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基　礎

アマンタジンの耐性

齋藤玲子，鈴木康司，李　丹娟，菖蒲川由郷，鈴木　宏

SAITO Reiko/新潟大学大学院医歯学総合研究科国際感染症医学講座公衆衛生学分野講師
SUZUKI Yasushi/同　国際感染症医学講座公衆衛生学分野
LI Danjuan/同　国際感染症医学講座公衆衛生学分野
SHOBUGAWA Yugo/同　国際感染症医学講座公衆衛生学分野
SUZUKI Hiroshi/同　国際感染症医学講座公衆衛生学分野教授

2005年以降，A型インフルエンザのアマンタジン耐性株がアジアを中心に急増し，2005/2006年は本邦でA/H3N2の耐性株増加が確認された．さらに2006/2007年はA/H3N2の耐性が持続しているばかりでなく，A/H1N1でもアマンタジン耐性株の増加がみられた．

KEY WORDS

- インフルエンザ
- アマンタジン
- 耐性
- サイクリング・プローブ法

はじめに

2005年以降，市中株A型インフルエンザでアマンタジン耐性頻度が世界的に急増した．本邦では2005/2006年冬にA/H3N2のアマンタジン耐性化率が60％を越え，ヒトからヒトへ伝播感染を起こしている事実がわれわれの調査により明らかになった．本稿ではこれまでの概要と，われわれが新たに開発した迅速診断法(サイクリング・プローブ法)による31位変異(Ser → Asn)アマンタジン耐性株検出法を紹介し，同法を使った2006/2007年シーズンの耐性頻度を報告する．合わせて高頻度耐性化を支える機序についての知見を紹介する．

1 2005/2006年までの状況

インフルエンザの治療・予防にはM2阻害薬とノイラミニダーゼ阻害薬が有効である．本邦ではM2阻害薬としてアマンタジン(シンメトレル®)が認可されている．アマンタジンは安価で安定であるが，投与後，中枢神経系の一過性の副作用が多くみられること，高い頻度で耐性株が出現することが問題である[1]．アマンタジンによるA型インフルエンザの耐性化は同薬剤が阻害するM2蛋白遺伝子の特定部位にアミノ酸変異(26，27，30，31位)が起

こることにより生じる[1]．これまでわれわれは，投与後に約3分の1の患者で耐性株が出現すること[2]，それにもかかわらず市中株中の耐性頻度は0～4％程度と低いことを報告してきた(図1)[3]．この傾向は日本各地や世界諸地域で同様であった[4][5]．

ところが，2004年以降中国においてA香港型(A/H3N2)インフルエンザのM2阻害薬(アマンタジン)耐性株の急増が報告され，状況が一変した[6]．それに引き続き，2005年にはアジア各国，オーストラリア，ニュージーランドにおいて耐性A/H3N2株の増加が確認された[7]．日本では，2005/2006年冬に全国6カ所(新潟，宮城，山形，群馬，長崎，福岡)でわれわれが調査を行ったところ，A/H3N2の65.3％がアマンタジン耐性であった(図1)[8]．同時流行のAソ連型(A/H1N1)に耐性株はなかった．このA/H3N2の耐性株はすべてM2遺伝子の31位がセリンからアスパラギンに変異した株(S31N)であった．患者にアマンタジン内服歴はなく，すべて耐性株の伝播感染によるものと思われた．奈良県でも同様に高率の耐性株が検出され[9]，われわれの調査と合わせ，2005/2006年シーズンは日本中でこれまでにないアマンタジン耐性株の大流行があったことが判明した．本邦のみならず，2005/2006年シーズンの米国で92.3％[10]，カナダでは76.5％[11]のA/H3N2株がアマンタジン耐性であったと報告され，耐性株流行はほぼ全世界的な傾向と考えられた．

2 サイクリング・プローブ法によるS31N変異株迅速検出法

このようなアマンタジン耐性株の急増を受け，当教室ではサイクリング・プローブ法(タカラバイオ)によるS31N耐性株の迅速診断法を開発した[12](図2)．サイクリング・プローブ法は定量PCRの変法で配列中の1塩基置換(SNPs)を特異的に検出する方法である．遺伝子増幅反応を起こすPCR用のプライマーを設定し，さらに検出部位を標的として(この場合M2遺伝子31位)，蛍光色素で標識したキ

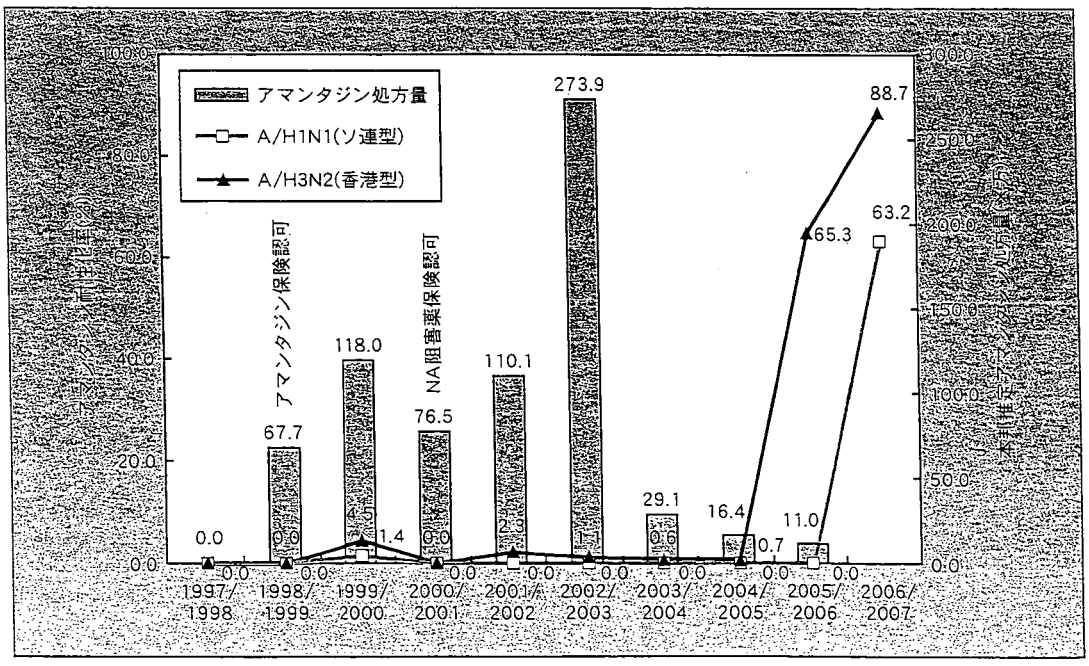


図1　市中株アマンタジン耐性A型インフルエンザ頻度と薬剤処方量

A香港型(A/H3N2)は2005/2006年以降，Aソ連型(A/H1N1)では2006/2007年以降アマンタジン耐性株が急増した．一方，日本国内のアマンタジンの処方量は2002/2003年の約300万人をピークとして減少している(2006/2007年シーズンの処方量は未発表)．

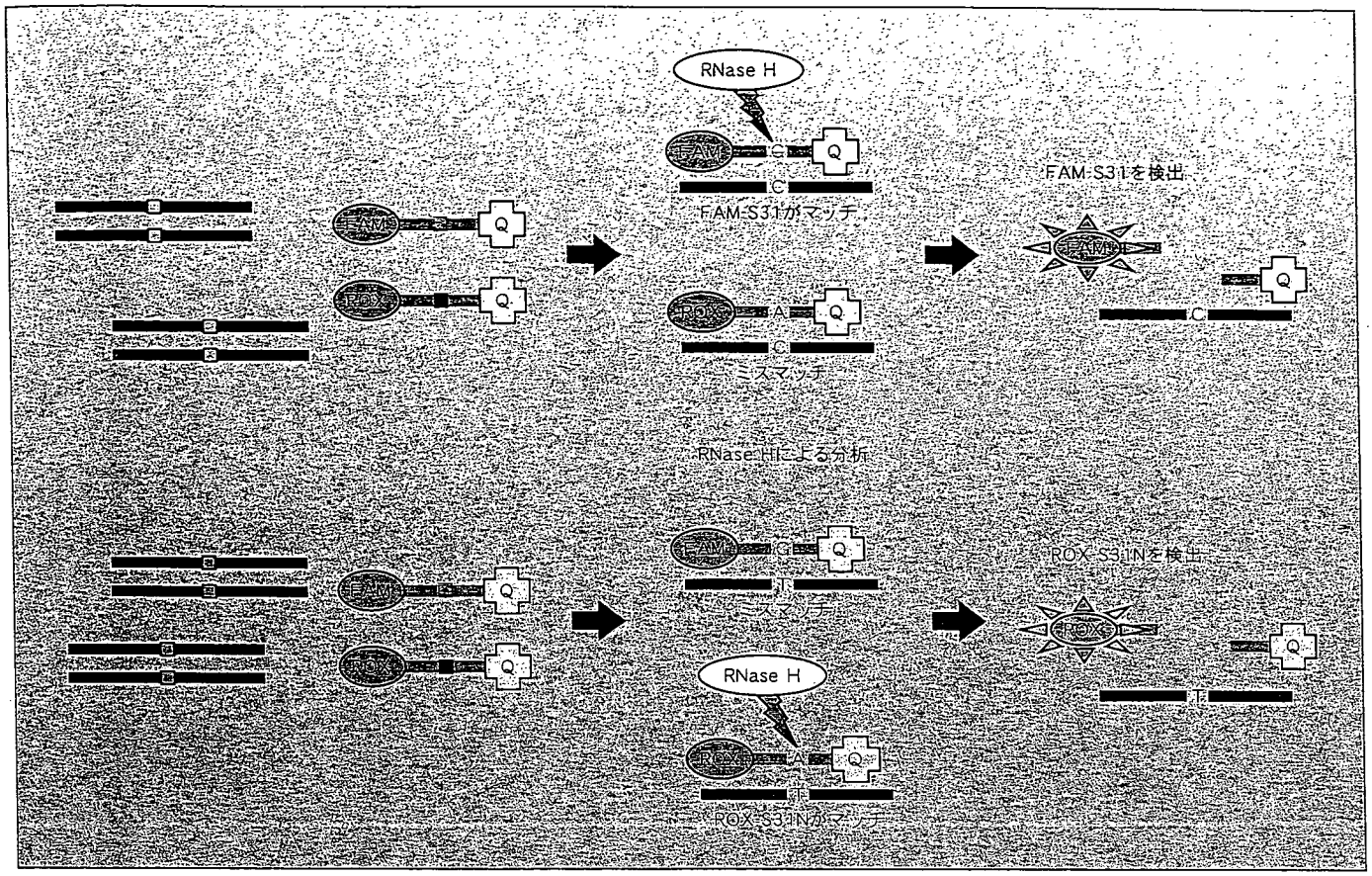


図2　サイクリング・プローブ法による S31N 変異の検出法(本文参照)

