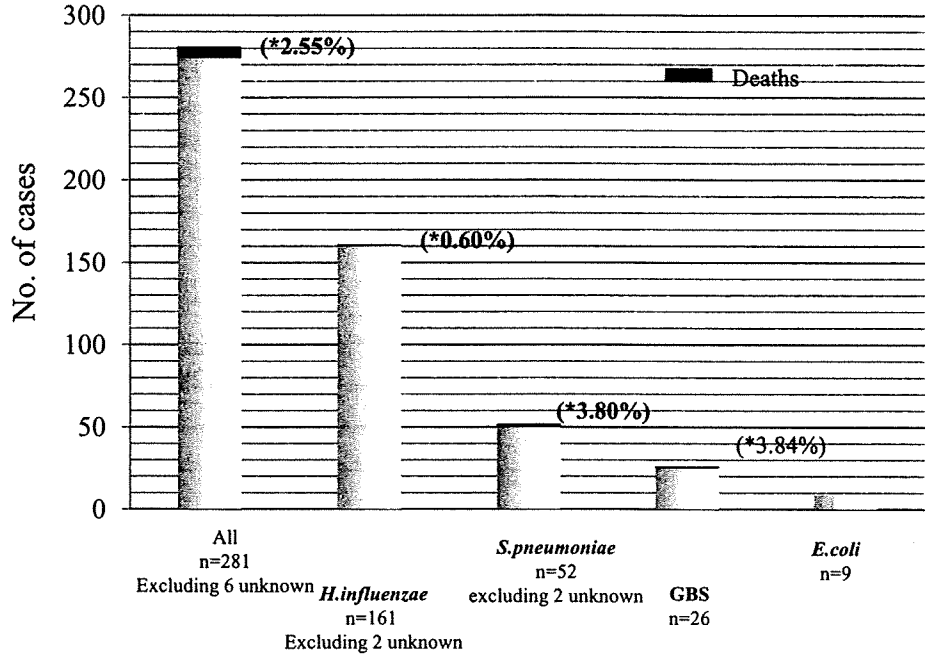
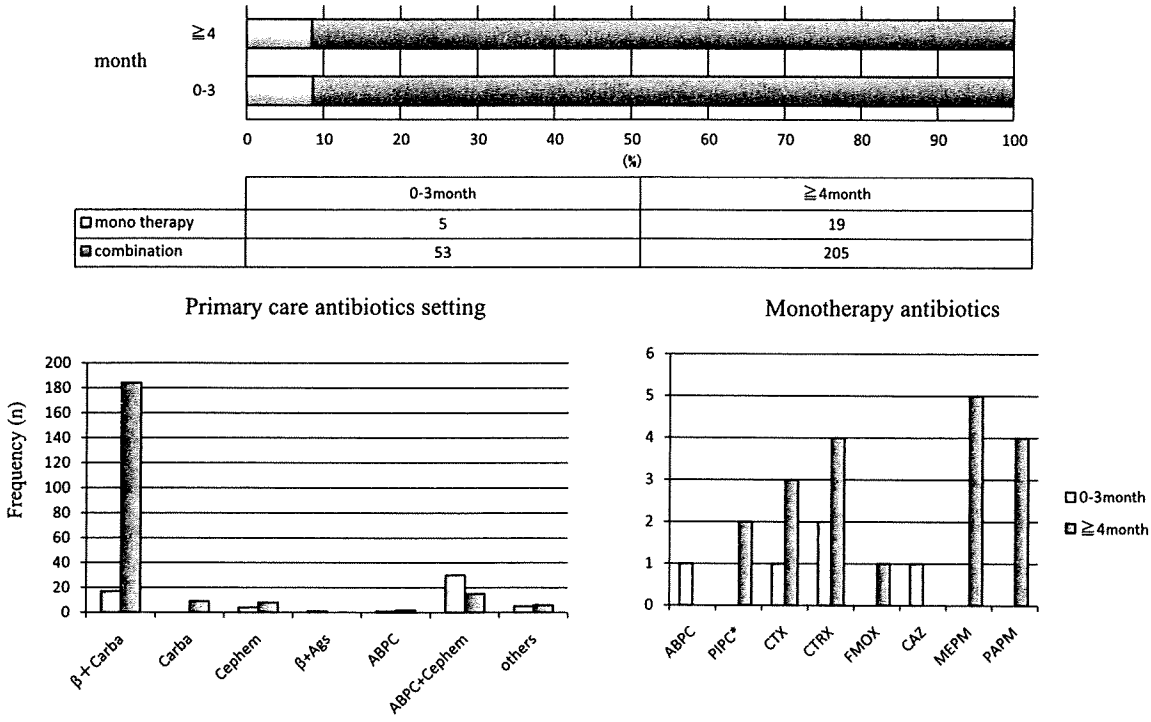


