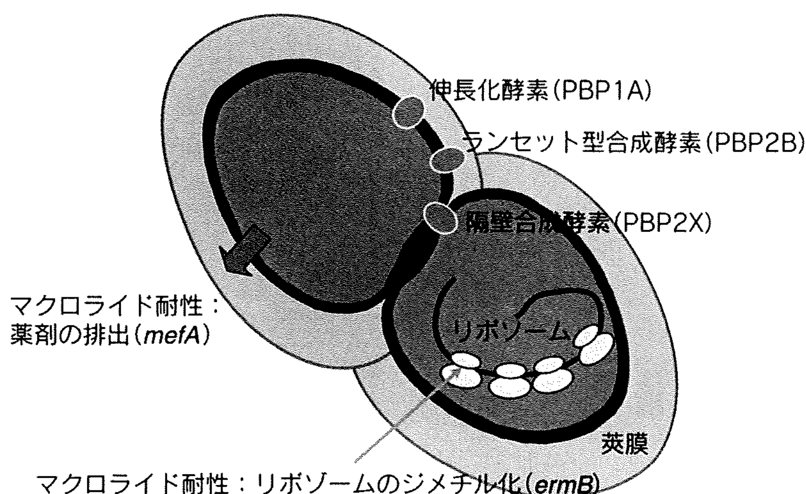

図2 肺炎球菌の薬剤耐性化のメカニズム
ペニシリン感性菌：gPSSP（PBP の遺伝子変異を認めない）
ペニシリン軽度耐性菌：gPISP（*pbp2x*, *pbp2b*, *pbp1a*+*2b*, *pbp2x*+*2b* などの遺伝子変異）
ペニシリン耐性菌：gPRSP（*pbp1a*+*2x*+*2b* 遺伝子変異）

下しているのである．ちなみに，耐性肺炎球菌に見出される遺伝子は，口腔内連鎖球菌の PBP 遺伝子と組み換えで生じたハイブリッド遺伝子である．正しくは“組み換え耐性遺伝子”であるが，ここでは便宜上単に“変異”と表現する．

マクロライド系薬剤耐性化には，23S rRNA をジメチル化する酵素をコードする *ermB* 遺伝子と，菌体内へ取り込まれたマクロライド系薬剤を異物として排出する蛋白をコードする *mefA* 遺伝子とが知られている．前者の遺伝子を保持するとマクロライド系薬剤すべてに高度耐性化し，後者の遺伝子を保持すると中等度耐性となるが，いずれかの遺伝子保持株は併せて 80%に達している．もはや肺炎球菌に対する第 1 選択薬としては，マクロライド系薬剤は使用できない．

本邦では，既存のニューキノロン系薬剤で小児の呼吸器感染症に認められた薬剤はないが，成人に多く使われているレボフロキサシンに耐性の肺炎球菌が世界的に増加しつつある．

Ⅳ．薬剤感受性と耐性遺伝子の関係

肺炎球菌に対する β-ラクタム系薬剤の感性/耐性の識別は，基準薬のペニシリン（PCG）の名前を付してよばれる．しかし，上述したように，耐性遺伝子を保持する株は多かれ少なかれ，すべての β-ラクタム系薬剤に対する感受性が低下している．

世界的に用いられる CLSI（Clinical and Laboratory Standard Institute）の基準では，PCG に 0.06 μg/ml 以下の MIC を示す株を感性菌（penicillin-susceptible *S. pneumoniae*：PSSP），0.125～1 μg/ml を軽度（中等度）耐性菌（penicillin-intermediate resistant *S. pneumoniae*：PISP），2 μg/ml 以上をペニシリン耐性肺炎球菌（penicillin-resistant *S. pneumoniae*：PRSP）とするよう勧告している．

しかし，**図 3** に示すように，耐性化には 3 つの PBP 遺伝子変異が主に関与しているため，菌がそのうちいくつを保持しているかで MIC は微妙に違ってくる．いずれの薬剤でも，感受性分布を描くとなだらかな 2 峰性を示し，感性/

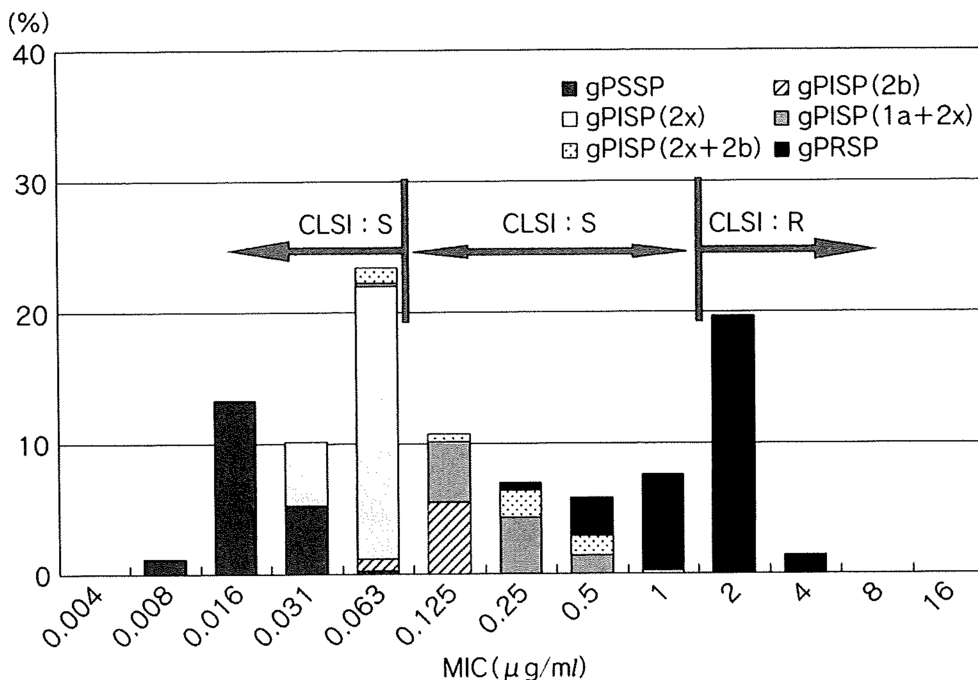

図3 肺炎球菌の PenicillinG 感受性（n＝496）
注：この成績には小児由来と成人由来とが半数ずつ含まれる.

耐性が明瞭に識別できない．そして，このような分布に CLSI の基準を当てはめると，分布と乖離していることが明らかである．むしろ遺伝子解析の方が 2 峰性分布と一致している．また，PCG では，わが国で 25%前後と高頻度に分離され，セフェム系薬剤に軽度耐性を付加する *pbp2x* 変異株が識別できないという問題を抱えている．臨床的にも CLSI の基準を当てはめることができないのは，欧米とわが国では投与量が大きく異なっていることによる．日本での通常投与量は欧米のほぼ半量であり，特に経口セフェム系薬剤投与で得られる血中有効濃度はせいぜい 1 μg/m*l* 前後，ペニシリン系薬剤よりもはるかに低いことを承知しておきたい．

CLSI の識別と区別するため，遺伝子変異に基づく結果には genotype を表す g を付して gPISP，あるいは gPRSP と表現するが，ちなみに gPSSP は 19.9%に過ぎず，gPISP が 48.3%，gPRSP の割合が 31.8%である．

図4には，参考までに主な注射用 β-ラクタム系薬剤の感受性累積分布を示す．注射薬のブレイクポイントを 1 μg/m*l* とすると，セフェム系薬剤では耐性菌をカバーしきれていない．化膿性髄膜炎のように，さらに良好な髄液移行が必要となる疾患には注意が必要となる．

V．侵襲性感染症由来肺炎球菌の莢膜型

図5には，2006 年の 1 年間に全国規模で収集された侵襲性感染症由来の肺炎球菌について，① 血清型別，② 遺伝子解析，③ 治療抗菌薬に対する感受性を正確に測定し，分離比率の高い莢膜型から順に並べてある．また，その中に占める gPSSP，gPISP および gPRSP を区別している．

これらの疫学研究には全国 180 医療機関の関係者が参加している．市中で発症し入院となった 20 歳以下の症例に限っているが，発症のピークは 1 歳にあり，2 歳までが 80%を占めている．疾患名としては敗血症がもっとも多く，次いで