メラプローブを設計する．キメラプローブはオリゴ DNA で形成され，SNPs の塩基部分のみを RNA に置換しておく．denature 後に温度を下げるとプローブが標的 DNA にアニールし，相補的な DNA と RNA が結合した際のみにその部分が RNaseH で切断され，クエンチャーがはずれ蛍光が検出される．31 位のセリン(AGT)が，耐性株ではアスパラギン(AAT)に 1 塩基置換を起こす．このことから，この部分を RNA に置換したプローブを設計し，感受性株(AGT)は FAM で標識し，耐性株(AAT)は ROX で標識し，FAM が検出されれば 31 位に変異はなく，ROX であれば S31N 変異ありと判定できるようにした．プライマーとプローブの違いから H1N1 と H3N2 の判別もできる．この方法を用い咽頭拭い液・培養検体から S31N 変異株を良好に検出し，シークエンスの結果とも一致していた．同法の導入により RNA 抽出を含め約 3 時間で S31N 耐性株検出が可能となり，繁忙時のシークエンスの手間を省くことが可能である．

3　2006/2007 年シーズン

2006/2007 年シーズンは本邦は例年よりインフルエンザ流行開始が遅く，A/H1N1，H3N2，B 型の混合流行であった．われわれは先シーズンと同様，日本各地で A 型インフルエンザのアマンタジン耐性調査を行った．新潟(よいこの小児科さとう，佐野医院)，群馬(川島内科クリニック)，京都(日比小児科，生嶋こどもクリニック，つなもと小児科，はしだ小児科，ふじわら

小児科)，長崎(田上病院，白髭内科，愛野記念病院，十善会病院，景浦内科)の各医療機関を受診したインフルエンザ患者より初診時に採取されたA型インフルエンザ株を使い，S31N変異をサイクリング・プローブ法により検出した．結果はA/H1N1では63.2％(55/87)，A/H3N2は88.7％(323/364)がS31N変異株アマンタジン耐性であった(図1)[12]．特記すべきことは，昨シーズンは皆無であったA/H1N1が今季は耐性率が非常に高くなったことである．また，昨シーズンには調査地によるH3N2耐性株頻度に差があり，九州地域は頻度が低い傾向にあったが，今年は調査地による差はなく，各地でH3N2耐性が高頻度であった(新潟80.7％，群馬90.0％，京都91.9％，長崎91.4％)．H1N1に関しては地域差がみられた(新潟55.9％，京都81.8％，長崎100.0％，群馬はH1N1検出なし)．なお，2006/2007年シーズンは流行開始が遅かったため，まだ検査が終わっていない検体もあるため，この数字は2007年6月現在のものである．

世界的には，オーストラリアWHO協力センターより，2006年にアジア，オセアニアで採取された株でA/H1N1の耐性が増加したとの報告がなされた[13]．オーストラリアの2006年の検体では40％(8/20)が耐性で，ニュージーランド14％(1/7)，マカオ82％(9/11)，台湾20％(1/5)，シンガポール10％(1/10)，タイ10％(1/10)，マレーシア0％(0/10)，カンボジア0％(0/3)，フィリピン0％(0/14)であり，全体としてH1N1の耐性率がアジア一円で増加傾向である．なかでもマカオの耐性率が高く，中国本土でも増加が著しいと推測される．これらの地域でH3N2の耐性は22～100％であり，2005年よりさらに割合が高まった．これまでのところ米国を含むほかの地域の情報が少ないが，通常インフルエンザが抗原性変異を起こした場合，1～2シーズン以内に全世界的に流行することが知られているため，全世界的にアマンタジン耐性が広がっていることは想像に難くない．

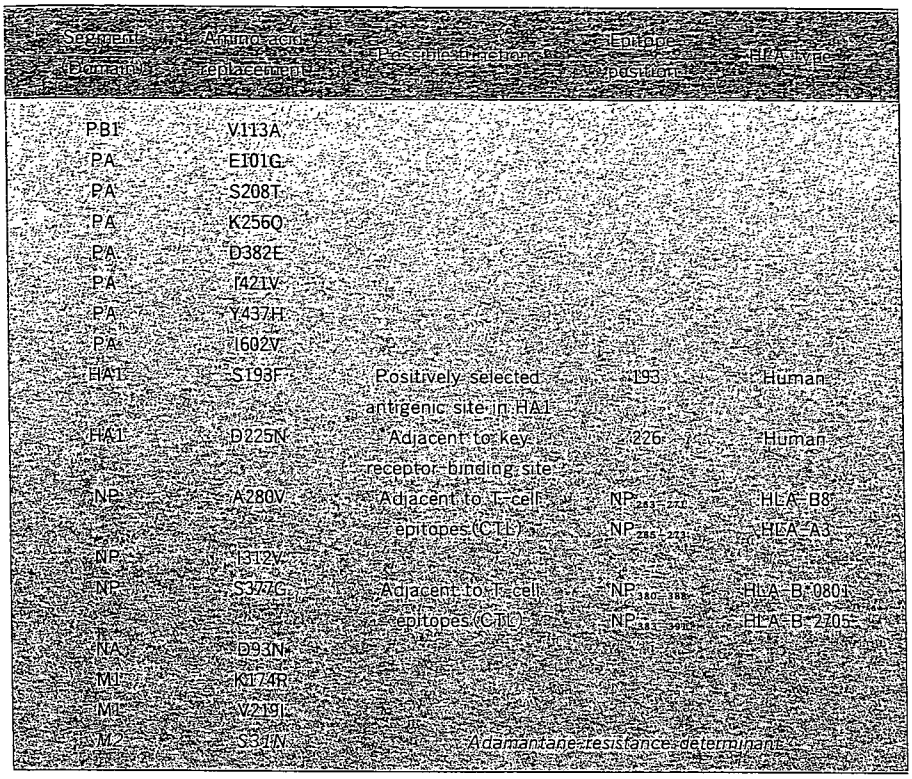
4 アマンタジン耐性化機序

このようなアマンタジン耐性株大流行はどのようにして起こったのであろうか．アマンタジン耐性株は*in vitro*(MDCK細胞)の状態でアマンタジン添加により容易に発生し，さらにアマンタジンを培養液中から除いても20代以上その表現型と遺伝子型が保たれる(自験データ)．これに対し，*in vivo*の状態では，耐性株は薬剤の選択圧で出現し，施設内や家族内といった限られた範囲でのみ伝播感染は起こすだけで，大流行を起こすことはなかった．これに対し，2005年以降報告されたアマンタジン耐性株は薬剤による選択ではなく，何らかの機序で耐性株が感受性株と同等あるいはそれ以上の伝播力を獲得したものと思われる．

近年増加しているアマンタジン耐性株H3N2は，そのほとんどがM2遺伝子の31位にSer→Asn(S31N)の変異をもつ．われわれはこれまで薬剤投与後にH3N2ではS31Nが，H1N1では27位の変異(V27A)が多いことを報告してきたが[2]，2004年以降の特徴はどのサブタイプでも耐性株にM2遺伝子の31位変異が圧倒的に多いことである．

このため，2005/2006年のH3N2のM2遺伝子以外のセグメントに注目し，このシーズンに流行したアマンタジン耐性株はHA遺伝子のHA1サブユニットの193位(Ser→Phe)と225位(Asp→Asn)に特有の変異があることを見出し本誌でも報告した[8][14][15]．この株は樹形図解析により特有のクレードを形成し，われわれはこの系統をClade Nと命名した．これまで確認しただけでも，米国，オセアニア，アジア各国でClade Nに属するウイルスが確認されており，国内では奈良県衛生研究所からも報告された[16]．この2つのHA変異部位はいずれも抗原決定基や，レセプター結合部位近傍で，このアミノ酸変異により伝播率が飛躍的に変わった可能性がある．しかし，その他の遺伝子の変異も大きく寄与している可能性が否めない．最近Simonsenらがニュージーランドのインフルエンザ遺伝子全シークエンスプロジェクトに供された株を中心にClade Nの遺伝子的特徴を解析した[17]．Clade Nアマンタジン耐性ウイルスは同時期に流行した感受性株インフルエンザと比して，ウイルス全ゲノム中に86の塩基変化がみられ，結果的に17のアミノ酸変異がみつかった(表1)．われわれが報告したHA以外にNPの4つの変異がヒトの免疫の抗原認識に関連する可能性がある．ほかのPB1，PA，NA，M1にみつかった変異について

表1　各セグメントにみられる Clade N に特徴的なアミノ酸変異



Segment	Amino acid replacement	Specific function	Epitope position	HLA type
PB1	V113A			
PA	E101G			
PA	S208T			
PA	K256Q			
PA	D382E			
PA	I421V			
PA	Y437H			
PA	I602V			
HA1	S193F	Positively selected antigenic site in HA1	193	Human
HA1	D225N	Adjacent to key receptor-binding site	226	Human
NP	A280V	Adjacent to T-cell epitopes (CTL)	NP$_{281-289}$	HLA-B8
			NP$_{265-273}$	HLA-A3
NP	I312V			
NP	S377G	Adjacent to T-cell epitopes (CTL)	NP$_{380-388}$	HLA-B*0801
			NP$_{383-391}$	HLA-B*2705
NA	D93N*			
M1	K174R			
M1	V219I			
M2	S31N	Adamantane-resistance determinant		