Fig. 6 Antibiotic use by age in initial treatment (2007-2008)



死亡は転医などの理由で予後が不明の6例を除く281例中7例（2.55%）で，年齢，性別，原因菌はTable 1に示した通りであった．死亡例の原因菌としては *S. pneumoniae* 2例（ペニシリン感性・耐性各1例），*H. influenzae* 1例(BLNAR)，GBS，*S. epidermidis*，*P. aeruginosa*，*B. cereus* 各1例であった．死亡例のうち基礎疾患の有無については *S. epidermidis* の症例は生後9日で脳腫瘍（基礎疾患が原因で死亡），*B. cereus* の症例は5歳3カ月で急性リンパ性白血病であったが，その他5例に基礎疾患は認められなかった．

Fig. 7　Comparison initial and second-line treatments by age

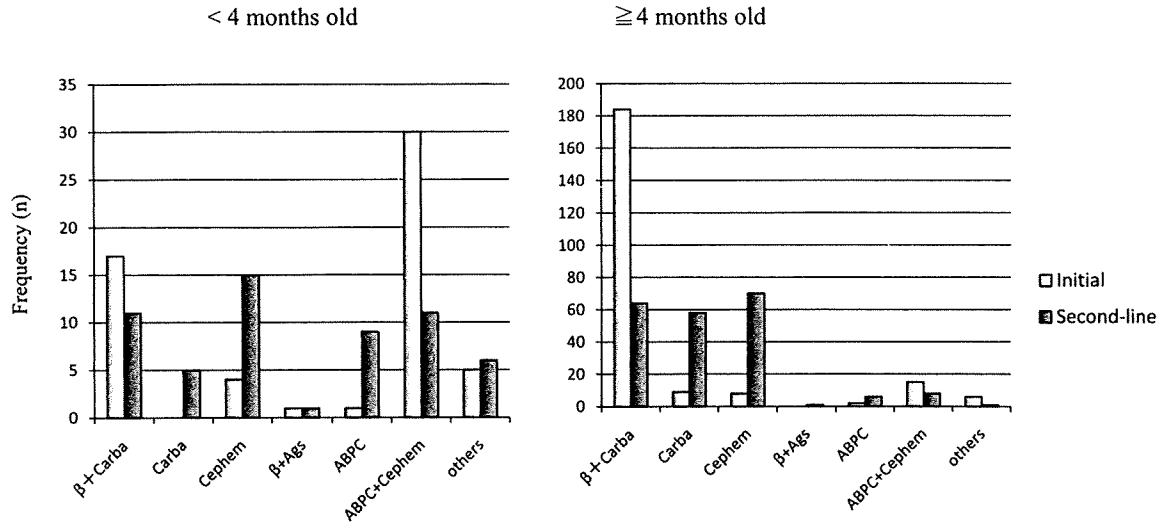


Fig. 8　Frequency of causative organisms by year

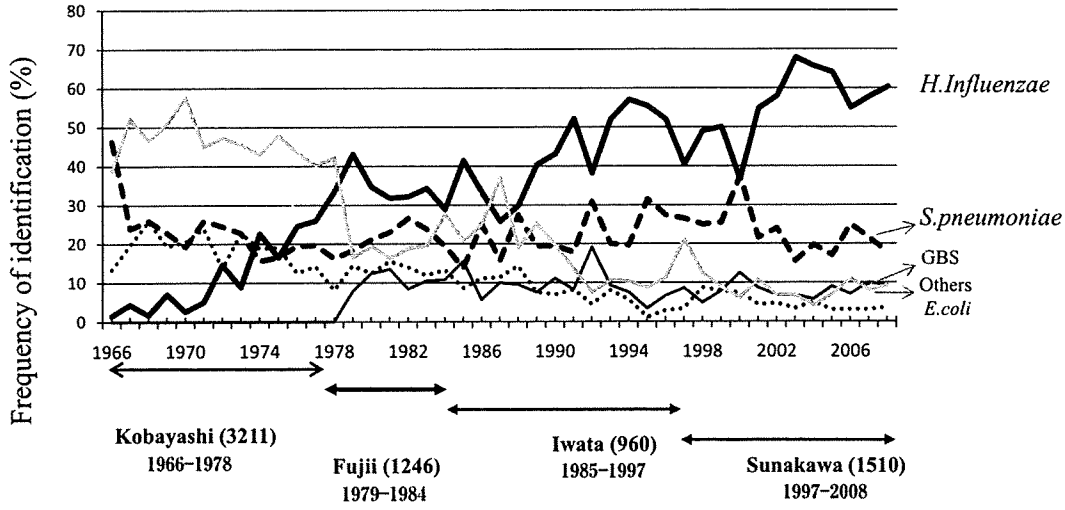


Table 1　Mortality

Age	Gender	Causative organism	Underlying disease
7 days	M	*P. aeruginosa*	
9 days	M	*S. epidermidis*	Brain tumor
1 month	F	GBS	
11 months	M	*S. pneumoniae* (Resistant strain)	
1.08 years	M	*H. influenzae* (BLNAR)	
3.1 years	F	*S. pneumoniae* (Sensitive strain)	
5.25 years	M	*Bacillus cereus*	ALL

2007-2008 (287 cases)

菌種別に死亡，後遺症の有無について解析を行ったが，後遺症については長期間観察している施設と入院期間だけの記載の施設があり，菌種別の死亡率のみを記した．全体の死亡率は 2.5% であり，*H. influenzae* は 0.6%，*S. pneumoniae* は 3.8%，GBS は前回の 10.5% から減少して 3.8% であった．菌種間に死亡率に差は認められなかった．

7．使用抗菌薬

1）初期治療抗菌薬の種類（Fig. 6）

初期治療に使用した抗菌薬の種類は，原因菌の頻度を考慮して 4 カ月未満と 4 カ月以上の年齢群で集計した．

平成22年 1 月20日

アンケート調査協力施設（回答時の施設名で記載）

所在地	病院名
＜北海道＞	旭川医科大学　市立札幌病院　網走厚生病院　市立小樽病院
