厚生労働科学研究費補助金　新興・再興感染症研究事業(平成17年度)
「新型の薬剤耐性菌のレファレンス並びに耐性機構の解析及び迅速・簡便検出法に関する研究」

分担研究課題：呼吸器感染症からの検査材料を用いた直接PCRによる原因菌の迅速検索法の確立

北里大学北里生命科学研究所　感染情報学研究室　生方　公子

【目的】 呼吸器感染症の主要な起炎菌において急速な耐性化が進行している。耐性菌増加の制御には，発症の初期診断においてその原因菌を迅速かつ正確に把握し，最も適切な抗菌薬を選択することが極めて重要である。そのことを目的として，呼吸器感染症の主要な病原細菌を 2 時間程度で検索でき，しかも省力化できる moleclar beacon(MB) probe を用いる real-time PCR 法の確立を考えた。菌種としては，市中から救命救急センター，あるいは外来受診から入院に至る症例の起炎菌を検索するという意図から，肺炎球菌，インフルエンザ菌，A 群溶血レンザ球菌，マイコプラズマ，クラミジア，レジオネラの 6 菌種を対象とした。まず，それらに特異的な MB プローブとプライマーを設計した。そして，成人や小児の喀痰や上咽頭ぬぐい液などの臨床検査材料を用い，培養法と real-time PCR 法の成績を比較し，その有用性を検証した。

【方法】 肺炎球菌は *lytA* 遺伝子，レジオネラは *mip* 遺伝子，他の 4 菌種は 16S rRNA 遺伝子上に MB probe とプライマーを設計した(Table1)。

TABLE　1. Primers and probes for real-time PCR

Species	Primer and probe	Primer[a] or probe sequence	Amplicon size
S. pneumoniae	Sense	5'-CAACCGTACAGAATGAAGCGG-3'	319
	Reverse	5'-TTATTCGTGCAATACTCGTGCG-3'	
	Probe	FAM-CGCGATCAGGTCTCAGCATTCCAACCGCCGATCGCG-BHQ1	
H. influenzae	Sense	5'-TTGACATCCTAAGAAGAGCTC-3'	167
	Reverse	5'-TCTCCTTTGAGTTCCCGACCG-3'	
	Probe	FAM-CGCGATCCTGACGACAGCCATGCAGCACGATCGCG-BHQ1	
S. pyogenes	Sense	5'-GAGAGACTAACGCATGTTAGTA-3'	317
	Reverse	5'-TAGTTACCGTCACTTGGTGG-3'	
	Probe	FAM-CGCGATCGCGACGATACATAGCCGACCTGGATCGCG-BHQ1	
M. pneumoniae	Sense	5'-GTAATACTTTAGAGGCGAACG-3'	225
	Reverse	5'-TACTTCTCAGCATAGCTACAC-3'	
	Probe	FAM-CGCGATACCAACTAGCTGATATGGCGCAATCGCG-BHQ1	
C. pneumoniae	Sense	5'-TGACAACTGTAGAAATACAGC-3'	248
	Reverse	5'-CTGTACTAACCATTGTAGCAC-3'	
	Probe	FAM-CGCGATCTCATCCTCGCCTTCCTCCTGGTGATCGCG-BHQ1	
L. pneumophila	Sense	5'-ACCGAACAGCAAATGAAAGA-3'	144
	Reverse	5'-AACGCCTGGCTTGTTTTTGT-3'	
	Probe	FAM-CGCGATCAGTACGCTTTGCCATCAAATCATCGCG-BHQ1	

臨床検査材料からの DNA 抽出には ExtragenII kit を用い，PCR には Mx3000P 機器を使用

した。DNAの抽出から結果を得るまでの所要時間は2時間以内である。

【結果】 6菌種に対する感度は，反応チューブ当り10^1〜10^6 コピー数の範囲にあった。菌量とCt値の間には高い相関がみられ，肺炎球菌:γ=0.9987, インフルエンザ菌:γ=0.9992, A群溶血レンサ球菌：γ=0.9984, マイコプラズマ：γ=0.9970, クラミジア：γ=0.9987, レジオネラ：γ=0.997 であった。また，設計したプライマーとプローブの特異性は高く，当研究室保有の口腔内および呼吸器系常在細菌には陽性反応を示さなかった。