（文献 17 より引用）

その機能は不明であり，今後の検討を要する．さらには，Clade N は遺伝子再集合体で，2004/2005 年に流行した株からの PB2，HA，NA，NS と，2002～2004 年にニューヨークで採取された Clade B という群の PB1，PA，NP，MP で構成されるという．Clade N になる前の群では M2 の S31N 変異はなかったため，遺伝子再集合により，アマンタジン耐性を維持できるバックボーンが作られたと推測される．いずれにせよ，より多くの地域の株で今後耐性化獲得に共通するメカニズムを検証する必要があろう．

おわりに

A/H1N1 と H3N2 双方で耐性頻度が急増していることから当面アマンタジンの臨床使用は勧められない．しかし，耐性ウイルスの臨床知見に関してはいまだに情報が少なく，倫理面を考えながら今後も検討を続ける必要があろう．このようなアマンタジン耐性株大流行は誰もが予想し得なかった事実であり，NA 阻害薬でもいつ何時同じ出来事が起こるかもしれない．耐性モニタリングを今後も継続し，臨床医に常に情報をフィードバックする体制を整えねばならない．

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アマンタジン耐性インフルエンザウイルス

＊新潟大学大学院医歯学総合研究科国際感染症医学講座・公衆衛生分野
齋藤玲子，鈴木康司，李　丹娟，鈴木　宏

インフルエンザの治療・予防にはM2阻害剤とノイラミニダーゼ阻害剤が有効である．2004年以降中国においてA香港型(A/H3N2)インフルエンザがM2阻害剤(アマンタジン)に対して耐性化したとされ，それにひきつづき世界各地で耐性株増加の報告が相次いでいる．我が国でも2005～06年冬にA/H3N2のアマンタジン耐性化率が60%を越え，人から人へ伝播感染を起こしている事実が明らかになった．今後も臨床治療方針に迅速に反映すべく耐性ウイルスモニタリングを強化していく必要がある．

はじめに

インフルエンザの予防・治療にはM2阻害剤とノイラミニダーゼ阻害剤が有効である．我が国ではM2阻害剤としてアマンタジン(シンメトレル®)，ノイラミニダーゼ阻害剤としてザナミビル(リレンザ®)およびオセルタミビル(タミフル®)が使用されている．日本は世界有数の抗インフルエンザ剤使用国であり，日常診療で処方される機会も多いだけに，薬剤の効果を減弱させてしまう耐性インフルエンザは大きな問題である．その中で2005～06年より，アマンタジン耐性A型インフルエンザの大流行がみられ注目を集めた．

Ⅰ．アマンタジンの作用と特徴

アマンタジンはA型インフルエンザ・ウイルス膜に存在するM2イオンチャンネルの阻害剤である[1]．元々，抗インフルエンザ剤として1960年代より米国で使用され始めたが，副作用が多いため，我が国では1975年にパーキンソン病・脳梗塞後遺症に対してのみ認可となり内科領域で多用されてきた．一方，A型インフルエンザに対しては香港での鳥インフルエンザA(H5N1)の流行を受け，1998年に追加保険適応となった．アマンタジンは安価で安定であるが，投与後，中枢神経系の一過性の副作用が多くみられること，約1/3の患者に耐性株が出現することが問題である[2]．

Ⅱ．アマンタジン耐性化機序

アマンタジンによるA型インフルエンザの耐性化は同薬剤が阻害するM2蛋白遺伝子の特定部位にアミノ酸変異(26，27，30，31位)が起こることにより生じる[2]．このアミノ酸変化により全てのA型インフルエンザ(ソ連型，香港型，鳥インフルエンザを含む)がアマンタジン耐性となる．尚，B型インフルエンザにはアマンタジンは作用しないため，B型のアマンタジン耐性株は存在しない．

本来，アマンタジン耐性株は自然条件では非常に少ない割合で感受性株に混在し，薬剤の投与による選択圧で優勢になると考えられている．これまでアマンタジン耐性株の伝播力は感受性株に比べて弱いとされ[3]，高齢者施設や家族などの密に接する集団での伝播感染が報告されるのみで，市中においてアマンタジン耐性株のみの流行が起きることはなかった[2]．このため，一般に市中株中の耐性頻度は世界的に低く0～10%程度に留まっていた[3～6]．日本でも当教室の調査により，A型インフルエンザ市中株(薬剤投与前)耐性頻度は2004～05年シーズンまで1～4%程度であった(図1)．

Ⅲ．アマンタジン耐性株の世界的大流行

ここ1，2年で，アマンタジン耐性株を取り巻く状況は一変した．中国，香港で2004年にアマンタジン耐性A/H3N2(A香港)型が市中株の70%を越したことを皮切りに[4]，2005年にはアジア各国，オーストラリア，ニュージーランドにおいて耐性A/H3N2株の増加が確認された[7]．日本では，2005年9月，長崎県で季節はずれの流行がアマンタジン耐性株で起きたことが我々の調査で判明し[8]，その後，2005～06年冬に全国6ヵ所(新

〒951-8510 新潟市旭町通1-757

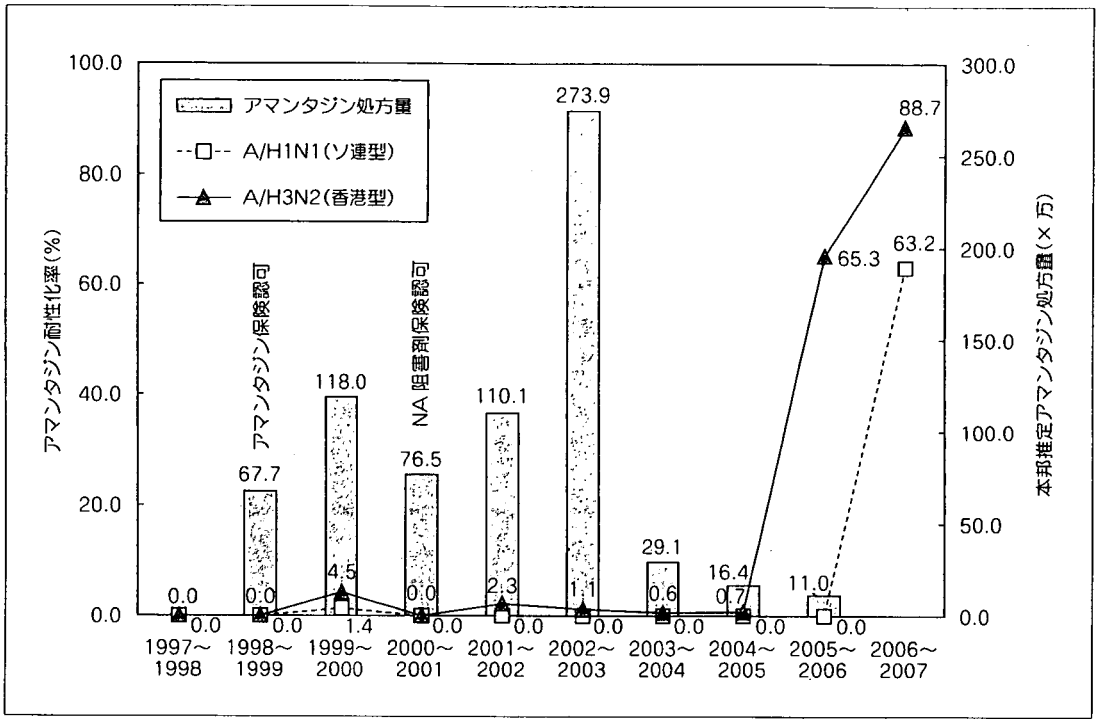

図1　市中株アマンタジン耐性A型インフルエンザ頻度と薬剤処方量

A型香港型(A/H3N2)は2005～06年以降，Aソ連型(A/H1N1)では2006～07年以降アマンタジン耐性株が急増した．一方，日本国内のアマンタジンの処方量は2002～03年の約300万人をピークとして減少している(2006～07年シーズンの処方量は未発表)．

潟，宮城，山形，群馬，長崎，福岡）で調査を行ったところ，A/H3N2の65.3%がアマンタジン耐性であった(図1)[9]．同時流行のAソ連型(A/H1N1)に耐性株はなかった．これらの患者にアマンタジン内服歴はなく，すべて耐性株の伝播感染によるものと思われた．年齢も2ヵ月児から106歳までと幅広く，アマンタジン耐性株があらゆる年齢層に伝播感染していたことが明らかになった．臨床経過については，初診時の症状にアマンタジン耐性株と感受性株で違いは無いものの，薬剤投与の効果については現在解析中である．奈良県でも同様に高率の耐性株が検出され[10]，我々の調査と合わせ，2005～06年シーズンは日本中でこれまでにないアマンタジン耐性株の大流行があったことが判明した．我が国のみならず，2005～06年シーズンには，米国で92.3%[11]，カナダでは76.5 %[12]のA/H3N2株がアマンタジン耐性であったと報告され，耐性株流行はほぼ全世界的な傾向と考えらる．

驚くべき事に，2006～07年も引き続きアマンタジン耐性株優位の状態が続いている．さらに，前年度まではみられなかったA/H1N1の耐性化率も増加している．当教室の調査では，2006～07年のA/H1N1株の63.2%，A/H3N2の88.7%がアマンタジン耐性であった．オーストラリアのグループからも同様な傾向が報告され[13]，世界的に依然A/H3N2で耐性株高値の状態が続いており，A/H1N1の耐性化も進んでいると考えられる．