＜青森県＞	青森県立中央病院
＜秋田県＞	秋田大学医学部
＜岩手県＞	盛岡赤十字病院
＜宮城県＞	仙台市立病院
＜山形県＞	山形県立中央病院
＜長野県＞	国立病院機構長野病院
＜新潟県＞	新潟大学医歯学総合病院
＜栃木県＞	国立病院機構栃木病院　足利赤十字病院　小山市立病院
＜群馬県＞	国立病院機構高崎病院　国立病院機構沼田病院　前橋赤十字病院　総合太田病院
＜茨城県＞	日立総合病院　水府病院
＜千葉県＞	千葉大学医学部
＜埼玉県＞	国立病院機構埼玉病院　埼玉社会保険病院
＜東京都＞	東京大学医学部　慶應義塾大学医学部　東京慈恵会医科大学　順天堂大学医学部　昭和大学病院 東邦大学医療センター大森病院　国立病院機構東京医療センター　都立大塚病院　済生会中央病院 公立福生病院　共済立川病院　東京女子医科大学東医療センター
＜神奈川県＞	横浜市立大学医学部　北里大学医学部　聖マリアンナ医科大学横浜市西部病院　東海大学医学部 昭和大学藤が丘病院　国立病院機構相模原病院　川崎市立川崎病院　横浜市立市民病院 伊勢原協同病院　平塚市民病院　平塚共済病院 海老名総合病院　横浜南共済病院　横須賀共済病院
＜静岡県＞	国立病院機構静岡医療センター　静岡県立総合病院　浜松赤十字病院
＜愛知県＞	名古屋市立大学医学部　藤田保健衛生大学　名鉄病院 国立病院機構名古屋医療センター　厚生連安城厚生病院
＜三重県＞	市立伊勢総合病院　山田赤十字病院
＜岐阜県＞	岐阜大学医学部　高山赤十字病院
＜石川県＞	金沢大学医学部
＜福井県＞	福井赤十字病院
＜京都府＞	京都大学医学部（小児科・ICU）国立病院機構舞鶴医療センター　京都第一赤十字病院　日本バプテスト病院
＜奈良県＞	大和高田市立病院
＜和歌山県＞	日本赤十字社和歌山医療センター
＜大阪府＞	大阪医科大学　大阪市立総合医療センター 住友病院　淀川キリスト教病院
＜兵庫県＞	国立病院機構姫路医療センター　兵庫県立塚口病院　神戸市立医療センター中央市民病院 公立八鹿病院明石市立市民病院　公立豊岡病院　赤穂市民病院　姫路赤十字病院　神鋼加古川病院
＜岡山県＞	川崎医科大学　川崎医科大学付属川崎病院
＜鳥取県＞	鳥取大学医学部
＜広島県＞	県立広島病院　広島市立舟入病院　吉田総合病院 広島赤十字・原爆病院　庄原赤十字病院 マツダ株式会社マツダ病院　尾道総合病院　広島通信病院　広島鉄道病院　JA 府中総合病院
＜島根県＞	国立病院機構浜田医療センター　島根県立中央病院　松江赤十字病院
＜愛媛県＞	愛媛大学医学部　愛媛県立中央病院
＜徳島県＞	徳島大学医学部
＜高知県＞	国立病院機構高知病院
＜福岡県＞	久留米大学医学部　産業医科大学　国立病院機構九州医療センター
＜佐賀県＞	国立病院機構嬉野医療センター
＜大分県＞	国立病院機構別府医療センター
＜熊本県＞	熊本大学医学部
＜長崎県＞	長崎大学医学部・歯学部
＜鹿児島県＞	鹿児島市立病院
＜沖縄県＞	沖縄県立中部病院　沖縄赤十字病院