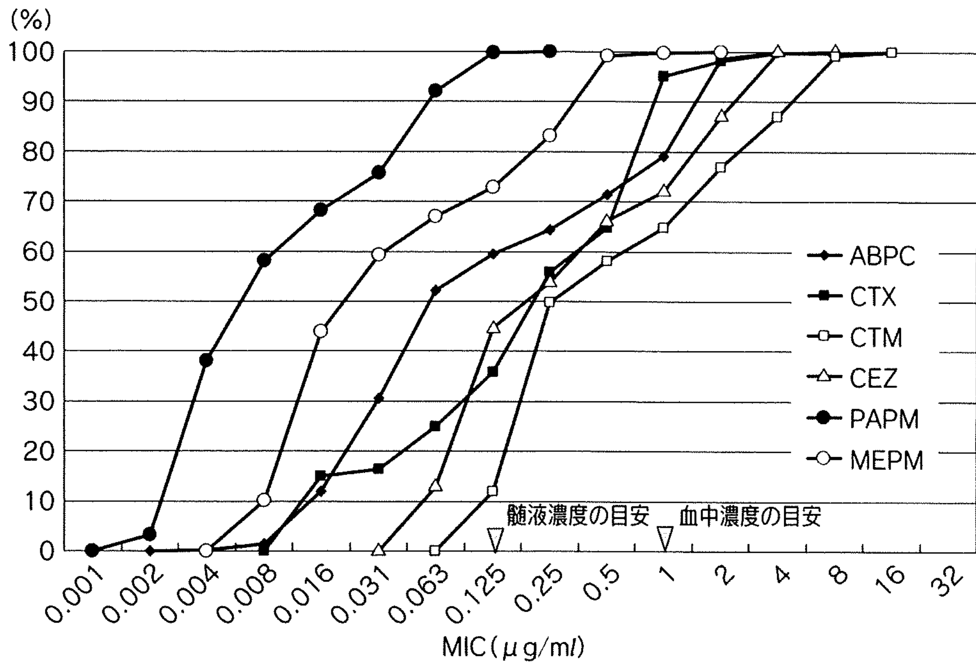

図4　肺炎球菌に対する主な注射用抗菌薬の感受性累積分布

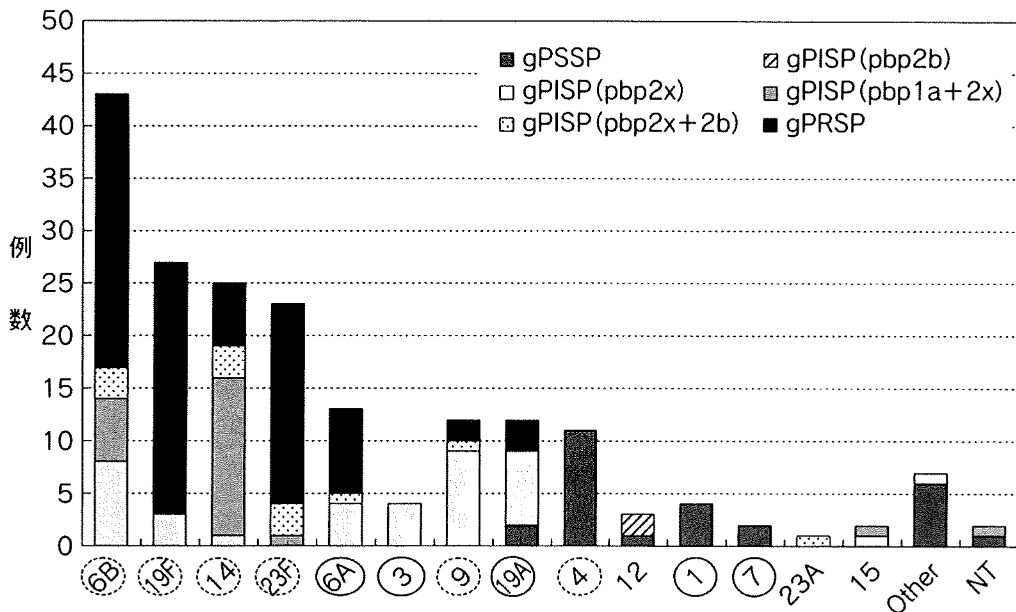

図5　小児侵襲性感染症由来株の莢膜型と耐性遺伝子との関係

注：図3の中の小児由来株についてのみ示す．◌は7PCVタイプ，○は13PCVにも含まれるタイプ．

化膿性髄膜炎と肺炎例である．その他に急性中耳炎の鼓膜切開液や蜂窩織炎の閉鎖性膿も多少含まれてはいるが，大半は血液あるいは髄液由来である．

成績をみると，日本で圧倒的に多い莢膜型は6B，次いで19F，14，23Fである．続いて6A，9，19A，4型も一定の比率で分離されている．このうち，7PCVに含まれる型には点線の丸印を付したが，18型のみがほとんど分離されていない．

この成績を元に分離株に対する7PCVのカバー率を算出すると約73.8%となる．特にPRSPの割合の高い6B，19F，14，23Fをカバーしていることは注目に値する．しかし，同一グループの中でも別の型，すなわち6Aや19Aの感染防御効果は乏しいことが，欧米におけるワクチネーション後の大規模疫学調査で明らかになってきている[10]．急性中耳炎でもっとも問題となるムコイド型コロニーを形成する3型もカバーされていないことと併せて，懸念される課題である．

そのような背景から，13PCVは構築されたと聞いているが，それらには1，3，5，6A，7F，19A型が追加されており，カバー率を試算すると92.1%とはるかに高い値となる．予防という視点で捉えると，本来は13価PCVによるワクチネーションが必要である．

おわりに

肺炎球菌の薬剤耐性化は，医療経済上わが国のみならず世界的な問題である．ドイツやオランダ，スイス，あるいは北欧の一部の国では依然としてPRSPの割合は低いが，米国でも30%となっており，ほとんどの国が無視しえない耐性率となっている．

感染症の予防に重点をおく欧米では，Hibワクチンにみられるように，肺炎球菌感染症の予防としても早くからワクチネーションが考えられてきた．小児用として開発された7PCVは欧米各国で定期接種が始まり，すでに5年以上が経過している．先の47回Interscience Conference Antimicrobial Agents and Chemotherapy（ICAAC：2007年9月，シカゴ市）においても，ワクチン導入後の莢膜型の変化に関する大規模疫学調査が多く報告されている．それらの成績をみると，ワクチン接種によって7PCVに含まれる型は70%から10%程度へと劇的に減少している．その反面，ワクチンに含まれない型，特に19A，15，35Bが増加しており，また今までほとんど認められなかった型が分離され始めていることが注目される．

しかし，7PCVがわが国で多く分離されるPRSPの大部分をカバーすることは紛れもない事実である．保育園児の増加，基礎疾患を有する児，難治性感染症の増加を考えると，早期に定期接種として導入されることが期待される．

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特集　感染症の新しい検査法と最近のトピックス

Ⅱ．各　論

呼吸器感染症原因微生物

－Real-time PCRによる迅速検索－

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Key Words
呼吸器感染症
原因微生物
Real-time PCR
迅速診断
市中肺炎

要旨

呼吸器感染症原因微生物の網羅的検索用として，われわれが構築した6菌種検索用のReal-time PCR用キットと，12種のウイルス検索用として構築しつつあるキットについて概説し，さらにそれらを小児市中肺炎に対して応用した際の成績について述べる．細菌検索に1.5時間，ウイルスには3時間を要しているが，省力化されておりルーチン検査に使用可能である．臨床的には入院時からevidenceに基づいた抗菌薬使用ができるメリットを有している．

はじめに

細菌の同定，あるいはその病原因子や薬剤耐性遺伝子の迅速検索法として，PCR法（従来型PCR）が応用され始めてから久しいが，それらをルーチン検査に使用するとなると，なかなか容易ではない．それにはいくつかの理由が考えられる．まず，症例の臨床所見から強く推定される起炎微生物（細菌とウイルスを含む）をPCR法で検索しても，単独検索で結果が陰性の場合，あらためて通常の培養検査に戻って実施せざるをえないといったことがあげられる．第二に，従来型PCRでは，DNAの増幅後に電気泳動や染色をして解析するといった手技上の煩雑さもネックとなっている．第三には，厳しさを増した医療経済の実態からは，どのように優れた方法であっても，検査コストが高ければそのルーチン化はほぼ困難といったこともあげられる．

このようないくつかの問題点をクリアする方法として，従来型PCR法にかわり，目的遺伝子のDNA増幅を行いながら，そのDNA鎖にさらに蛍光標識probeを結合させ，蛍光発色量を自動的に測定して検索する方法が開発され，急速にその応用範囲が広がっている．

本稿では，われわれが呼吸器感染症原因微生物の網羅的検索用として開発した細菌検索用キット[1)]と，構築中のウイルス検索用キット[2)]を用いるReal-time PCR法について，その具体的な方法と成績の一部を述べたい．