アマンタジン耐性株はどのような機序で大流行を起こしたのであろうか．我が国のアマンタジン処方量はNA阻害剤の認可後2002～03年シーズン以降減っているのに対し(図1)，問題の耐性株は全て初診の時点でM2遺伝子の31位に変異を持つ耐性ウイルスであるため，患者に薬剤を投与したために大流行したとは到底考えがたい．このアマンタジン耐性株は中国起源説が唱えられており，SARS，鳥インフルエンザ以降中国で一般の風邪薬の一成分として配合され，耐性株増加の原因を作ったといわれる．しかし，それだけでは耐性株は激増しないため，M2遺伝子の耐性変異を保ちつつ通常のインフルエンザと同じ伝播力を発揮する遺伝子変異が起こったか，また

は遺伝子再集合により有利な遺伝子を獲得したものと思われるが[9,14]．詳細な機序については今後の検討が待たれる．

おわりに

これまでにないアマンタジン耐性化が急速に進み，我が国の2006～07年シーズンはほとんどのA/H3N2と，約半数のA/H1N1は耐性であった．臨床的に同薬剤の使用は勧められないが，一方でアマンタジン耐性株の臨床知見は乏しく，今後倫理的な問題をふまえた上で症例の積み重ねと解析が必要と思われる．

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SHORT COMMUNICATION

Antigenic variation in *Bordetella pertussis* isolates recovered from adults and children in Japan

Hyun-Ja Han[a], Kazunari Kamachi[a,*], Kenji Okada[b], Hiromi Toyoizumi-Ajisaka[a], Yuko Sasaki[a], Yoshichika Arakawa[a]

[a] *Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011, Japan*

[b] *Division of Pediatrics, National Hospital Organization Fukuoka National Hospital, Yakatabaru 4-39-1, Minami-ku, Fukuoka 811-1394, Japan*

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KEYWORDS
Bordetella pertussis;
Adult;
Allelic variant;
PFGE profile

Summary Recently, the incidence of reported pertussis cases of adults has dramatically increased in Japan. In the present study, we analyzed seven *Bordetella pertussis* isolates recovered from adults in Japan using pulsed-field gel electrophoresis (PFGE) and sequencing of their antigenic and virulence-associated proteins, compared with those from children. PFGE analysis demonstrated that the adult strains were closely related to the child strains (78–100% genetic similarity). On the other hand, the genotyping revealed that 71% (5/7) of the adult strains and 47% (25/53) of the child strains had the same combination of antigenic/virulence-associated allelic variants (*ptxS1B/prn1/fim2-1/fim3A/fhaB1/tcfA2*) as the Japanese vaccine strain Tohama, respectively. In comparison to the child strains, there was no apparent antigenic and genetic shift in the adult strains. Our result suggests that (i) there is no *B. pertussis* circulating strain specific to adults and (ii) the antigenic/virulence-associated proteins are unrelated to the rise in adult pertussis incidence in Japan.

Introduction

Bordetella pertussis, a highly communicable gram-negative coccobacillus, causes whooping cough which has been a major acute respiratory infection resulting in severe childhood illness and infant death [1]. Although, childhood pertussis immunization programs have been contributing to the reduction in morbidity and mortality in infants and children, the incidence of the disease in the U.S. has increased over the past 20 years, most notably in previously immunized adults and adolescents [2–4]. Recently, pertussis adults/adolescents are thought to be the primary reservoir of *B. pertussis* and play a crucial role in the transmission of the microbe to children, especially unvaccinated young infants [5–7]. In Japan, the incidence of reported pertussis cases of adults has dramatically increased from

* Corresponding author. Tel.: +81 42 561 0771; fax: +81 42 561 7173.
E-mail address: kamachi@nih.go.jp (K. Kamachi).

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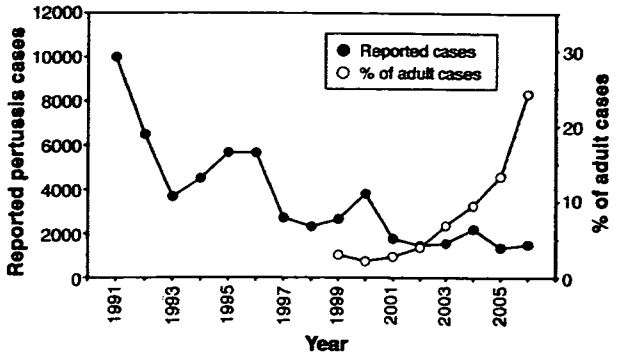

Figure 1 Reported pertussis cases and % of adult cases (≥20 years old) in Japan. The data were obtained from Infectious Disease Surveillance data of the Ministry Health, Labor and Welfare of Japan. The adult cases before 1998 were not monitored.

2002 to the present, although there has been no significant change in the diagnostic method. Based on the Infectious Disease Surveillance data of the Ministry of Health, Labor and Welfare of Japan, the pertussis incidence rates of adults (≥20 years old) were 4.0, 6.9, 9.5, 13.4 and 24.3% of the total reported cases in 2002–2006, respectively (Fig. 1). Although, high attack rates of pertussis have been observed in adults/adolescents even in the high vaccinating countries, it is not clear whether the adult/adolescent pertussis and *B. pertussis* recently circulating strain are associated with each other.

During the last decade, antigenic and genetic divergences in *B. pertussis* circulating strains have been observed in many countries [8–16]. The antigenic variants have been found in the S1 subunit of pertussis toxin (*ptxS1*) and pertactin (*prn*), which are important virulence factors of *B. pertussis*. In the United States, Canada, Russia and European countries, vaccine-types *ptxS1B* and *prn1* alleles of circulating strains have been replaced mainly with nonvaccine-types *ptxS1A* and *prn2*, respectively [8,9,14]. In Japan, the antigenic shifts have been observed from the mid-1990s, and two *ptxS1* (*ptxS1A* and *ptxS1B*) and 4 *prn* (*prn1*, *prn2*, *prn3* and *prn12*) allelic variants have been identified so far [17,18]. The novel variant, *prn12*, was found in one isolate collected in 1991 (GenBank accession no AB278117). Besides the antigenic shifts, the genetic divergence of *B. pertussis* virulence factors, tracheal colonization factor (*tcfA*), filamentous hemagglutinin (*fhaB*) and serotype 2 and 3 fimbriae (*fim2* and *fim3*), has also been found in several countries [13,19–21]. It has been suggested that the shifts in *B. pertussis* recently circulating strains have been induced as a vaccine-driven evolution [9,11]. However, it is unclear whether or not the divergence in the antigenic/virulence-associated proteins is indeed implicated in the increase of the adult/adolescent pertussis patients.

The aims of the present study were (i) to determine the genetic similarity in *B. pertussis* isolates recovered from adults and children by pulsed-field gel electrophoresis (PFGE) analysis, and (ii) to investigate the divergence in the antigenic/virulence-associated allelic variants in the adult strains and to compare this with the child strains. We also wanted to investigate the relationship between the allelic variation and the adult pertussis.

Materials and methods

Isolates

Seven and 53 *B. pertussis* clinical isolates recovered from adults (≥20 years old) and children [0–11 year(s) old], respectively, were investigated in the present study. All of the isolates were collected from nonepidemiologically related cases of pertussis during the period of 1991–2007 in Japan. The number of available adult strains from the laboratory collection of the National Institute of Infectious Diseases (NIID, Japan) was 7 due to the difficulty in isolating the organism from previously immunized persons, whereas the 53 child strains were randomly selected from the laboratory collections so as to reflect the same temporal distribution. Among the adult strains, three (BP173, BP183 and BP313) were recovered from individual familial infection cases, but the clinical associations of the others (BP241, BP239, BP245 and BP316) were unknown (Table 1). The isolates were cultured on Bordet-Gengou agar (Difco) supplemented with 1% glycerol and 15% defibrinated horse blood and incubated at 37 °C for 2–3 days.

PFGE analysis

PFGE was performed on a CHEF Mapper apparatus (Bio-Rad, Hercules, CA, USA) according to standardized recommenda-

Table 1 *Bordetella pertussis* isolates recovered from adults in Japan

Isolate	Isolation year	PFGE type	MLST	Patient		
				Age	Sex	Infection route
BP173	1991	A	MLST-1	32	Female	Household contact
BP183	1991	A	MLST-1	20	Male	Household contact
BP241	2003	A	MLST-1	27	Male	NA[b]
BP239	2004	B	MLST-2	38	Female	NA
BP245	2004	A	MLST-1	73	Female	NA
BP313	2006	A	MLST-1	58	Female	Household contact
BP316	2007	A	MLST-3	53	Female	NA

[a] Precise age unknown.
[b] Data not available.

tions for typing of *B. pertussis* [22]. The run condition was selected by the autoalgorithm mode of the system with a size range of 20–300 kb. The PFGE patterns were analyzed by the unweighted pair-group method with arithmetic averages (UPGMA) using Diversity Database version 1.1 software (PDI, Inc).

Genotyping

PtxS1 and *prn* of *B. pertussis* isolates were genotyped as described previously [18]. For *fim2*, *fim3* and *tcfA*, the complete open reading frames were sequenced [21]. On the other hand, *fhaB* was sequenced between positions 2250 to 2750, which contains the single polymorphic site identified previously [21]. Sequence reactions were carried out with BigDye terminator v.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), and the products were sequenced on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

Multilocus sequence typing (MLST)

Among 60 *B. pertussis* isolates tested, allelic variation was observed only in the three genes, *ptxS1*, *prn* and *fim3*. These combined allelic profiles were used to define MLSTs.

Statistic analysis

The chi-square test was employed. A value of $P < 0.05$ was considered to be significant.

Results

Genetic relationship between *B. pertussis* adult and child strains

To investigate the genetic relationship between adult and child strains, their PFGE profiles were analyzed in 7 and 53 clinical isolates recovered from adults and children, respectively. As shown in Fig. 2, 36 PFGE profiles were identified among the adult and child strains, and the profiles were classified into two major groups (type A and B) using UPGMA. The adult strains showed 6 different PFGE profiles, but the strains could be classified into the two groups; 6 isolates (BP173, BP183, BP241, BP245, BP313 and BP316) were type A and 1 isolate (BP239) was type B (Table 1 and Fig. 2). Of the 7 adult strains, 2 isolates (BP183 and BP316) had the same PFGE profile and 3 isolates (BP183, BP313 and BP316) had PFGE profiles to specific adults. However, the phylogenetic analysis of PFGE profiles demonstrated that all of the adult strains were closely related to the child strains (78–100% genetic similarity).