初期治療薬剤の選択にあたっては，髄液の塗沫・染色やメニンギートキット（日本ビオメリュー）などの原因抗原検出のための迅速診断キットを用いて原因菌を推定して治療を開始する施設と，複数の抗菌薬を組み合わせて広域に対応する施設が見られたが，併用で開始する施設が多く見られた．原因菌としてGBS，*E. coli* が多い4カ月未満では，従来の標準的治療法とされているABPC＋セフェム（cefotaxime：CTX：ceftriaxone：CTRX）を組み合わせた使用が29/53（54.7%）と半数を占め，続いてカルバペネム＋β-lactamの併用が16/53(30.1%)であった．ABPC＋aminoglycosideは1例であった．*H. influenzae* や *S. pneumoniae* が原因として多くなる4カ月以降に関しては，PRSPを考慮したカルバペネム＋β-lactamの併用が184/205（89.7%）と前回の73%に比べさらに増加し，標準的治療とされてきたABPC＋セフェムの併用は15/205（7.3%）であった．セフェム単独は8/205（3.9%）と半減し，PRSPに効果が優れているカルバペネム単独は9/205（4.4%）であった．

2）最終治療（Fig. 7）

最終治療は原因菌の種類・薬剤感受性が判明した後に選択する例が多く，その結果に従って投与することから，併用→単独への変更が多く見られた．今回の調査では4カ月未満ではABPC＋セフェム併用例の約2/3がABPCまたはセフェムの単独使用に，4カ月以上ではカルバペネム＋β-lactam併用例の約2/3がセフェムまたはカルバペネムの単独に変更されていた．

8．ステロイド薬の併用

ステロイド薬の併用の有無について，記載がされていた3カ月未満の症例50例中20例が併用あり（40%），3カ月以上の症例230例中205例が併用あり（91.4%）で，3カ月以上の症例でステロイド薬を使用する例が有意に多かった．ステロイド使用の有無による死亡，後遺症の有無と種類，原因菌別効果を調べたが，特に特徴は認められなかった．ステロイド薬の投与日数は1日投与5例，2日120例（60.6%），3日32例，4日36例，5日以上5例で，2日投与が主流であった．

考　察

今回287例の症例が集積された．小児髄膜炎の発生頻度について，年間の小児入院症例数1,000に対する割合は，2007年1.54，2008年1.62人で，1997年調査開始以降増減はみられないが，約20年前の藤井の報告[3]の3.1～4.0に比べ明らかに減少した（$p<0.001$）．細菌性髄膜炎の定点報告数も，過去9年間定点あたり0.01～0.03を前後し，増減がみられていない[11]．男女比は1.26：1であり，従来[1)~10)]の報告と大きな違いはみられず，年齢分布についても1997年以降大きな変化はみられなかった．