Real-time PCR法

1．原　理

図1には，当該市販キットに用いられているReal-time PCRの原理を示す．従来型PCR法は，検索したい遺伝子の一部にsense primerと

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reverse primer からなる一対の primer を設計し，DNA を増幅させた後に電気泳動で DNA 断片を確認するが，Real-time PCR 法では，さらに増幅される DNA 断片中の 20 bp 程度の塩基配列と相補的な probe を設計する．タカラバイオ（株）から市販されているキット（CycleavePCR®呼吸器感染症起因菌検出キット）には，キメラ probe が使用されているが，molecular beacon（MB）probe でもその原理は同じである．つまり，20 bp 程度の相補的塩基配列の両端に，蛍光物質（FAM:6-carboxyfluorescens など）と，その発色を抑えるクエンチャー（black hole quencher 1）をそれぞれ結合させたものを合成する．増幅される DNA 断片に結合していない状態では，蛍光色素とクエンチャーが近くに位置するため，蛍光発色が抑えられている．一方，増幅が開始されて，その二本鎖 DNA が高温域で一本鎖 DNA に変性し，次いで反応液の温度が下り始めると，直鎖状になった probe が相補的 DNA 部分に結合し始める．結果的に，蛍光色素とクエンチャーが離れ，発色する．Real-time PCR 機器は，この蛍光量の増加をサイクルごとに自動的に測定する仕組みとなっている．

2. DNA/RNA の抽出と Real-time PCR プロトコール

PCR に際しては，検査材料からいかに簡単で効率よく，かつ短時間で DNA と RNA とを抽出できるかにかかっている．図2に示すように，DNA/RNA が抽出できる EXTRAGEN Ⅱ 核酸抽出キット〔東ソー（株)〕を用いると，20 分程で抽出でき，それらは細菌検索とウイルス検索の両方に用いることができる．

まず細菌検索について述べる．同時検索菌種は *Streptococcus pneumoniae*（肺炎球菌），

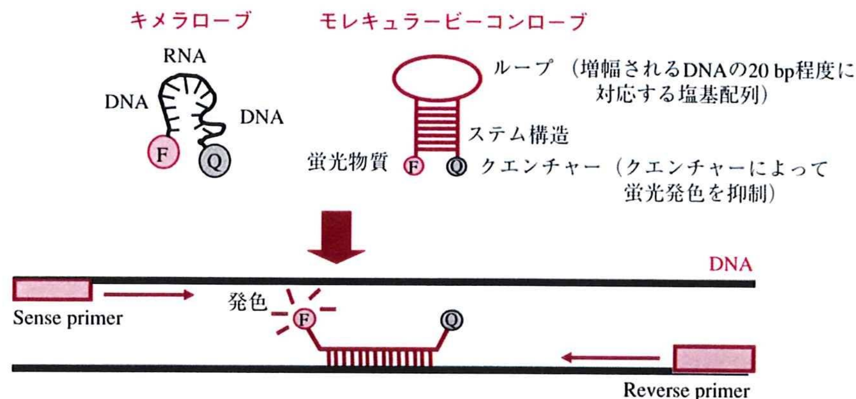

図1　蛍光プローブを用いる Real-time PCR 法

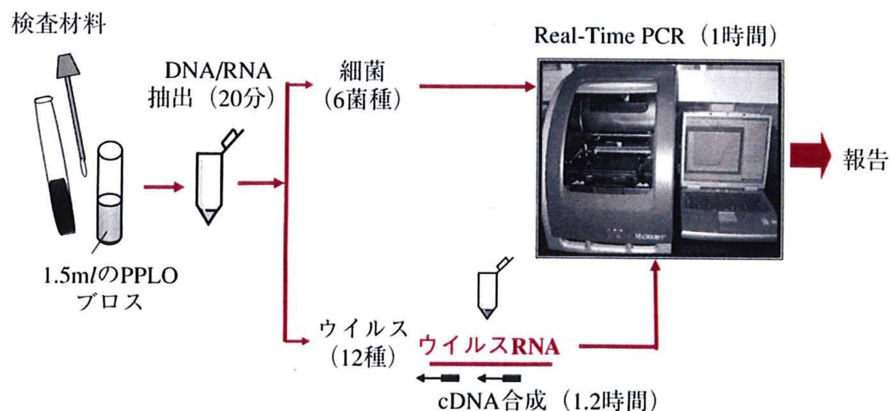

図2　Real-time PCR による細菌/ウイルスの迅速検索プロトコール

Haemophilus influenzae（インフルエンザ菌），β溶血レンサ球菌，*Mycoplasma pneumoniae*（マイコプラズマ），*Chlamydophila pneumoniae*（クラミジア），そして*Legionella pneumophila*（レジオネラ）の6種である．β溶血レンサ球菌は2種の遺伝子で*Streptococcus pyogenes*と，そのほかのβ溶血性レンサ球菌を区別，レジオネラも同様に2種遺伝子検索で*L. pneumophila*とそのほかのレジオネラを区別している．対象菌種は，小児と成人の市中感染症，とくに肺炎例において，起炎菌としての可能性の高いものを選択した．黄色ブドウ球菌のように，健常人の保菌率が高く，起炎菌か否かの判断がむずかしいものは対象としていない．

ちなみに，肺炎球菌の検索遺伝子は自己溶解酵素をコードする*lytA*遺伝子，インフルエンザ菌，マイコプラズマ，クラミジアは*16S rRNA*遺伝子，レジオネラはマクロファージ炎症性蛋白をコードしている*mip*遺伝子と*16S rRNA*遺伝子，β溶血性レンサ球菌はstreptolysin O遺伝子と*16S rRNA*遺伝子である．

これらの反応液は，96ウエルのPCR用プレートにあらかじめ分注しておき，症例数に応じカットして用いる．PCRは各ウエル内に抽出DNAサンプルを2μlずつ加えた後スタートする．検体受領から結果判明までの所要時間は1.5時間であるが，サンプル中の菌量が多ければ，1時間以内に結果が判明する．ちなみに，このPCRはチューブあたり1～10コピーの菌量が，35～38サイクルで陽性反応を示す感度に設定されている．従来型PCRに比べ，増幅されたDNAにさらに結合するprobeを使用することから，特異度が高く，検体中の微生物の定量も，**図3**に示したマイコプラズマにみられるように可能である．

3. Real-time PCRによるウイルス検索

呼吸器系ウイルスの検索であるが，現在，**表**に示す12種ウイルスを同時に検索できるまでに構築しつつある．すなわちAdenovirus（Adeno），Influenza virusAとB（FluA, FluB），RSV

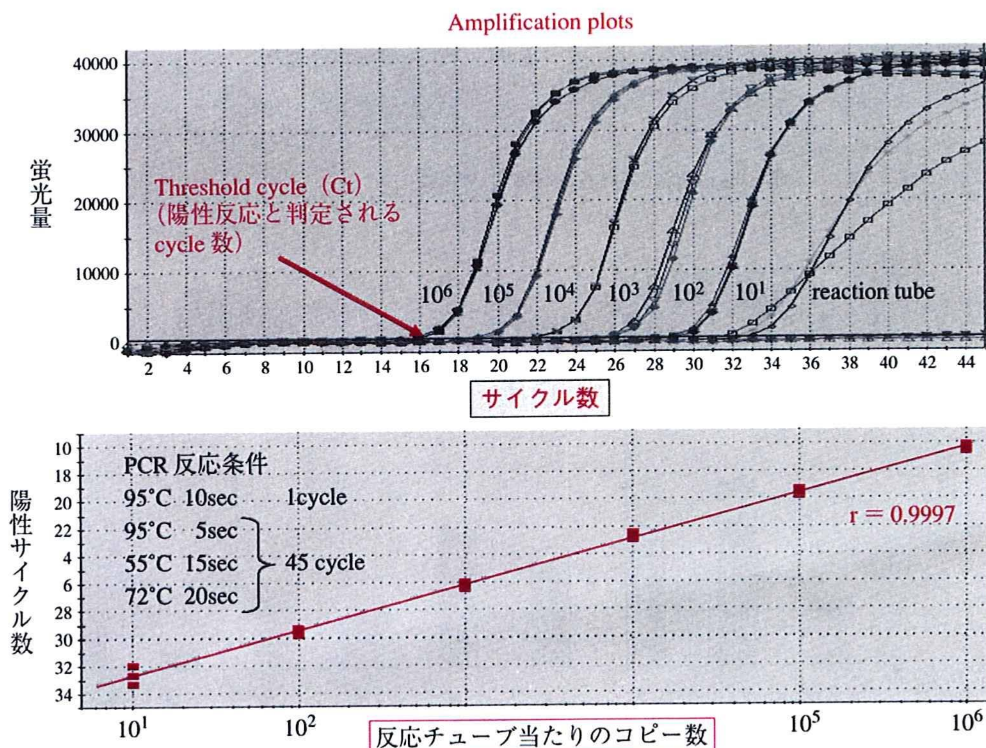

図3 ***Mycoplasma pneumoniae*の陽性反応**

（subgroupAとB），Parainfluenza virus1, 2, 3（PIV1, PIV2, PIV3），Rhinovirus, Enterovirus, Coronavirus, Human metapneumovirus（hMPV）[3]，およびHuman bocavirus（HBoV）[4]である．AdenoとEnterovirusはすべてが検索できるわけではなく，その一部のタイプのみである．また，これらウイルスの検索にはcDNA合成が必要となるので，結果判明までにやや時間がかかることになる（図2参照）．