Distribution of antigenic and virulence-associated allelic variants in *B. pertussis* adult and child strains

To identify the antigenic/virulence-associated allelic variants in the adult strains, we carried out sequencing analyses of *ptxS1*, *prn*, *fim2*, *fim3*, *fhaB* and *tcfA* genes. Table 2 shows

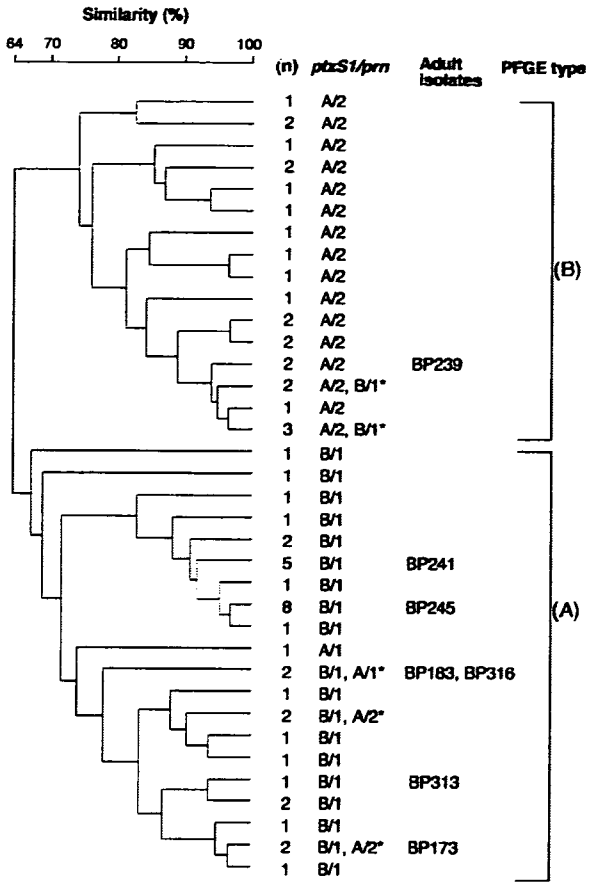

Figure 2 Phylogenetic tree of PFGE profiles of *Bordetella pertussis* isolates recovered from adults and children. The dendrogram was calculated by UPGMA. The adult isolates are indicated by their strain number (BP173, BP183, BP239, BP241, BP245, BP313 and BP316). The regions including the *ptxS1* and *prn* repeat were sequenced, and the combination of *ptxS1* and *prn* alleles is shown as *ptxS1/prn*, e.g., B/1 indicates *ptxS1B/prn1*. * One isolate had different *ptxS1/prn* alleles in the PFGE profile: BP173, B/1; BP183, B/1; BP316, A/1.

the distribution of the antigenic/virulence-associated allelic variants between the adult and child strains. Among the adult strains, *ptxS1B* and *ptxS1A* were detected in 71.4% ($n = 5$) and 28.6% ($n = 2$) of the isolates, respectively, and *prn1* and *prn2* in 85.7% ($n = 6$) and 14.3% ($n = 1$), respectively. For *fim3* gene, only *fim3A* was detected in the adult strains. On the other hand, among the child strains, *ptxS1B* and *ptxS1A* were detected in 54.7% ($n = 29$) and 45.3% ($n = 24$) of the isolates, *prn1* and *prn2* in 56.6% ($n = 30$) and 43.4% ($n = 23$), and *fim3A* and *fim3B* in 84.9% ($n = 45$) and 15.1% ($n = 8$), respectively. The distributions of *ptxS1*, *prn* and *fim3* alleles between the adult and child strains were not statistically significant ($P = 0.42$, 0.14, and 0.27, respectively), although the number of adult strains was somewhat low for meaningful analysis. Interestingly, *fim3B* was first observed in 1994 in Canada and then in Taiwan and Russia [8,19,20], whereas the variant was first identified from 2004 in Japan.

Table 2 Comparison of *Bordetella pertussis* antigenic and virulence-associated allelic variants between adult strains and child strains

Gene	Allele	Type[a]	% Frequency (*n*)	
			Adult strain (*n*=7)	Child strain (*n*=53)
ptxS1	*ptxS1B*	Vaccine	71.4 (5)	54.7 (29)
	ptxS1A	Nonvaccine	28.6 (2)	45.3 (24)
prn	*prn1*	Vaccine	85.7 (6)	56.6 (30)
	prn2	Nonvaccine	14.3 (1)	43.4 (23)
fim2	*fim2-1*	Vaccine	100 (7)	100 (53)
fim3	*fim3A*	Vaccine	100 (7)	84.9 (45)
	fim3B	Nonvaccine		15.1 (8)
fhaB	*fhaB1*	Vaccine	100 (7)	100 (53)
tcfA	*tcfA2*	Vaccine	100 (7)	100 (53)

[a] The Japanese vaccine strain Tohama harbors *ptxS1B*, *prn1*, *fim2-1*, *fim3A*, *fhaB1* and *tcfA2* alleles.

For *fim2*, *fhaB* and *tcfA* genes, each variant (*fim2-1*, *fhaB1* and *tcfA2*) was identified among the adult and child strains. There was no allelic variation in *fim3*, *fhaB* and *tcfA*. The Japanese vaccine strain Tohama harbored *ptxS1B*, *prn1*, *fim2-1*, *fim3A*, *fhaB1* and *tcfA2* alleles.

MLSTs of *B. pertussis* adult and child strains

Three genes (*ptxS1*, *prn* and *fim3*) were used to define MLSTs. Among 60 *B. pertussis* isolates tested, five MLSTs (MLST-1 to -5) were identified by the combined allelic profiles (Table 3). Three (MLST-1 to -3) and 5 MLSTs (MLST-1 to -5) were found in the adult and child strains, respectively. MLST-1 was predominant in both strains. Five (71.4%) of 7 adult strains and 25 (47.2%) of 53 child strains were MLST-1, respectively. Among the adult strains, MLST-1 strains were found from 1991 to 2006, and MLST-2 and MLST-3 in 2004 and 2007, respectively (Table 1). The differences in frequencies of the MLSTs between adult and child strains were not statistically significant ($P=0.28$). The Japanese vaccine strain Tohama was MLST-1.

Discussion

In the present study, we demonstrated that (i) PFGE profiles of adult strains were closely related to those of child strains (78–100% genetic similarity), and (ii) there were no significant differences in the distribution of the antigenic/virulence-associated allelic variants between the adult and child strains. These results indicate that *B. pertussis* adult strains are indistinguishable from the child strains by molecular typing methods. To estimate the possibility of acquisition of novel virulence factor(s) in adult strains, we also performed a comparative proteomic analysis of the adult and child strains. However, no apparent difference was observed in their protein profiles (data not shown). Our observations strongly suggest that there is no *B. pertussis* circulating strain specific to adults, and that the circulating strains have an ability to infect not only children but also adults.

Previously, De Schutter et al. [23] reported that Belgian *B. pertussis* adult strains, recovered from familial infections in the period of 1999–2002, harbored nonvaccine-types *ptxS1A* and *prn2* (or *prn3*) alleles. However, in European countries and the United States, the genetic shifts observed in *ptxS1* and *prn* alleles had already occurred since the 1990s [8,9,14]. The most circulating strains therefore harbored *ptxS1A* and *prn2* (or *prn3*) alleles in those countries. In contrast, approximately half of the Japanese circulating strains, collected in the period of 1991–2007, harbored vaccine-types *ptxS1B* and *prn1* alleles, indicating that the shifts have now progressed in Japan (this study). Therefore, the present study showed the actual relationship between the antigenic shift and *B. pertussis* adult strains, i.e., there was no apparent antigenic shift in the adult strains. In a previous report, Mastrantonio et al. [24] demonstrated that the frequency of vaccine-type *prn1* allele in strains recovered from unvaccinated children was higher than that of vaccinated children. They suggested that *B. pertussis* strains harboring *prn1* allele might be more affected by vaccine-induced immunity than the strains harboring nonvaccine-type *prn2* or *prn3* allele. However, in the present study, no such difference in the distribution of *prn* allelic variants was observed between adult and child strains.

In Japan, the acellular pertussis vaccines introduced in 1981 have successfully controlled pertussis in children. However, the incidence of reported pertussis cases of adults has considerably increased from 2002 to the present. The waning of vaccine-acquired immunity and decreased oppor-

Table 3 MLST frequencies between *Bordetella pertussis* adult strains and child strains

MLST	Alleles	Type[a]	% Frequency (*n*)	
			Adult strain (*n*=7)	Child strain (*n*=53)
MLST-1	*ptxS1B/prn1/fim3A*	Vaccine	71.4 (5)	47.2 (25)
MLST-2	*ptxS1A/prn2/fim3A*		14.3 (1)	35.8 (19)
MLST-3	*ptxS1A/prn1/fim3A*		14.3 (1)	1.9 (1)
MLST-4	*ptxS1A/prn2/fim3B*			7.5 (4)
MLST-5	*ptxS1B/prn1/fim3B*			7.5 (4)

[a] The Japanese vaccine strain Tohama is MLST-1.

tunities for boosting of immunity because of reduced levels of circulation of *B. pertussis* have been proposed as possible reasons for adult/adolescent pertussis [25]. However, the Japanese adult pertussis cannot be thus explained, since U.S. adult/adolescent pertussis has steadily increased during the last two decades [2–4]. The reason for the rising pertussis incidence in Japanese adult population has remained unclear. Further analyses are warranted to determine the cause of the adult pertussis.

In conclusion, *B. pertussis* isolates recovered from Japanese adults and children were indistinguishable by molecular typing methods. In Japan, the antigenic and genetic shifts, especially *ptxS1* and *prn* alleles, would be unrelated to the rise of the adult pertussis incidence.