原因菌については，過去の報告と同様に生後3カ月まではGBS，*E. coli*が多く，その後*H. influenzae*，*S. pneumoniae*が主要原因菌となっていた．

我が国の小児細菌性髄膜炎の原因菌の変遷を見ると（Fig. 8），小林の調査[1]の初期には*H. influenzae*の占める割合が極めて低い数字であり，1970年代に入り増加に転じ，その後年々比率が増し[1)~10)]，現在では約2/3を占めている．*S. pneumoniae*は1966年に約50%を占めていたが，翌年以降20～30%を維持している．

GBSは検査が一般化した1979年以降から報告が見られ1992年の19.1%が最高で，その後は10%前後を推移している．*E. coli*は1990年代以降10%以下の数値であった．

Hibワクチンの導入以降の海外で，原因菌として頻度の少なくなった*H. influenzae*は，ワクチンの未承認であった我が国では2005～2006年の調査で全体の55%を占め，相変わらず第一位であった．その上BLNARなどの耐性菌が増加している点[12]からも，2007年承認されたHibワクチンのリスクの高い乳幼児に対する早期の普及が望まれる．

薬剤耐性化が問題となっている*H. influenzae*と*S. pneumoniae*の各施設で実施した薬剤感受性は，ABPC耐性の*H. influenzae*は1997年に比べ有意に増加し，今回調査の2007年55.5%，2008年51.3%で，最も高かった2003年の70.4%に比べ年々減少の方向にあるといえる．ペニシリン耐性の*S. pneumoniae*も2007年72%，2008年56.5%であり，*H. influenzae*と同様に2004年の83%に比べ年々減少の方向にあった．現在我々が実施している，全国の小児由来*H. influenzae*と*S. pneumoniae*の薬剤感受性サーベイランスでも2007年にはPRSP，BLNARの割合が減少の方向にあり，今後抗菌薬の適正使用と耐性菌の出現についての検討を行っていきたい．

治療に関して，抗菌力の面からは耐性菌を含めて*H. influenzae*に対してはセフェム系のCTRXまたはCTXおよびカルバペネム系のmeropenem（MEPM）が，*S. pneumoniae*に対してはカルバペネム系で髄膜炎に適応を有するpanipenem/betamipron（PAPM/BP）とMEPMが最も優れておりガイドラインにも記載されている[13)14)]．

初期治療について原因菌の頻度から4カ月未満，4カ月以上に分けて検討したところ，1997年～2000年は当時標準的治療とされていたABPC＋CTX　or CTRXが最も好んで使用されていた．PRSPやBLNARなどの耐性菌の蔓延が小児科医の間で広く認識されて以来，治療に関して変化がみられるようになった．*H. influenzae*や*S. pneumoniae*の分離頻度が少ない4カ月未満ではβ-ラクタム＋カルバペネムの組み合わせは増加の傾向にあるものの，2007～2008年で17例（29.3%）であったが，ABPC＋セフェムは1997～2000年51例（61%），2003～2004年には19例（43%）に減少したものの，2007～2008年再び30例（51%）となり，最も好まれる初期治療の組み合わせとなっている．一方*H. influenzae*や*S. pneumoniae*の多い4カ月以上の小児に対しては，これら耐性菌を念頭に置いたカルバペネム＋セフェムの組み合わせが年々増加し，2007～2008年には184例（82%）となっていた．*S. pneumoniae*に対して抗菌力が若干劣るABPC＋セフェムの組み合わせは1997～2000年の202例（59.6%）から2007～2008年には15例（7.5%）に減少していた．セフェム単独使用も1997～2000年の50例（14.7%）から2007～2008年には8例（3.6%）と減少した．

初期治療が併用で開始されても，その約2/3の症例では原因菌の薬剤感受性の結果ABPC，セフェム，カルバペネムのいずれかの薬剤単独投与に変更されており，抗菌薬の適正使用の考えが浸透しつつあることが

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うかがえた.