現在，cDNAの合成にはReverTra Ace®を含むTOYOBO（株）の試薬を用いているが，手法的にはいろいろ開発されつつあり，2ステップ法でも将来逆転写反応部分が15分程度まで短縮できそうである．近い将来ウイルス検索も2時間以内に終了できよう．

小児の市中肺炎例への応用[5]

図4には，中山らが（財）博慈会記念総合病院小児科において1年間に経験した117例のCAP例に対し，Real-time PCRを用いて検索した起炎菌の成績と年齢の関係を示す．これらの成績は，外来受診でCAPと診断され，入院となっ

表　検索対象としたウイルス

ウイルス（略称）	核酸の種類	増幅する目的遺伝子
1. Adenovirus	DNA	ヘキソン（hexon）
2. Influenza virus A（FluA）	RNA	非構造蛋白（NS1）
3. Influenza virus B（FluB）	RNA	核蛋白質（NP）
4. RSV（subgroup A, B）	RNA	F蛋白（F）
5. Parainfluenza virus 1（PIV1）	RNA	ヘマグルチニン・ノイラミニダーゼ（HN）
6. Parainfluenza virus 2（PIV2）	RNA	ヘマグルチニン・ノイラミニダーゼ（HN）
7. Parainfluenza virus 3（PIV3）	RNA	ヘマグルチニン・ノイラミニダーゼ（HN）
8. Rhinovirus	RNA	非翻訳領域（5' NCR）
9. Enterovirus	RNA	非翻訳領域（5' NCR）
10. Coronavirus	RNA	スパイク糖蛋白（S）
11. Human metapneumovirus（hMPV）	RNA	核蛋白質（NP）
12. Human bocavirus（HBoV）	DNA	非構造蛋白（NP-1）

Adenoは16/51血清型を増幅する
Enteroはcoxsackie A9, A16, B5, B6, echo 6, 11, 30, entero 71を増幅する

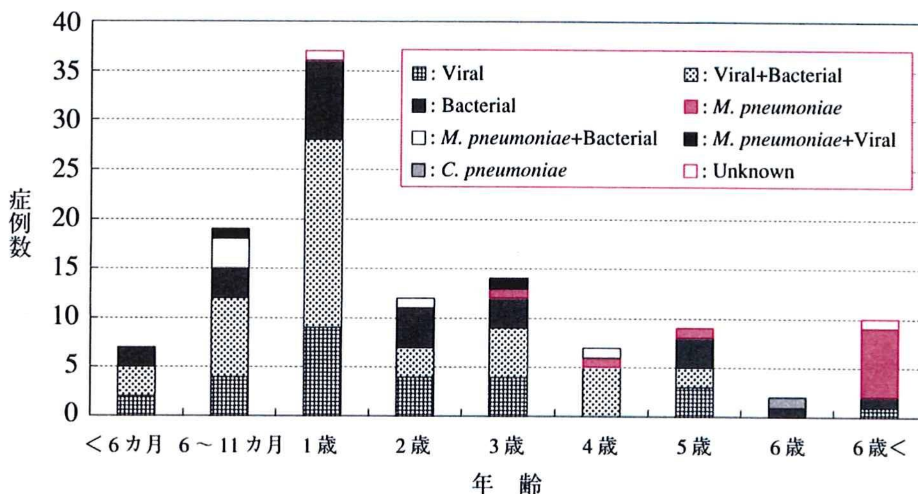

図4　年齢別にみた小児 市中肺炎例の原因微生物（n = 117）

た症例から治療開始前に上咽頭ぬぐい液を採取し，PCRと並行して培養も実施し，その精度の検証も行った正確な成績である．マイコプラズマ，クラミジア，ウイルスの一部については，抗体価測定を実施してPCRとの一致の有無を調べている．細菌単独感染21.4％，細菌とウイルスの混合感染38.5％，マイコプラズマ単独感染8.5％，マイコプラズマと細菌，あるいはウイルスの混合感染7.7％，クラミジア感染0.9％，ウイルス単独感染23.1％の割合となっている．

合計すると，細菌関与例は全体の72.6％，ウイルスのそれは61.5％，マイコプラズマは16.2％，クラミジアは0.9％となった．これらは，Juvenら[6]の報告ときわめて近い．

また，細菌の中では肺炎球菌がもっとも多く，インフルエンザ菌はその1/3程度であった．ウイルスは単独感染時にはRSVが多く，次いでAdeno，PIV，FluAであったが，細菌との混合感染例ではPIVが多く，次いでRSV，FluA，Adeno，Rhino，hMPVが検出されていた．

誌面の関係でここには示さないが，CAP例におけるそれぞれの起炎微生物別に，入院時のるWBCとCRPの関係をみると，Adenoを除くウイルス単独感染例群，ウイルスと細菌の混合感染群，細菌単独感染群，マイコプラズマ感染群，マイコプラズマと細菌の混合感染群では，それぞれのWBCとCRPの中央値間に有意な差が認められている[5]．

まとめ

市中感染症をひきおこす細菌，とくに呼吸器感染症の原因菌である肺炎球菌[7]，インフルエンザ菌[8]，そしてマイコプラズマ[9]やβ溶血性レンサ球菌においても，次々とその耐性化が問題となってきている．最大の原因は，起炎微生物を特定しないままに抗菌薬が投与され，治療のエンドポイントも不確実なままに使用されるといったことがあげられる．

本来，抗菌薬投与にあたっては，起炎微生物を特定し，もっとも適切な抗菌薬を使用することが原則である．この最終ゴールに到達するためには，培養検査からの脱却が必要であるが，ここで述べたReal-time PCR法はそれに近づくための有力な手段のひとつとなりうると考える．そして，このような網羅的な検索法を実施することが，患者にとってもまた医療経済のうえからも，結局はcost-effectiveであることを，最後に強調しておきたい．

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第12回ウイルソン病研究会学術集会のお知らせ

会　期　平成20年5月10日（土曜）14:00～18:00（予定）
会　場　東邦大学医療センター大森病院 臨床講堂（大田区大森西6-11-1）
代表幹事　青木継稔（東邦大学）
参加費　2,000円
演題募集　Wilson病をはじめMenkes病など銅代謝異常症に関する症例報告，研究報告（中間的なものでも可）など
演題名・施設名・演者名（共同演者がある場合は発表者に○印），連絡先（住所，TEL，FAX），発表者メールアドレスを記入しe-mailにて事務局までご送付ください．なお演題申込後1週間を過ぎても事務局より返信がない場合には電話にてご連絡ください
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Distribution of *emm* type and antibiotic susceptibility of group A streptococci causing invasive and noninvasive disease

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To determine the prevalence of macrolide antibiotic and levofloxacin resistance in infections with *Streptococcus pyogenes* (group A streptococcus or GAS), strains were collected from 45 medical institutions in various parts of Japan between October 2003 and September 2006. Four hundred and eighty-two strains from patients with GAS infections were characterized genetically. Strains were classified into four groups according to the type of infection: invasive infections (n=74) including sepsis, cellulitis and toxic-shock-like syndrome; acute otitis media (AOM; n=23); abscess (n=53); and pharyngotonsillitis (n=332). Among all strains, 32 *emm* types were identified; *emm1* was significantly more common in invasive infections (39.2 %) and AOM (43.5 %) than in abscesses (3.8 %) or pharyngotonsillitis (10.2 %). *emm12* and *emm4* each accounted for 23.5 % of pharyngotonsillitis cases. Susceptibility of GAS strains to eight β-lactam agents was excellent, with MICs of 0.0005–0.063 μg ml^{-1}. Macrolide-resistant strains accounted for 16.2 % of all strains, while the percentages of strains possessing the resistance genes *erm*(A), *erm*(B) and *mef*(A) were 2.5 %, 6.2 % and 7.5 %, respectively. Although no strains with high resistance to levofloxacin were found, strains with an MIC of 2–4 μg ml^{-1} (17.4 %) had amino acid substitutions at either Ser-79 or Asp-83 in ParC. These levofloxacin-intermediately resistant strains included 16 *emm* types, but macrolide-resistant strains were more likely than others to represent certain *emm* types.

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INTRODUCTION

Streptococcus pyogenes (group A streptococcus or GAS) is well recognized as the most common pathogen causing pharyngotonsillitis in school-age children. In addition, GAS is well known as a cause of impetigo, necrotizing fasciitis and other infections (Cunningham, 2000). In the late 1980s, toxic-shock-like syndrome caused by GAS was reported in Europe, Japan and the USA, being termed 'flesh-eating bacteria' in the popular press (Cunningham, 2000; Stevens *et al.*, 1989).