Acknowledgements

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Genetic Characteristics and Clonal Dissemination of β-Lactamase-Negative Ampicillin-Resistant *Haemophilus influenzae* Strains Isolated from the Upper Respiratory Tract of Patients in Japan▽

Muneki Hotomi,[1] Keiji Fujihara,[1] Dewan S. Billal,[1] Kenji Suzuki,[2,3] Tadao Nishimura,[2] Shunkichi Baba,[2] and Noboru Yamanaka[1,2]*

Department of Otolaryngology-Head and Neck Surgery, Wakayama Medical University, Wakayama, Japan[1]; Surveillance Subcommittee, Japan Society for Infectious Diseases in Otolaryngology[2]; and Department of Otolaryngology, Second Affiliated Hospital, Fujita Health University, Nagoya, Japan[3]

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We evaluated the recent prevalence of antimicrobial-resistant *Haemophilus influenzae* isolated from the upper respiratory tracts (URT) of patients in Japan. Mutations in the *ftsI* gene, which encodes penicillin binding protein 3 (PBP3), and the clonal dissemination of the resistant strains were also investigated. A total of 264 *H. influenzae* isolates were collected from patients with URT infections. According to the criteria of the Clinical and Laboratory Standards Institute for the susceptibility of *H. influenzae* to ampicillin (AMP), the isolates were distributed as follows: 161 (61.0%) susceptible strains (MIC ≤ 1 μg/ml), 37 (14.0%) intermediately resistant strains (MIC = 2 μg/ml), and 66 (25.0%) resistant strains (MIC ≥ 4 μg/ml). According to PCR-based genotyping, 172 (65.1%) of the isolates had mutations in the *ftsI* gene and were negative for the β-lactamase (*bla*) gene. These 172 isolates were thus defined as genetically β-lactamase-negative ampicillin-resistant (gBLNAR) strains. The *ftsI* mutant group included 98 (37.1%) strains with group I/II mutations in the variable mutated region (group I/II gBLNAR) and 74 (28.0%) strains with group III mutations in the highly mutated region (group III gBLNAR). Eighty-seven (33.0%) of the isolates were genetically β-lactamase-negative ampicillin-susceptible (gBLNAS) strains. The group III gBLNAR strains showed resistance to β-lactams. Only five strains (1.9%) were positive for a *bla* gene encoding TEM-type β-lactamase. The three clusters consisting of 16 strains found among the 61 BLNAR strains (MIC ≥ 4 μg/ml and without the *bla* gene) showed identical or closely related DNA restriction fragment patterns. Those isolates were frequently identified among strains with a MIC to AMP of 16 μg/ml. The current study demonstrates the apparent dissemination and spread of a resistant clone of *H. influenzae* among medical centers in Japan. The gBLNAR strains show a remarkable prevalence among *H. influenzae* isolates, with the prevalence increasing with time. This fact should be taken into account when treating URT infections.

Haemophilus influenzae is a frequently isolated bacterium responsible for various infections of the respiratory tract, including acute otitis media, sinusitis, acute purulent exacerbation of bronchitis, and pneumonia (14, 23, 29, 34). Since the first reports of ampicillin-resistant strains of *H. influenzae* in the United States in 1974, the major mechanism of antimicrobial resistances of *H. influenzae* has been considered to be related to either TEM-1 or ROB-1 types of β-lactamase (4–6, 35). The prevalence of β-lactamase-producing strains in the United States has increased progressively: up to 15.2% in 1983 and 1984, 36.4% in 1994 and 1995, and 31.3% in 1997 and 1998 (4–6). The issue was further complicated in the 1980s by the identification of β-lactamase-negative ampicillin (AMP)-resistant (BLNAR) strains (21). The BLNAR strains generally continued to be isolated at low frequencies in the 1980s (24, 25). However, a surveillance study conducted in Japan in the 1990s showed a marked 19.5% increase in the frequency of BLNAR strains (28). Systematic surveillance studies are essential tools in the effort to define trends in the antimicrobial resistance of bacteria.

The mechanism of resistance in the BLNAR strains involves decreased affinities of penicillin binding proteins (PBPs) for β-lactam antibiotics (26, 30). Among the several PBPs of *H. influenzae*, alterations in PBP3-mediating septal peptidoglycan synthesis during cell division are essential for developing resistance (2, 4). Recent studies characterizing mutations of the *ftsI* gene encoding PBP3 classified the BLNAR strains into three groups based on deduced amino acid substitutions (36). These are the group I strains, with the substitution of Arg-517 for His-517 (Arg-517-His) near the conserved Lys-Thr-Gly (KTG) motif; the group II strains, with the substitution Asn-526-Lys; and the group III strains, with the substitution of three amino acid residues (Met-377, Ser-385, and Leu-389) positioned near the conserved Ser-Ser-Asn (SNN) motif for Ile-377, Thr-385, and Phe-389, respectively, in addition to the substitution Asn-526-Lys (3, 9, 10, 17, 36). Group II BLNAR strains were further divided into four subgroups: subgroup IIa, with the substitution Asn-526-Lys without the substitution for Ala-502; subgroup IIb, with the substitution Val-502-Ala; subgroup IIc, with the substitution Thr-502-Ala; and subgroup IId, with the substitution Val-449-Ile. Of the various missense mutations of the *ftsI* gene, resistance to β-lactam antibiotics

* Corresponding author. Mailing address: Department of Otolaryngology-Head and Neck Surgery, Wakayama Medical University, 811-1 Kimiidera, Wakayama-shi, Wakayama, 641-8509, Japan. Phone: 81-73-441-0651. Fax: 81-73-446-3846. E-mail: ynobi@wakayama-med.ac.jp.
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TABLE 1. Oligonucleotide primers used in this study

Primer	Gene	Sequence	Product size (bp)
PBP3-S	*ftsI*	Forward 5′-GATACTACGTCCTTTAAATTAAG-3′ Reverse 5′-GCAGTAAATGCCACATACTTA-3′	551
PBP3-BLN	*ftsI*	Forward 5′-TTCAAGTAACCGTGGTGTGAC-3′ Reverse 5′-GCAGTAAATGCCACATATTTC-3′	465
BLP	*bla*	Forward 5′-TAAGAGAATTATGCAGTGCTGCC-3′ Reverse 5′-TCCATAGTTGCCTGACTCCCC-3′	458
P6	*p6*	Forward 5′-ACGATGCTGCAGGCAATGGT-3′ Reverse 5′-TCCATAGTTGCCTGACTCCCC-3′	198
CPSB	*cps* b	Forward 5′-ACGATGCGCAGGCAATGGT-3′ Reverse 5′-CATCAGTATTACCTTCTACTAAT-3′	224

largely depends on the substitutions Arg-517-His, Asp-526-Lys, Ser-385-Thr, and Leu-589-Phe. Intermediate AMP resistance is commonly found in groups I and II; however, isolates in group III are associated with a higher level of AMP resistance (3, 10, 36).

In 2003, the Japanese Society of Infectious Diseases in Otolaryngology conducted its fourth nationwide surveillance to define the causative pathogens of infectious diseases of the upper respiratory tract (URT) and their contemporary resistance status in Japan. In this report, we present the first part of the surveillance data regarding *H. influenzae*, including the genetic characteristics and the clonal pattern of BLNAR strains.

(This work was presented at the 45th Interscience Conference on Antimicrobial Agents and Chemotherapy in Washington, DC, 16 to 19 December 2005.)

MATERIALS AND METHODS

Populations. Between January and May 2003, the Japanese Society of Infectious Diseases in Otolaryngology conducted the fourth nationwide surveillance of the bacterial pathogens responsible for otolaryngological infections. Informed consent was obtained from patients or their parents or guardians according to the guidelines of the institutional review board on clinical samples. Duplicate isolates from the same patients were excluded from the study to prevent repeated isolates.

Identification of *H. influenzae*. Specimens were collected aseptically with small cotton swabs and placed in an anaerobic transport jar (Eiken Chemical Co., Tokyo, Japan). All strains were identified at the Mitsubishi Kagaku Bio-Clinical Laboratories (Tokyo, Japan). *H. influenzae* strains were identified and confirmed by colony morphology, Gram staining, growth in chocolate agar but not in blood agar, the catalase test, and the X and V factor requirement. Production of β-lactamase was examined using a nitrocefinase disc (Nippon Becton Dickinson Company Ltd., Tokyo, Japan). Until the study began, the isolates were stocked in litmus milk broth (Difco Laboratories, Detroit, MI) containing 10% glycerol at −80°C. Serotyping of *H. influenzae* was determined by a slide agglutination procedure using type b antiserum (Denka Seiken, Tokyo, Japan). The method used to identify *H. influenzae* in this study could not accurately distinguish these strains from *Haemophilus haemolyticus*. Thus, some of the *H. influenzae* strains may in fact be *H. haemolyticus* (27).

Antimicrobial susceptibility. Antimicrobial susceptibilities were determined by measuring the isolates' MICs at Mitsubishi Kagaku Bio-Clinical Laboratories using the broth microdilution method according to the procedure set forth by the Clinical and Laboratory Standards Institute (CLSI) (2a). The 10 antibiotics tested in this study were AMP, clavulanate-amoxicillin, cefpodoxime, cefditoren, cefuroxime, clarithromycin, azithromycin, telithromycin, levofloxacin, and meropenem. The breakpoints recommended by the CLSI were used to define susceptibility patterns.