ステロイド薬併用の有無に関する調査では，3カ月未満では併用は40%と低いが3カ月以降はほとんどの症例がステロイドが併用され，2日間投与が標準的な治療として広く知られていることがうかがわれた．残念ながら今回の調査からステロイド併用の意義に関する結果は得られなかったが，アンケートの質問内容について再度検討する必要があると考えられた．

今後は *H. influenzae* や *S. pneumoniae* に対してより有効な薬剤の開発が望まれるとともに，我々自身が抗菌薬の投与量，投与間隔，投与時間などPK-PD理論に基づいた投与法について検証し，有効性・安全性はもとより耐性菌発現防止にも注意をはらう必要性を痛感した．更に，これら耐性菌の伝播が保育園を介して行われていること，髄膜炎の発症年齢を考慮すると，我が国においても，既に海外で感染予防効果が十分に確認されている *H. influenzae*, *S. pneumoniae* のワクチン[15)~17)]による予防法が早期に確立されるべく努力していく必要がある．

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Childhood Bacterial Meningitis Trends in Japan from 2007 to 2008

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We surveyed pediatrics bacterial meningitis epidemiology from January 2007 to December 2008 in Japan, with the following results: Cases numbered 287-160 male and 127 female-equivalent to 1.54-1.62 of 1,000 pediatric hospitalization per year. Children under 1-year-old accounted for the highest number of cases, which decreased with increasing age. *Haemophilus influenzae* was the most common cause of infection, followed by *Streptococcus pneumoniae*, group B streptococcus (GBS), and *Escherichia coli*. GBS and *E. coli* were major pathogens in children under 4 months of age, while *H. influenzae* and *S. pneumoniae* mainly accounted for those over 4 months of age. Susceptibility tests showed that 51% of *H. influenzae* isolates and 56.5% of *S. pneumoniae* isolates in 2008 were drug-resistant.

Ampicillin combined with cephem antibiotics effective against GBS, *E. coli*, and Listeria, were mainly used to initially treat those under 4 months of age. In those over 4 months of age, carbapenem antibiotics are effective against PRSP and cephem antibiotics against *H. influenza*.

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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*		*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.