M protein encoded by the *emm* gene (Fischetti, 1989) has been exploited for M typing in epidemiological studies (Tewodros & Kronvall, 2005). M typing has progressed from identification methods using antisera to analysis of *emm* gene sequences of the N-terminal hypervariable region using the Centers for Disease Control and Prevention (CDC) database (Beall *et al.*, 1996). Results for GAS surveillance by Rogers *et al.* (2007) demonstrated that *emm1* predominated in invasive infections compared with other noninvasive infections.

Macrolide and levofloxacin resistance have gradually increased in GAS isolates, although the isolates remain uniformly susceptible to β-lactam antibiotics. Clonal spread of levofloxacin-resistant GAS has been reported (Malhotra-Kumar *et al.*, 2005), and possible implications for public health have been pointed out.

Recently, penicillin-intermediately resistant *Streptococcus agalactiae* that has amino acid substitutions in penicillin-binding protein 2X has appeared in Japan (Kimura *et al.*, 2008). Therefore, attention to annual trends of β-lactam susceptibility of GAS is also necessary.

In this study, we aimed to clarify (i) *emm*-type distributions in invasive strains compared with noninvasive isolates; (ii) susceptibility of these strains to 11 β-lactam antibiotics, four macrolides, clindamycin and levofloxacin;

Abbreviations: AOM, acute otitis media; CDC, Centers for Disease Control and Prevention; FQ, fluoroquinolone; GAS, group A streptococcus.

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and (iii) resistance genes for macrolides, lincosamides and streptogramin B (MLS_B), and levofloxacin.

METHODS

Strains. GAS isolates from patients with GAS infection were collected from clinical laboratories belonging to 45 general hospitals. A total of 482 isolates were sent to our laboratory (Kitasato Institute for Life Sciences, Kitasato University) between October 2003 and September 2006. After reidentification following the *Manual of Clinical Microbiology* (Ruoff *et al.*, 2003), strains were stored at −80 °C in 10 % skim milk (Becton Dickinson) until subsequent testing.

For the purposes of this study, isolates were divided into four groups according to the modified classification of Rogers *et al.* (2007). The invasive infection group (total $n=74$) included septicaemia ($n=34$), cellulitis ($n=13$), septic arthritis ($n=8$), meningitis ($n=4$), necrotizing fasciitis ($n=5$), toxic-shock-like syndrome ($n=4$), pneumonia ($n=3$), mastitis ($n=2$) and pleurisy ($n=1$) isolates. In this group, isolates defined tentatively as GAS were obtained from blood, joint fluid or pleural fluid. The other GAS infections were classified in abscess ($n=53$), pharyngotonsillitis ($n=332$) and acute otitis media (AOM) ($n=23$) groups.

Characterization of the resistance mechanism. PCRs for detection of the *erm*(A), *erm*(B) and *mef*(A) genes mediating macrolide resistance were performed as described previously (Sunaoshi *et al.*, 2004).

Strains possessing the *erm*(A) gene express an inducible macrolide, lincosamide and streptogramin B resistance ($iMLS_B$) phenotype (Seppälä *et al.*, 1998) and strains possessing the *erm*(B) gene express a constitutive MLS_B ($cMLS_B$) phenotype. The $iMLS_B$ phenotype strains by the target site modifications due to methylase activity show high resistance to 14- and 15-membered ring macrolides but are susceptible or intermediately resistant to clindamycin without induction (Giovanetti *et al.*, 1999). The $cMLS_B$ phenotype strains by methylase activity show high resistance to all macrolides and clindamycin without induction. By contrast, strains possessing the *mef*(A) gene express a 14- and 15-membered ring macrolide resistance (M) phenotype by an active drug efflux pump (Giovanetti *et al.*, 1999; Roberts *et al.*, 1999).

For the *gyrA*, *gyrB*, *parC* and *parE* genes involved in fluoroquinolone (FQ) resistance, PCR was carried out for 40 cycles under the conditions of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 60 s. Analytical primer sets for FQ resistance, shown in Table 1, were designed to detect these genes in GAS strains (GenBank accession nos NC_002737, NC_008023, NC_008024). The PCR product was then purified using the QIAquick PCR Purification kit (Qiagen). Sequencing was performed using the BigDye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems), with assessment of results using the Applied Biosystems 3130 Genetic Analyzer.

***emm* gene typing.** Typing of the *emm* gene was performed as follows.

Extraction of template DNA was done using the established procedure (Ubukata *et al.*, 1996). In brief, one colony of GAS growing on a sheep blood agar culture plate was picked and suspended in lysis solution, which comprised 0.1 M Tris/HCl (pH 8.0), 4 μg proteinase K, 0.225 % Tween 20 and 0.225 % Nonidet 40. This was incubated at 60 °C for 20 min and then at 90 °C for 10 min.

The PCR for *emm* genotyping was carried out according to minor modifications of the method described previously by Beall *et al.* (1996). The resulting PCR fragments were purified and sequenced in the same way as described above.

Table 1. Primers for PCR and sequencing for quinolone resistance

Primer name	Primer sequence (5′→3′)	Amplicon size (bp)
gyrA		
gyrA-s	TTTGCCAGATGTGCGTGATG	446
gyrA-r	TGTTAGTTGCCATCCCAACG	
gyrB		
gyrB-s	ATTGGGCAACTCAGAAGTGG	504
gyrB-r	GTTCTAATATGAGCGCCATCC	
parC		
parC-s	ATTATGGGAGAACGCTTCGG	442
parC-r	AAGCTGCTGGTAAAACGGTG	
parE		
parE-s	CTCATCTAGTTCGCAAAGCC	781
parE-r	TTATCCTCGATCCACTGACG	

The first 240 bases of the 5′ end of the *emm* gene sequences were compared with those in the CDC *S. pyogenes emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm). An *emm* type showing over 98 % homology with a CDC reference strain was identified as that particular *emm* type.

Antimicrobial susceptibility tests. Susceptibility testing of GAS strains was carried out by the 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) and with a final inoculum of 5×10^5 c.f.u. ml^{-1}. Oral antimicrobial agents employed in this study were ampicillin, amoxicillin, cefdinir, cefpodoxime, cefditoren, cefcapen, faropenem, tebipenem, clarithromycin, azithromycin, josamycin, clindamycin, telithromycin and levofloxacin. Parenteral agents were cefotaxime, panipenem and meropenem. These antimicrobial agents were obtained from the respective pharmaceutical manufacturers.

Statistical analysis. Statistical analysis was performed using Microsoft Excel Statistics 2006 for Windows (Social Survey Research Information). The chi-square test was used to assess significance of differences involving categorical variables.

RESULTS AND DISCUSSION

Age distribution of the patients with GAS infection

Fig. 1 shows the patient age distribution in the four groups of GAS infection cases described in Methods.

Invasive cases ($n=74$) occurred mostly in patients over 20 years old (85.1 %) as opposed to in children, especially in older adults between the ages of 50 and 70 years old. Important underlying conditions such as diabetes mellitus, liver dysfunction, renal dysfunction and medical treatment for cancer were noted in 56.4 % of those adults. Decreased immunity in these cases may have contributed synergisti-

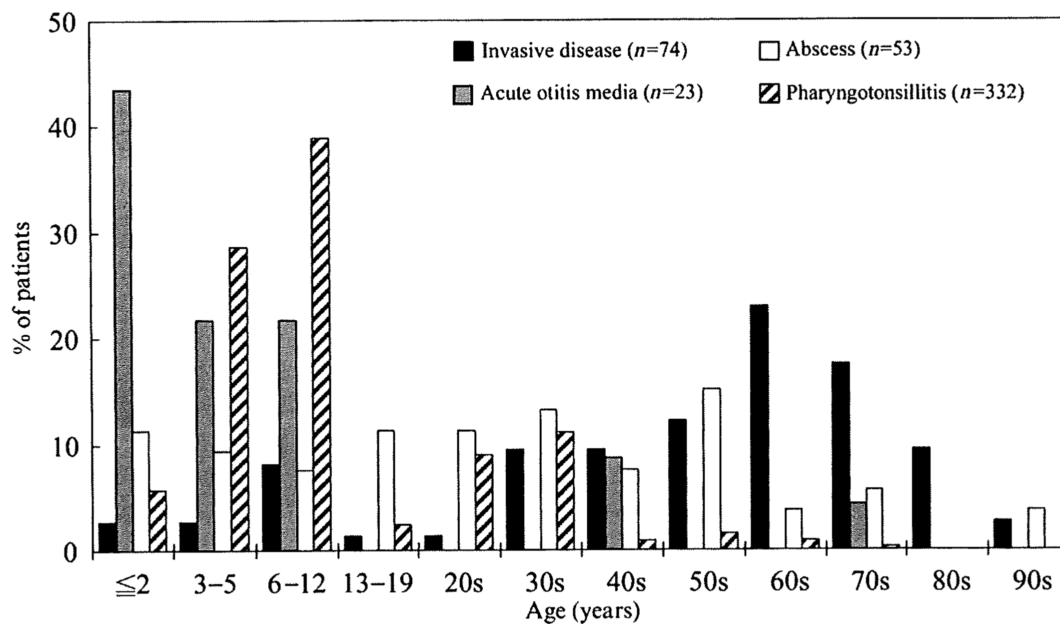

Fig. 1. Age distribution of patients with *Streptococcus pyogenes* infection. Patients were classified into four groups. Black columns indicate patients with invasive infections; finely stippled columns, those with AOM; open columns, those with abscess; diagonally hatched columns, those with pharyngotonsillitis.

cally with GAS virulence factors to an increased incidence of invasive infections.