PCR-based genotyping. The oligonucleotide primers used in this study are listed in Table 1. Primers for the *ftsI* gene were used to amplify both the variable mutated locus (Asn-526 or Arg-517; primer set PBP3-S) and a highly mutated locus (Ser-385; primer set PBP3-BLN) (9, 11, 30). To determine the strain having a β-lactamase gene, the *bla* locus was amplified by specific primers (9). To confirm that the isolated pathogen was *H. influenzae*, the P6 gene was identified (9). Outer membrane protein P6 is a member of the class of outer membrane proteins known as peptidoglycan-associated lipoproteins, which are highly conserved among *H. influenzae* isolates. To identify the type b *H. influenzae* strain, the *cps* b locus, which encodes type b capsular polysaccharides, was examined by PCR (9). A single colony of *H. influenzae* on chocolate agar plates was lysed in 30 μl of lysis solution (1 M Tris, pH 8.9, 4.5% [vol/vol] NP-40, 4.5% [vol/vol] Tween 20, and 10 mg/ml proteinase K) for 10 min at 60°C and for 5 min at 94°C in a programmable thermal cycler (Gene Amp PCR System 9700; Perkin-Elmer, Norwalk, CT). The reaction mixture (total volume, 50-μl) consisted of 2 μl of bacterial lysate, 0.8 μl of a 10 mM deoxynucleoside triphosphate mixture, 0.1 μl of *Taq* DNA polymerase, 2.5 μl of 10× PCR buffer, 0.5 μl of 25 mM $MgCl_2$, 5.0 μl Q-solution (QIAGEN, Valencia, CA), and 0.125 μl (100 μM) each of primer and distilled water. The mixture was subjected to denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and then further extension at 72°C for 10 min. The amplified DNA fragments were analyzed using 3% agarose gel electrophoresis. On the basis of the PCR-based genotyping, the *H. influenzae* strains were classified into four genotypes: genetically β-lactamase-negative AMP-susceptible (gBLNAS) strains, without amino acid substitutions in the *ftsI* gene and β-lactamase (*bla*) gene; genetically BLNAR (gBLNAR) strains, with an amino acid substitution in the *ftsI* gene; genetically β-lactamase-positive ampicillin-resistant (gBLPAR) strains, with the *bla* gene but without an amino acid substitution in the *ftsI* gene; and genetically β-lactamase-positive amoxicillin-clavulanate-resistant (gBLPACR), with the *bla* gene and an amino acid substitution in the *ftsI* gene. The gBLNAR strains were further divided into two subgroups: group I/II gBLNAR strains, with an amino acid substitution in the variable mutated locus of *ftsI* (at Asn-526 or Arg-517), and group III gBLNAR strains, with an amino acid substitution in the highly mutated locus of *ftsI* (at Ser-385). The strains positive for the *bla* gene were also further divided into three groups based on amino acid substitutions in the *ftsI* gene: strains with group I/II amino acid substitutions in the *ftsI* gene (group I/II gBLPACR), strains with group III amino acid substitutions in the *ftsI* gene (group III gBLPACR), and strains without amino acid substitutions in the *ftsI* gene (BLPAR) (Fig. 1). In this study, we have designated PCR-based genotypes gBLNAS, gBLNAR, gBLPACR, and gBLPAR to distinguish them from phenotypes, which are written without the introductory "g."

DNA restriction fragment polymorphism analyzed by PFGE. The restriction fragment polymorphisms of SmaI-digested chromosomal DNA from *H. influenzae* isolates were evaluated by pulsed-field gel electrophoresis (PFGE) (12). PFGE patterns were analyzed by using Fingerprinting software (Bio-Rad Laboratories, Hercules, CA).

RESULTS

Characteristics of *H. influenzae* isolates. A total of 264 *H. influenzae* isolates were collected from 264 patients during the period of surveillance. The patients ranged in age from 0 to 83 years old, with 143 males and 121 females (Table 2). Among the isolates, 66 (25.0%) were from middle ear fluid, 77 (29.1%) were from the nasopharynges of patients with acute otitis media, 58 (22.0%) were from the crypts of the palatine tonsils of patients with pharyngotonsillitis, and 63 (23.9%) were from the nasal discharges or sinus aspirates of patients with acute rhi-

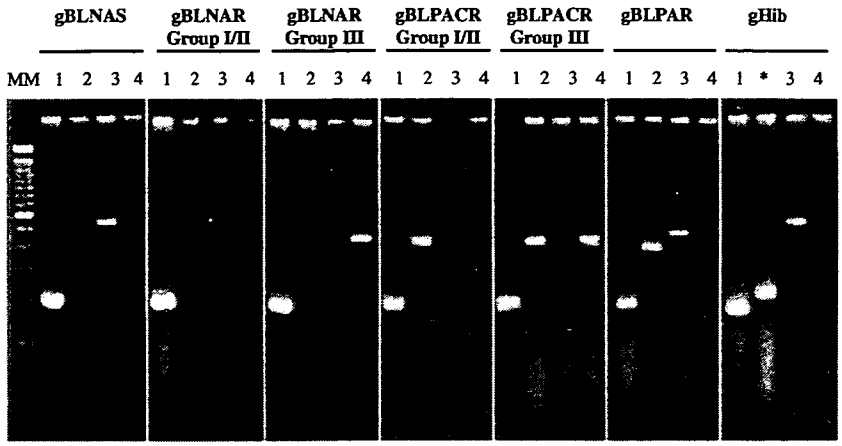

FIG. 1. PCR-based genotypes of *H. influenzae*. Lane 1, P6 gene encoding outer membrane protein P6; lane 2, *bla*; lane 3, *pbp3-S*; lane 4, *pbp3-BLN*; lane *, *cps* b encoding type b capsular polysaccharides. MM, molecular weight marker.

nosinusitis. The isolates from middle ear fluid, sinus aspirates, and tonsillar crypts represented true infections, while the remaining isolates from the nasopharynx and nasal discharge might have represented colonization of the upper airway. There were 37 (14.0%) isolates collected in university hospitals, 84 (31.8%) collected in general hospitals, and 143 (54.2%) collected in private clinics.

Out of the 264 *H. influenzae* isolates, 259 (98.1%) were *bla* gene negative. Only five (1.9%) strains had *bla* genes encoding TEM-type β-lactamase. According to the criteria for the susceptibility of *H. influenzae* to AMP set forth by the CLSI, the 259 strains negative for the *bla* gene were divided into three groups: 161 (62.2%) susceptible strains (MIC ≤ 1 μg/ml), 37 (14.3%) intermediately resistant strains (MIC = 2 μg/ml), and 61 (23.5%) resistant strains (MIC ≥ 4 μg/ml). Serotype b *H. influenzae* was identified in only two isolates. The phenotypic and genotypic tests completely agreed as to species level identification, β-lactamase production, and capsular type. There were no significant differences in the distributions of resistant strains based on gender, age, disease, and type of clinic.

PCR-based genotypes and susceptibility to AMP. The prevalence of each PCR-based genotype among the 264 *H. influenzae* isolates was as follows: 87 (33.0%) were gBLNAS strains, 98 (37.0%) were group I/II gBLNAR strains, 74 (28.0%) were group III gBLNAR strains, 1 (0.4%) was a group I/II gBLPACR strain, 2 (0.8%) were group III gBLPACR strains,

TABLE 2. Sources of clinical isolates of *H. influenzae*

Characteristic	Total no. (%) of isolates	No. (%) with susceptibility to AMP			*P* value[a]
		Susceptible	Intermediate	Resistant	
Total	264	161 (61.0)	37 (14.0)	66 (25.0)	
Gender					
Male	143 (54.2)	91 (63.6)	16 (11.2)	36 (25.2)	NS
Female	121 (45.8)	70 (57.9)	21 (17.4)	30 (24.8)	
Age (yr)					
0–2	108 (40.9)	56 (51.9)	19 (17.6)	33 (30.6)	NS
3–5	38 (14.4)	27 (71.1)	5 (13.2)	6 (15.8)	NS
6–12	19 (7.2)	12 (63.2)	1 (5.3)	6 (31.6)	NS
13–20	15 (5.7)	10 (66.7)	1 (6.7)	4 (26.7)	NS
21–50	72 (27.3)	48 (66.7)	9 (12.5)	15 (20.8)	NS
≥51	12 (4.5)	8 (66.7)	2 (16.7)	2 (16.7)	NS
Specimens type					
Middle ear fluid	66 (25.0)	34 (51.5)	14 (21.2)	18 (27.3)	
Nasopharyngeal secretion	77 (29.2)	46 (59.7)	10 (13.0)	21 (27.3)	NS
Nasal discharge/sinus aspirate	63 (23.9)	38 (60.3)	9 (14.3)	16 (25.4)	NS
Tonsil swab	58 (22.0)	43 (74.1)	4 (6.9)	7 (19.0)	NS
Type of clinic					
University medical center	37 (14.0)	23 (62.2)	7 (18.9)	11 (18.9)	NS
General hospital	84 (31.8)	49 (58.3)	14 (16.7)	21 (25.0)	NS
Private clinic	143 (54.2)	89 (62.2)	16 (11.2)	38 (26.6)	NS

[a] NS, nonsignificant.

TABLE 3. Correlation between PCR-based genotyping and susceptibility to AMP

PCR-based genotype	No. of isolates	No. of isolates with MIC to AMP (μg/ml) of:										MIC (μg/ml)			Susceptibility (%)[a]		
		≤0.12	0.25	0.5	1	2	4	8	16	32	≥64	MIC_{50}	MIC_{90}	Range	S	I	R
gBLNAS	87	3	35	46	3							0.5	0.5	0.12–1	100	0	0
Group I/II gBLNAR	98	5	23	21	18	23	6	2				0.5	2	0.12–8	68.4	23.5	8.1
Group III gBLNAR	74				7	14	18	24	10	1		4	16	1–32	9.5	18.9	71.6
Group I/II gBLPACR	1										1	128	128	128	0	0	100
Group III gBLPACR	2										2	>128	>128	>128	0	0	100
gBLPAR	2										2	64	>128	64–>128	0	0	100
Total	264	8	58	67	28	37	24	26	10	1	5	0.5	8	0.12–>128			

[a] S, susceptible; I, intermediate; R, resistant.

and 2 (0.8%) were gBLPAR strains (Table 3). Forty-nine strains (18.6%) had group I/II mutations apparently affecting PBP3, although their AMP MICs were at or below the CLSI susceptible breakpoint. The MIC_{50}s of AMP for the group I/II gBLNAR strains were similar to those for the gBLNAS strains, while the MIC_{90}s of AMP for the group I/II gBLNAR strains were higher than those for the gBLNAS strains. The MIC_{50}s and MIC_{90}s of AMP for the group III gBLNAR strains were 8 times and 32 times higher than those for the gBLNAS isolates, respectively.