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

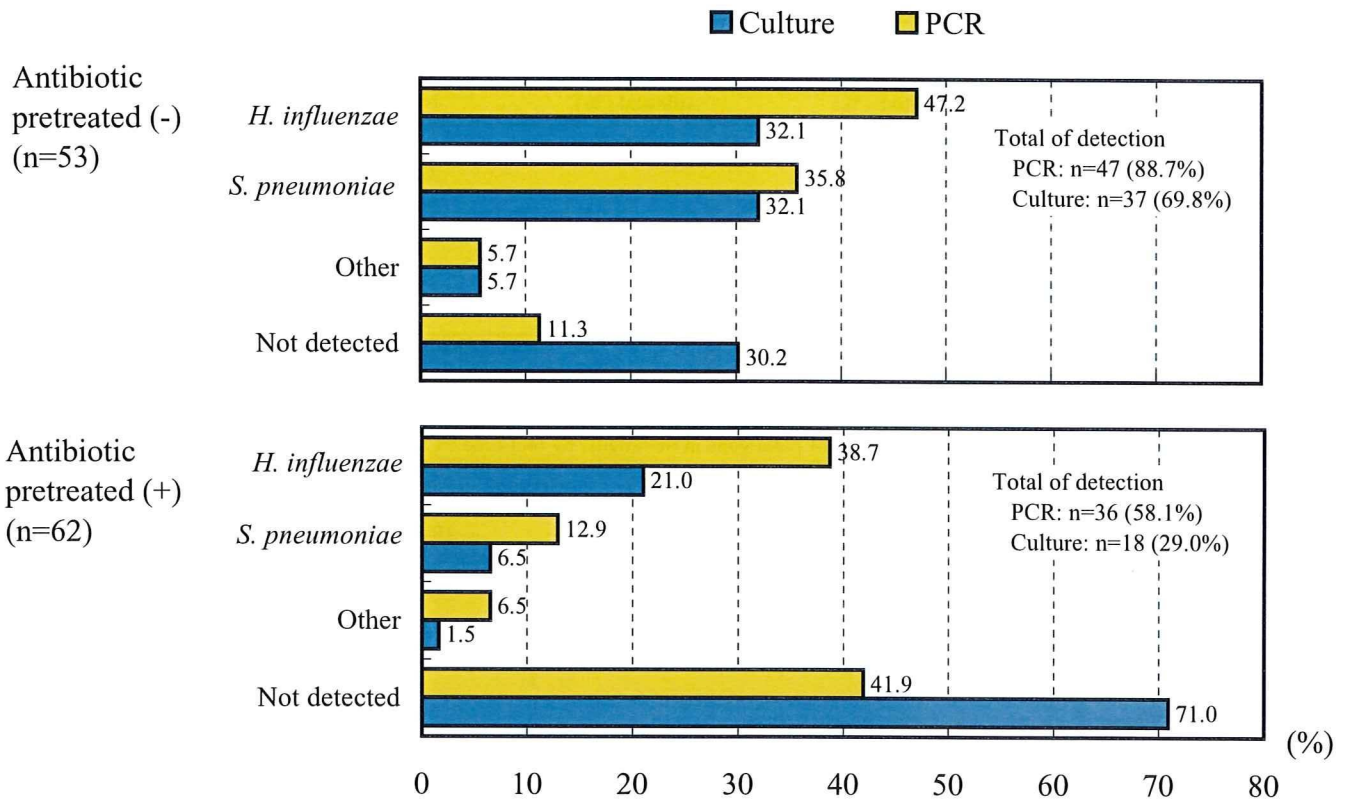

Fig. 1. Influence of prior antibiotics on the detection of causative pathogens by real-time PCR or culturing

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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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′-TATTCGCTTAGAAAATTAAAAACAGG-3′ and an ABI PRISM 3130/3130xl Genetic Analyser (Applied Biosystems). The first 300 bases of the 5′ end of the *emm* gene were compared with those in the CDC *emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strep-blast.htm). An *emm* type showing more than 98 % identity with a CDC reference strain was identified as that particular *emm* type.

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

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

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

RESULTS AND DISCUSSION

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

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

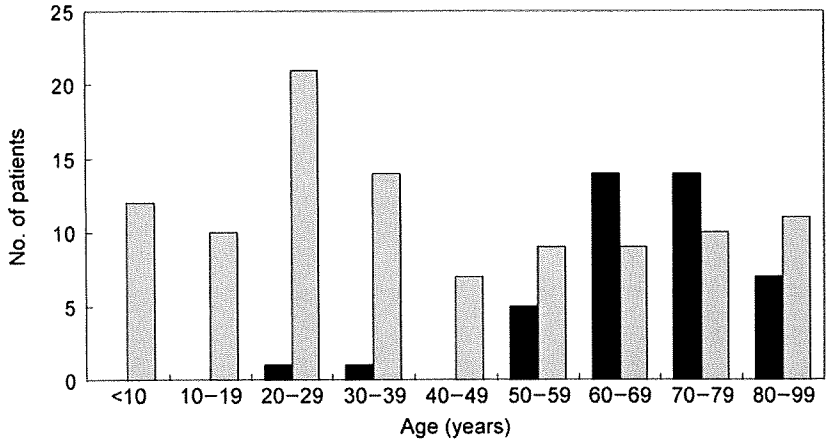

Fig. 1. Age distribution of patients with *Streptococcus dysgalactiae* subsp. *equisimilis* infection. In patients with invasive infection (black bars; n=42), causative agents were isolated from blood (n=32), synovial fluid (n=6) and tissue (n=4). In patients with non-invasive infections (grey bars;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