Both AOM cases (n=23) and pharyngotonsillitis cases (n=332) mainly involved children between 3 and 12 years old, which accounted for 43.5 % and 67.4 %, respectively, although 20.1 % of the pharyngotonsillitis patients were 20–30 years old.

Abscess cases (n=53), in which GAS was isolated from either draining or nondraining localized abscesses, were distributed across all age brackets.

Statistically significant differences in age distribution were recognized between the invasive group and each of the other three groups (invasive vs AOM, P <0.0001; invasive vs abscess, P=0.0003; invasive vs pharyngotonsillitis, P <0.0001).

Typing for *emm*

Results of *emm* typing of GAS isolates in four groups, that is, invasive (A), AOM (B), abscess (C) and pharyngotonsillitis (D) groups, are listed in Table 2. Although GAS isolates included a variety of *emm* types, the predominant *emm* types in each group differed. In the invasive group, *emm1* was prominent, accounting for 39.2 % of cases, while other *emm* types generally accounted for 10 % of cases or fewer. Similarly, *emm1* was most frequent in the AOM group (43.5 %), followed by *emm12* (30.4 %).

In the abscess group, *emm28* and types described as 'other' in Table 2 accounted for 22.6 % and 26.4 % of cases each, while *emm1* was responsible for only 3.8 %. In the pharyngotonsillitis group, *emm4* and *emm12* accounted for 23.5 % of cases each, in contrast to 10.2 % for *emm1*.

A significant difference in prevalence of *emm* types was noted between the invasive group and each of the other three groups (invasive vs AOM, P=0.0895; invasive vs abscess, P=0.0013; invasive vs pharyngotonsillitis, P <0.0001).

Susceptibilities to β-lactam agents

All β-lactam agents showed sharp distributions for all GAS, indicating that none of these agents showed decreased efficacy against GAS.

In data not shown here, MIC_{90} values for GAS were excellent in the following order: tebipenem (0.002 μg ml^{-1}) > cefditoren=cefcapen (0.008 μg ml^{-1}) > amoxicillin=cefdinir=cefpodoxime (0.016 μg ml^{-1}) > ampicillin=faropenem (0.031 μg ml^{-1}) for oral β-lactams; and panipenem=meropenem (0.008 μg ml^{-1}) > cefotaxime (0.016 μg ml^{-1}) for parenteral β-lactams. All isolates remained uniformly susceptible to β-lactam antibiotics.

Susceptibilities to macrolides and resistance genes

Table 3 shows the MIC ranges, MIC_{50} and MIC_{90} of clarithromycin, azithromycin, josamycin, telithromycin and clindamycin for GAS according to the macrolide-resistance genes identified. Of all 482 isolates, strains

Table 2. Relationship between streptococcal diseases and *emm* types of isolates

Results of chi-square tests were as follows. A:B, $P=0.0895$; A:C, $P=0.0013$, A:D, $P<0.0001$. Values in parentheses are percentages for each classified group. AOM, acute otitis media.

emm type	Groups classified as:				No. of isolates
	Invasive (A)	AOM (B)	Abscess (C)	Pharyngotonsillitis (D)	
1	29 (39.2)	10 (43.5)	2 (3.8)	34 (10.2)	75
3	2 (2.7)		2 (3.8)	13 (3.9)	17
4	2 (2.7)	2 (8.7)	1 (1.9)	78 (23.5)	83
6			3 (5.7)	15 (4.5)	18
11	4 (5.4)		2 (3.8)	20 (6.0)	26
12	5 (6.7)	7 (30.4)	4 (7.5)	78 (23.5)	94
28	5 (6.7)	1 (4.3)	12 (22.6)	33 (9.9)	51
49	8 (10.8)		4 (7.5)	1 (0.3)	13
58	4 (5.4)		3 (5.7)	7 (2.1)	14
75	3 (4.1)	2 (8.7)	1 (1.9)	9 (2.7)	15
87	2 (2.7)		2 (3.8)	2 (0.6)	6
89	1 (1.4)	1 (4.3)	3 (5.7)	12 (3.6)	17
Other	9 (12.2)		14 (26.4)	30 (9.0)	53
Total	74	23	53	332	482

possessing *erm*(A), *erm*(B) and *mef*(A) represented 2.5 % ($n=12$), 6.2 % ($n=30$) and 7.5 % ($n=36$), respectively.

Table 3. MIC distributions and resistance genes identified by PCR in *Streptococcus pyogenes* strains

Antimicrobial agent	MIC range (μg ml^{-1})	MIC_{50}	MIC_{90}
Clarithromycin			
Resistance gene (−)	0.031–2	0.063	0.125
erm(A)	2–16	4	16
erm(B)	>64	>64	>64
mef(A)	2–16	8	16
Azithromycin			
Resistance gene (−)	0.125–4	0.25	1
erm(A)	32–>64	>64	>64
erm(B)	>64	>64	>64
mef(A)	4–32	8	32
Josamycin			
Resistance gene (−)	0.125–2	0.5	0.5
erm(A)	0.5–8	4	8
erm(B)	>64	>64	>64
mef(A)	0.125–0.5	0.25	0.5
Telithromycin			
Resistance gene (−)	0.008–0.25	0.031	0.063
erm(A)	0.063–0.125	0.063	0.063
erm(B)	2–>64	>64	>64
mef(A)	0.25–1	0.5	1
Clindamycin			
Resistance gene (−)	0.031–0.25	0.125	0.25
erm(A)	0.25–>64	0.5	8
erm(B)	>64	>64	>64
mef(A)	0.031–0.25	0.063	0.25

Except for telithromycin, the *erm*(A) gene related to $iMLS_B$ phenotype decreased the susceptibilities of GAS to macrolides and clindamycin 8–128-fold compared with susceptible strains. GAS with the *erm*(B) gene related to $cMLS_B$ phenotype showed high resistance to macrolides, telithromycin and clindamycin (MIC $\geqslant 64$ μg ml^{-1}). GAS strains with the *mef*(A) gene, which mediate an M phenotype, showed slight decreases in susceptibility, from 4- to 8-fold, to clarithromycin, azithromycin and telithromycin, but maintained undiminished susceptibility to josamycin and clindamycin.

In Japan, the prevalence of macrolide resistance in GAS has remained at 5–7 % for a long time. In the survey in 2003, of the total of 533 GAS strains collected from all over the country, 0.5 % had the *erm*(A) gene, 3.2 % the *erm*(B) gene and 4.9 % the *mef*(A) gene (Sunaoshi *et al.*, 2004). Macrolide resistance appears to increase gradually.

Table 4 shows the correlation between the *emm* type and macrolide-resistance genes. Of the GAS strains typed to *emm1*, 21.3 % had the *mef*(A) gene, which was prevalent in invasive infections, in contrast to the *emm* status of strains with *erm*(A) and *erm*(B) genes.

High clindamycin resistance mediated by the *erm*(B) gene was detected at 2.7 % in invasive strains.