PCR-based genotypes and antimicrobial susceptibilities to other antibiotics. The susceptibilities of gBLNAS and gBLNAR isolates of *H. influenzae* to other antibiotics are listed in Table 4. Most of the gBLNAS isolates were susceptible to β-lactams and exhibited relatively reduced susceptibilities (MIC_{50} = 8 μg/ml) to clarithromycin. On the other hand, the MIC_{50}s of clavulanate-amoxicillin and cephalosporin for the group III gBLNAR isolates were 4 to 64 times higher than those for gBLNAS isolates. Most of the group III gBLNAR isolates were susceptible to azithromycin, telithromycin, and meropenem. Among cephalosporins, cefditoren was the most active agent tested, with a MIC_{50} and MIC_{90} of 0.12 and 0.5 μg/ml for the group III gBLNAR isolates, respectively. The group I/II gBLNAR isolates usually showed susceptibilities similar to those of the gBLNAS isolates. Levofloxacin was the most potent antimicrobial agent against *H. influenzae* isolates.

Genetic distribution of BLNAR strains by PFGE. PFGE was used to evaluate the clonal disseminations of the BLNAR strains by detecting the restriction enzyme polymorphism of chromosomal DNA. PFGE analysis of the 61 BLNAR isolates showed that 16 (26.3%) isolates shared either identical or highly similar (≥80%) banding patterns (Fig. 2). Both of these clusters had MICs to AMP of 4 μg/ml and were *bla* negative. One cluster (cluster A) was made up of a group I/II gBLNAR strain and its related clones, which mostly showed MICs to AMP of 16 μg/ml. Another cluster (cluster B; 100% identical) was a group III gBLNAR strain and its related clones, which mostly showed MICs to AMP of 4 to 8 μg/ml. The last cluster (cluster C) had five isolates, and all shared ≥80% pattern similarity. In contrast to strains with MICs to AMP of 16 μg/ml, most of strains with MICs to AMP of 4 to 8 μg/ml showed various PFGE patterns (similarity ≤ 70%). All of the BLNAR strains in the three clusters were isolated in different clinics and different places in Japan.

DISCUSSION

The BLNAR strains of *H. influenzae* are becoming the latest problem for medical doctors who have to deal with the antimicrobial resistance of *H. influenzae* (7, 16). In the current surveillance, we focused on BLNAR strains isolated from the URT and investigated the clonal dissemination of the resistant strains in Japan. The BLNAR strains were identified in 25.0% of *H. influenzae* samples isolated from the URT, with *H. influenzae* strains having the *bla* gene being identified in only five (1.9%) isolates. Interestingly, in the United States and Europe, the BLNAR strains were uncommon and represented less than 1 to 2% of *H. influenzae* strains until the early 1990s, with their prevalence gradually increasing from 2.5% in 1994 to 10.1% 1995 (4–6, 16). In Japan, the BLNAR strains were not identified before 1984 and then had a prevalence of only 2.1% in 1988 and 5.0% in 1991 (32). The strains with MICs for AMP of 1.0 μg/ml then increased from 23.1% to 37.8% from 1996 to 1999 (32). Furthermore, a prospective prevalence study in Japan in 1999 showed that 55.1% of the *H. influenzae* strains were BLNAS, 3% were BLPAR, 26.4% were intermediate-resistant strains, and 13.2% were BLNAR (35). The increase in the percentage of BLNAR strains has led to serious problems in the treatment of infectious disease in Japan (26, 31–33). Unfortunately, there is still limited information about the precise prevalence and dissemination of antimicrobial-resistant pathogens.

The detection of BLNAR strains with decreased susceptibilities to β-lactams is controversial. The CLSI definition of BLNAR strains consists of those strains with no detectable β-lactamase and with MICs for AMP of 4 μg/ml. Strains with MICs for AMP of 2 μg/ml are defined as having intermediate, or indeterminate, resistance because such strains can show themselves to be susceptible, intermediate, or resistant to AMP if retested another day or by another method. There is no universal consensus concerning the breakpoint of BLNAR, with several different values having been proposed (1, 15). Kim et al. reported antimicrobial susceptibilities by Etest and showed reduced susceptibilities to AMP (256 μg/ml) for BLNAR strains (18). However, the Etest sometimes fails to exhibit the actual MIC, especially for BLNAR strains, while the test is useful to determine the antimicrobial susceptibilities of BLNAS strains (1a). An effort to acquire a genetic understanding of the intricacies of the resistance mechanism led to the development of reliable tests for detecting BLNAS strains.

TABLE 4. Antimicrobial susceptibilities of *H. influenzae* isolates to other antibiotics according to PCR-based genotypes

Antimicrobial agent	PCR-based genotype	No. of isolates	MIC (μg/ml)			Susceptibility (%)[a]		
			MIC_{50}	MIC_{90}	Range	S	I	R
Clavulanate-amoxicillin	gBLNAS	87	0.5	1	0.25–2	100	0	0
	Group I/II gBLNAR	98	1	4	0.25–16	92.9	NA	7.1
	Group III gBLNAR	74	8	32	2–32	26.7	NA	73.3
	Total	259	1	16	0.25–32			
Cefpodoxime	gBLNAS	87	0.12	0.12	≤0.06–0.12	100	NA	0
	Group I/II gBLNAR	98	0.25	2	≤0.06–8	90.8	NA	9.2
	Group III gBLNAR	74	8	16	≤0.06–64	9.1	NA	91.9
	Total	259	0.25	8	≤0.06–64			
Cefditoren	gBLNAS	87	≤0.06	≤0.06	≤0.06–0.12	NA	NA	NA
	Group I/II gBLNAR	98	≤0.06	0.12	≤0.06–0.5	NA	NA	NA
	Group III gBLNAR	74	0.25	0.5	≤0.06–8	NA	NA	NA
	Total	259	≤0.06	0.5	≤0.06–8			
Cefuroxime	gBLNAS	87	1	2	0.25–16	97.7	1.1	1.2
	Group I/II gBLNAR	98	2	16	0.25–64	66.3	12.3	21.4
	Group III gBLNAR	74	64	128	0.25–>128	1.4	4.1	94.6
	Total	259	2	128	0.12–>128			
Clarithromycin	gBLNAS	87	8	16	2–32	79.3	17.2	3.5
	Group I/II gBLNAR	98	8	16	2–32	75.5	22.5	2.0
	Group III gBLNAR	74	8	16	4–16	60.8	39.2	0
	Total	259	8	16	2–32			
Azithromycin	gBLNAS	87	2	4	0.25–8	98.8	NA	1.2
	Group I/II gBLNAR	98	2	4	0.5–16	95.9	NA	4.1
	Group III gBLNAR	74	2	4	0.5–8	97.3	NA	2.7
	Total	259	2	4	0.25–16			
Telithromycin	gBLNAS	87	2	4	0.5–8	97.7	2.3	0
	Group I/II gBLNAR	98	2	4	0.5–8	93.9	6.1	0
	Group III gBLNAR	74	2	4	1–8	87.8	12.2	0
	Total	259	2	4	0.5–8			
Levofloxacin	gBLNAS	87	≤0.06	≤0.06	≤0.06	100	0	0
	Group I/II gBLNAR	98	≤0.06	≤0.06	≤0.06–0.12	100	0	0
	Group III gBLNAR	74	≤0.06	≤0.06	≤0.06–0.5	100	0	0
	Total	259	≤0.06	≤0.06	≤0.06–0.5			
Meropenem	gBLNAS	87	≤0.06	≤0.06	≤0.06–0.12	100	0	0
	Group I/II gBLNAR	98	0.12	0.25	≤0.06–0.5	100	0	0
	Group III gBLNAR	74	0.25	0.5	≤0.06–2	90.5	0	9.5
	Total	259	0.12	0.5	≤0.06–2			

[a] S, susceptible; I, intermediate; R, resistant; NA, not available.

Thus, in this study, we used a PCR-based method to determine *H. influenzae* genotypes. This method allowed us to evaluate mutations of the *ftsI* gene, which encodes PBP3. This, in turn, led to a better understanding of the genetic characteristics of the pathogen.

The group I/II gBLNAR strain has mutations at the Asn-526 locus in the *ftsI* gene. The group III gBLNAR strain has a Thr-385-Ser substitution in the *ftsI* gene around the SSN motif, in addition to a Lys-526 substitution that mostly affects the increases of MICs (3, 17–19, 22, 31, 36). The Asn-526 locus in the *ftsI* gene may not affect the PBP3 structure at the β-lactam binding site and thus may not result in phenotypic amipicillin resistance, whereas the Thr-385-Ser substitution may have a direct effect. The Asn-526 region may not represent a significant mutation site. For PCR-based genotyping, we used primers for the *ftsI* gene to amplify the variably mutated locus of Asn-526 in BLNAS strains and the highly mutated locus of Ser-385 frequently identified in BLNAR strains (9, 11). We found that the Asn-526 locus can be amplified in BLNAS strains while the highly mutated Ser-385 locus in the BLNAR strains failed to be amplified. On the other hand, the latter locus frequently showed similar mutations among BLNAR strains.

According to the PCR-based genotyping of *H. influenzae*, gBLNAR strains were highly prevalent in Japan (65.1%), and about 86.9% of gBLNAR strains were classified as group III gBLNAR. About 62.2% of intermediately AMP-resistant strains were also classified as group I/II gBLNAR strains, and about 41.6% of AMP-susceptible strains were classified as group I/II gBLNAR strains. The AMP-susceptible isolates with an amino acid substitution in the *ftsI* gene have the potential to develop further resistance to penicillin and cephalosporin.