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

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

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

Susceptibility to 12 agents

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

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

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

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

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

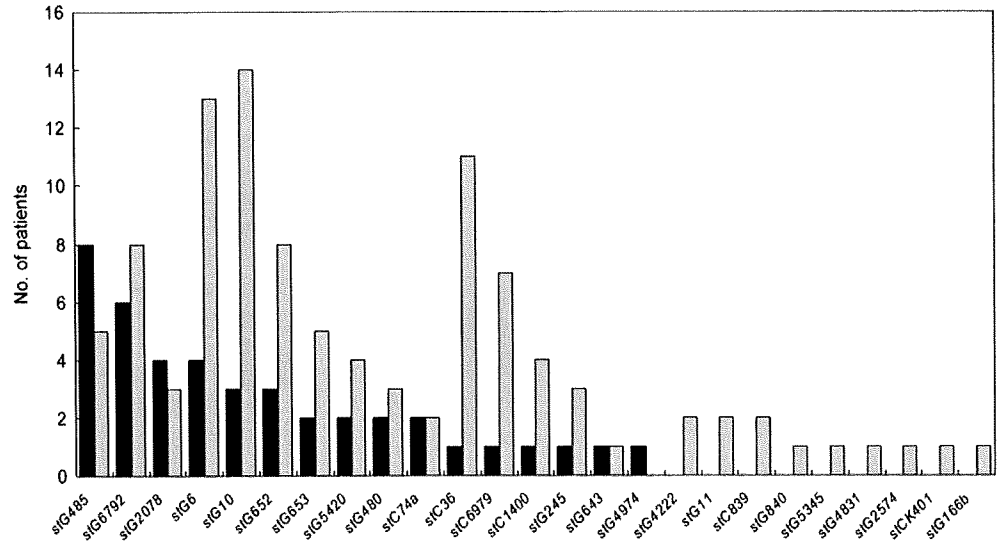

Fig. 2. Comparison of *emm* types in *Streptococcus dysgalactiae* subsp. *equisimilis* isolates separated into invasive (black bars; $n=42$) and non-invasive (grey bars; $n=103$) infections. The distribution of *emm* types was significantly different between the two groups (χ^2 test, $P<0.001$).

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

Typing of *emm* and PFGE profile

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

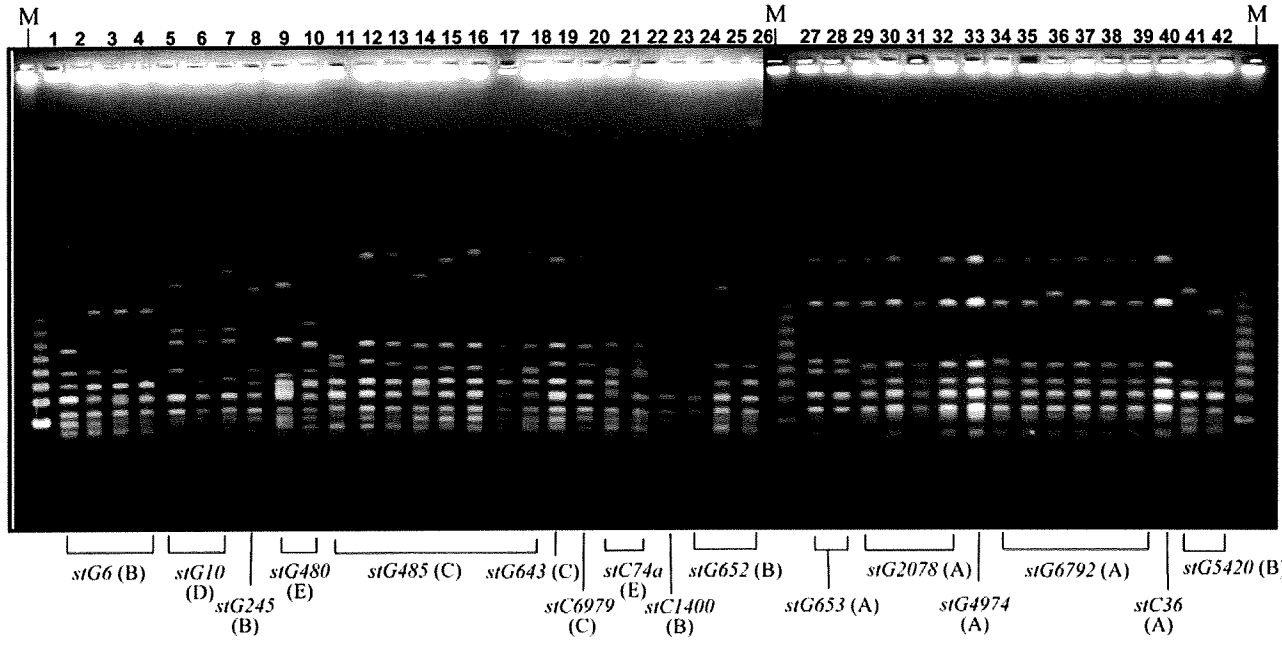

Fig. 3. PFGE profiles and their classification into five clones of chromosomal DNA from *Streptococcus dysgalactiae* subsp. *equisimilis* isolates from patients with invasive infection. Chromosomal DNAs were digested with *Sma*I. Capital letters in parentheses represent clones. Lanes: M, size marker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