Susceptibility to levofloxacin and mutations of the target gene

Table 4 also shows the *emm* type distribution of GAS strains ($n=71$) which were intermediately resistant to FQ. Although no strain was identified as showing high resistance, strains with an MIC of 2–4 μg ml^{-1} accounted for 17.4 % ($n=84$) of all isolates. Eighty-five per cent of

Table 4. Correlation of *emm* type and resistance genes of *Streptococcus pyogenes* strains

Values in parentheses are percentages for each *emm* type.

emm type	No. of isolates	Macrolide resistance			Fluoroquinolone resistance *parC* mutant*
		erm(A)	*erm*(B)	*mef*(A)	
1	75		3 (4.0)	16 (21.3)	5 (6.7)
3	17				1 (5.9)
4	83			6 (7.2)	1 (1.2)
6	18				17 (94.4)
11	26				20 (76.9)
12	94		11 (11.7)	7 (7.4)	3 (3.2)
28	51	3 (5.9)	14 (27.4)		2 (3.9)
49	13				4 (30.8)
58	14	6 (42.8)			3 (21.4)
75	15			7 (46.7)	1 (6.7)
87	6				5 (83.3)
89	17		2 (11.8)		2 (11.8)
Other	53	3 (5.7)			7 (13.2)
Total	482	12 (2.5)	30 (6.2)	36 (7.5)	71 (14.7)

*Ser-79 and Asp-83 in ParC encoded by *parC* changed to Phe, Ala, or Tyr and Asn.

strains with an MIC of at least $\geqslant 2\ \mu g\ ml^{-1}$ ($n=71$) had an amino acid substitution at the Ser-79 or Asp-83 position in quinolone resistance-determining regions (QRDRs) encoded by *parC*. Ser-79 was changed to Phe-79, Ala-79 or Tyr-79 in 93.0 % ($n=66$), while Asp-83 was changed to Asn-83 in 7.0 % ($n=5$). There was no amino acid substitution affecting FQ resistance in *gyrA*, *gyrB* and *parE* genes.

Genotypic levofloxacin-intermediately resistant GAS strains belonged to 16 *emm* types, although *emm6* and *emm11* were prominent at 94.4 % and 76.9 %, respectively, as described previously (Orscheln *et al.*, 2005). This finding suggests that genotypically FQ-intermediately resistant GAS is selected under the pressure of exposure to FQs including levofloxacin. *In vitro* experiments have indicated that all *emm* types seem equally prone to induction of FQ resistance (Billal *et al.*, 2007).

Recently, we isolated a GAS strain, showing high resistance to FQ, from an adult patient (29 years old) with pharyngotonsillitis in September 2007. The strain possessed amino acid substitutions in QRDRs of both *gyrA* and *parC*, which had already been reported in several countries (Malhotra-Kumar *et al.*, 2005; Reinert *et al.*, 2004; Richter *et al.*, 2003; Rivera *et al.*, 2005; Yan *et al.*, 2000).

According to the market research for oral antibiotics (Fujita *et al.*, 2007) in Japan, oral FQs, including four respiratory FQs, have been prescribed for adult outpatients aged $\geqslant 15$ years at the highest rate of 50 %, followed by oral cephalosporin antibiotics at the rate of 43 %, which is higher than that for penicillins. The status of the current usage of FQ causes concern that the incidence of GAS and *Streptococcus pneumoniae* strains possessing high FQ resistance might increase in the near future.

On the other hand, macrolides to which 14-membered ring macrolides and azalides belong have been widely prescribed to reduce inflammation in patients with diffuse panbronchiolitis, chronic bronchitis and chronic sinusitis at low doses for long periods, in addition to being used in the treatment of community-acquired respiratory tract infections. Such long-term non-chemotherapeutical usage of macrolides may result in a decrease in usefulness of macrolides for infectious disease and be related to selection and spread of macrolide resistance in *S. pneumoniae* and *S. pyogenes*.

In conclusion, continuous molecular epidemiological surveillance of GAS is necessary to ensure proper use of antimicrobial agents.

ACKNOWLEDGEMENTS

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Simultaneous and Rapid Detection of Causative Pathogens in Community-acquired Pneumonia by Real-time PCR (1167)

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Introduction

Community-acquired pneumonia (CAP) is still a major threat to individuals, especially children and compromised hosts such as senior citizens and people with underlying chronic diseases. The main causative pathogens in CAP are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Mycoplasma pneumoniae*, but the rates change according to age and the underlying disease.[1,2] Recently, *Chlamydophila pneumoniae* and *Legionella pneumophila* have frequently been identified as causative pathogens of CAP.[3–5] In clinical practice, empirical chemotherapy with broad spectrum antibiotics must be started based on clinical symptoms, chest X-rays, and clinical examinations, considering the severity of the symptoms.

On the other hand, the increase of resistant strains in the CAP pathogens is a worldwide health problem.[6] One effective measure to prevent the new emergence of resistant bacteria is to use only the sufficient quantity of the most appropriate antibiotics to eliminate target pathogens. We have developed a method to simultaneously identify the six pathogens in CAP by real-time PCR with high sensitivity and specificity, thus reducing analysis time and improving cost performance, toward the goal of the rapid and precise detection of causative agents.[7]

In this paper, I will describe an outline of the new identification system using real-time PCR assay for six CAP pathogens, *S. pneumoniae*, *H. influenzae*, *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, and *Streptococcus pyogenes*.

Methods

Extraction of DNA from clinical samples

Samples of nasopharyngeal and sputum from pediatric and adult patients who were diagnosed with CAP at their first visit to a hospital, were collected after obtaining informed consent from patients themselves or from their family members.

Firstly, the samples were suspended in 1.5 ml of PPLO broth and centrifuged at 2,000×g for 5 min at 4°C to collect bacterial cells, together with epithelial and polymorphonuclear leukocyte cells. The supernatant was discarded, and the harvested pellet was resuspended in 150 μl of DNase- and RNase-free H_2O. All DNA samples were extracted using EXTRAGEN II kit. The process of DNA extraction was completed in 20 minutes.

Identification of pathogens by real-time PCR

The six sets of bacterial specific primers and molecular beacon (MB) probes used in this real-time PCR have been reported by us.[7] These primers and MB probes were designed on appropriate genes for each pathogen; the *lytA* gene was selected for *S. pneumoniae*, the *mip* and 16S rRNA genes for *L. pneumophila*, the *slo* and 16S rRNA genes for *S. pyogenes*, and the 16S rRNA gene for the other three pathogens were selected as a target, respectively.

As shown in Fig. 1, all of the MB probes were labeled with a fluorescent reporter, 6-carboxy-fluorescein (FAM) at the 5'-end, and labeled with a black hole quencher 1 (BHQ-1) at the 3'-end.

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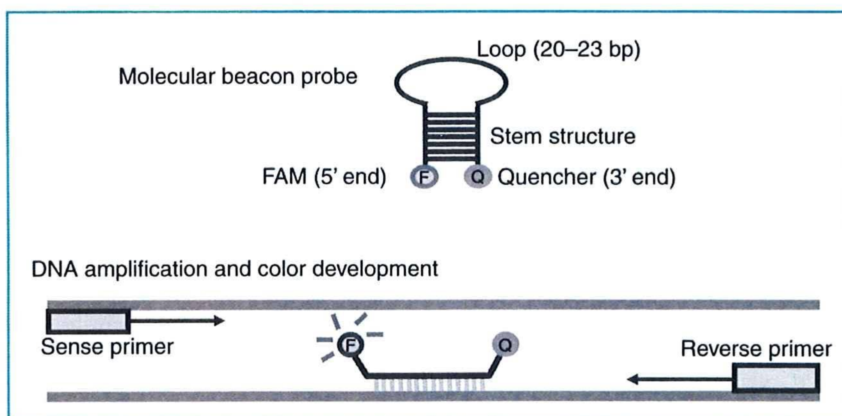

Fig. 1 Principle of real-time PCR using a molecular beacon probe

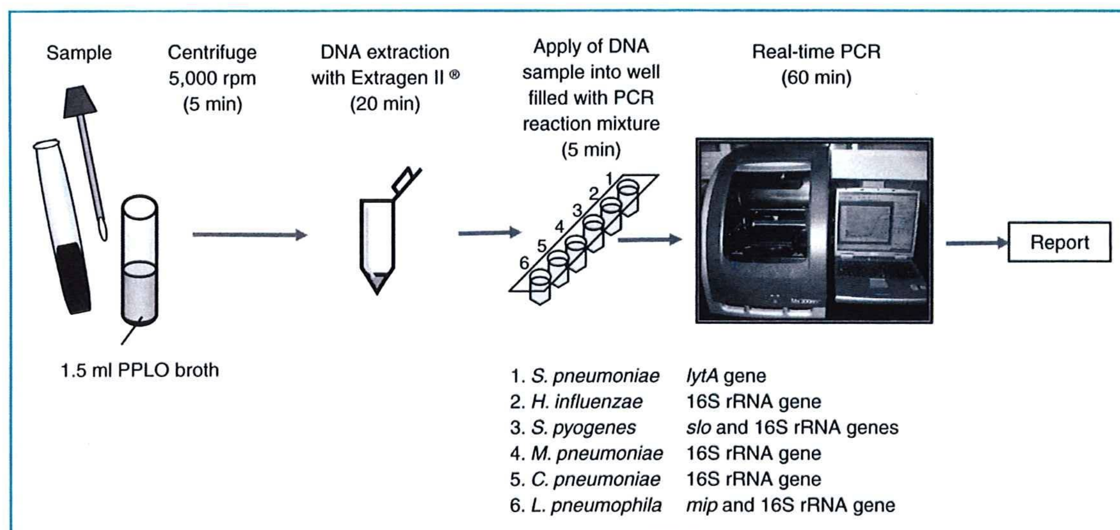

Fig. 2 Protocols of six pathogens detection from CAP samples by real-time PCR

The reporters and quenchers were connected to stem structure with short oligonucleotides. The central region of about 20 bp, which is shown as a loop, corresponds to the sequences of each target gene. The color development of FAM occurred by an attachment of the oligonucreotides to correspondent sequences of single stranded DNA at the denature stage.

The overall protocol of real-time PCR assay constructed by us is shown in Fig. 2. Reaction mixtures corresponding to six CAP pathogens were employed in six wells of one strip. After the addition of a 2 μl DNA extraction to the six wells, the strip was placed on the real-time PCR instrument (Thermal cycler Dice, TP800, TAKARA BIO INC.), and DNA amplification was immediately started at 40 cycles of PCR with conditions at 95°C for 15 sec, at 50°C for 30 sec, and at 75°C for 30 sec per cycle, followed by 95°C for 30 sec. The results for every one cycle of the PCR were displayed on the screen of a personal computer connected to a PCR instrument. All processes in this protocol were completed in 1.5 hours.

Results

Sensitivity and specificity of real-time PCR

The Ct (threshold cycle) value for a positive

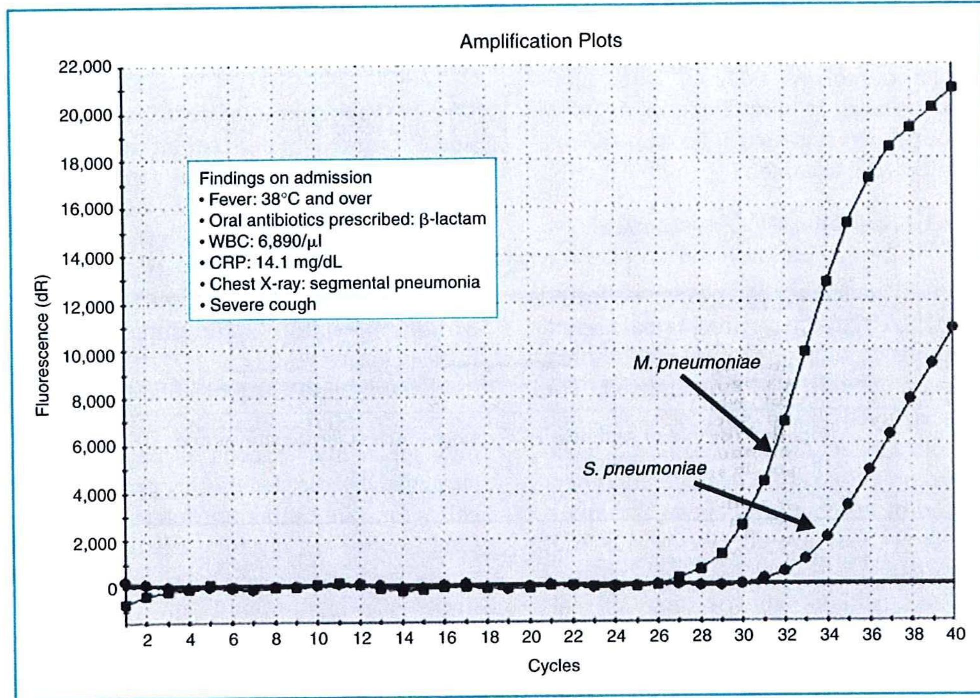

Fig. 3 PC screen after real-time PCR assay for sputum from adult patient with CAP

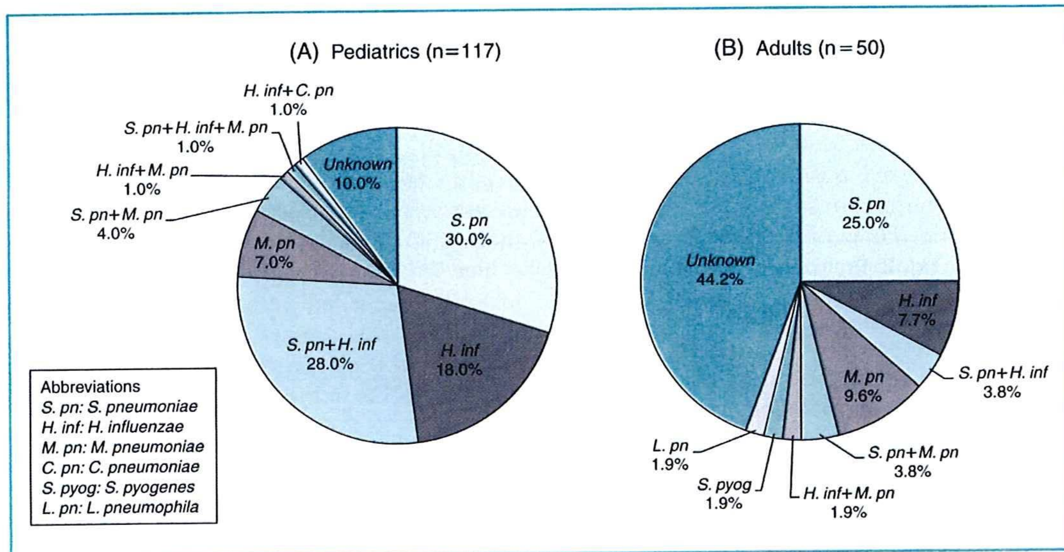

Fig. 4 Bacterial pathogens identified by real-time PCR in pediatric and adult patients with CAP

result was defined as the point at which the horizontal threshold line was crossed. The sensitivities of PCR assay for six CAP pathogens were high, from 2 copies for *S. pneumoniae* to 18 copies for *S. pyogenes* per reaction tube. The correlation coefficient between Ct and bacterial cell counts was high from r = 0.9970 of *S. pyogenes* to r = 0.9992 of *S. pneumoniae*.

The specificity of the six MB probe and primer sets were examined against 27 gram-positive and -negative microorganisms selected from laboratory stock cultures as amplification negative controls. From those organisms, no non-specific positive results were obtained.

Application of real-time PCR for clinical samples

Figure 3 shows the result of sputum collected from a typical CAP case in a young female adult. She was diagnosed with segmental pneumonia, having a severe cough, and hospitalized. The PCR results displayed on the PC screen suggested a mixed infection of *M. pneumoniae* and *S. pneumoniae*. Based on these results, medical treatment was conducted by combination chemotherapy.

Figure 4 shows the results of real-time PCR applied to pediatric patients (A) and adults (B) with CAP. The sensitivity and specificity of real-time PCR assay for *S. pneumoniae*, *H. influenzae*, *S. pyogenes*, and *M. pneumoniae* for 150 clinical samples was determined, comparing them with the results of conventional culture. Both the relative sensitivity and the specificity of this PCR was over 90% for all six pathogens.

Culture assay of *C. pneumoniae* has not been performed routinely in clinical laboratories, but has instead been determined by the antibody titer in acute phase and the convalescence phase. In all of *C. pneumoniae* cases identified serologically, the real-time PCR gave positive results.

Although the culture for sputum was negative, *L. pneumophila* pneumonia was identified by PCR in the case of an adult patient. Later, *L. pneumophila* serogroup 5 was actually detected in the water from the patient's bathroom.

Discussion

One of the measures to decrease healthcare costs and to improve benefit for patients with bacterial infection is to identify the causative pathogens rapidly and precisely, thus enabling the most appropriate antibiotic to be selected at the beginning of hospitalization. In the case of patients who were treated by antibiotics prior to hospitalization, the culture method may sometimes give false-negative results.

By real-time PCR molecular assay, it is possible to detect microorganisms with high sensitivity and specificity,[8–10] even if bacteria have been damaged by the antibiotics pretreatment. Basically, the simultaneous detection of the main CAP pathogens described here is desirable for rapid diagnosis of CAP and for the selection of the appropriate antibiotics.

To determine causative pathogens, sputum has been employed in the case of adults. However, expectoration is impossible for infants and children. Alternatively, nasopharyngeal secretions are readily obtainable from children with respiratory tract infections (RTIs), but the test results must be analyzed carefully because healthy children also carry *S. pneumoniae* and *H. influenzae* in nasopharyngeal secretions.[11]

Our data demonstrates that real-time PCR with pathogen-specific MB can detect microorganisms in a few hours, and thus by this assay it is possible to assess the time course of empirical chemotherapy, thus supporting infection management.

Finally, we also expect that the real-time PCR technique described here could be expanded into a multiplex real-time PCR to detect several RTI causative viruses as a general diagnostic method for lower RTIs in the near future.

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