429 症例から得られた臨床検査材料に対する肺炎球菌、インフルエンザ菌、A 群溶血レンサ球菌、マイコプラズマに対する real-time PCR の結果は，小児では、肺炎球菌 203 例、インフルエンザ菌 169 例、マイコプラズマ 49 例、クラミジア 3 例、A 群溶血レンサ球菌 3 例が陽性であった。成人 40 例では、肺炎球菌 14 例、インフルエンザ菌 4 例、マイコプラズマ 5 例、A 群溶血レンサ球菌 1 例、レジオネラ 1 例が陽性であった。

各菌種の感度と特異度は、それぞれ肺炎球菌が96.2%と93.2%、インフルエンザ菌が95.8%と95.4%、A群溶血レンサ球菌が100%と100%、マイコプラズマが100%と95.4%であった。ちなみに，マイコプラズマの感度と特異度を抗体価上昇の有無と比較すると，real-time PCRの感度と特異度は90.2%と97.9%となった。

クラミジアの培養は当研究室では実施していないが，real-time PCR で陽性であった 6 症例のクラミジア抗体価はいずれも有意に上昇していた。

レジオネラは、肺炎が疑われた成人患者の 1 例のみで real-time PCR で陽性であったが，抗菌薬が既に使用されていたため，培養は陰性であった。しかし，同時期の風呂水から本菌が検出された。

なお，肺炎球菌 (r=0.9910)とインフルエンザ菌 (r=0.9953) について培養後の菌数と Ct 値との関係をみると，その相関性は高かった。

【考察】 real-time PCR は高い感度と特異度で細菌を検出することが可能であり、抗菌薬が前投与されていても直後であれば検索可能である。私どもが構築した real-time PCR はひとつのモデルと考えており，菌種をさらに増やすことによって有用性がさらに高まると考える。特に，従来の PCR 法とは異なって，感度と特異度が一段と向上しており，加えて PCR 機器にセットした後は，機器そのものが蛍光量の変化を読み取ってくれるので，人手をほとんど必要としない点がルーチン検査に耐えうると考えている。

また，省力化できたことで臨床検査室への導入も可能であり，入院時に最も的確な抗菌薬を選択でき，入院期間の短縮による医療費コストの削減という視点からも有用であると結論された。

β-ラクタム剤に対する耐性度が低下した変異株を生じる肺炎球菌の解析

国立感染症研究所・細菌第一部・和田昭仁

肺炎球菌は、呼吸器感染症の主要な起炎菌であるばかりでなく中耳炎、敗血症、髄膜炎の起炎菌としても重要であり、この菌のβ－ラクタム剤に対する耐性度の上昇は治療上の問題となっている。この耐性は主として細胞壁を合成するペニシリン結合蛋白(PBP)の変異によるものであるが、類似したPBP遺伝子変異を持つ菌が異なる耐性度をしめす場合があり、β－ラクタム剤に対する高度耐性化にはPBP遺伝子以外のどのような変異が必要なのか、詳細は不明のままである。今回、われわれは小児の咽頭から分離されたペニシリン耐性肺炎球菌の解析をおこなったので報告する。

【方法】菌の培養にはヒツジ血液寒天 EX (Nissui)をもちいた。MIC は、CLSI (NCCLS) M7-A6, M100-S15 に準拠し(37°C, 22 h, 大気)、ドライプレート(栄研化学)をもちいて測定した。血清型は Statens Seruminstitut の群別ならびに因子血清をもちいて膨潤法により判定した。競合 PBP アッセイのために、マイクロビーズ法により菌体を破砕して調製した膜画分をもちい、これと、蛍光ラベルペニシリン（Bocillin FL, Molecular Probe）および非ラベル PCG の反応性を測定した。PBP 遺伝子の塩基配列解析のために、PBP1a, PBP2x, PBP2b の遺伝子 *pbp1a, pbp2x, pbp2b* の一部を PCR にて増幅し、その配列を決定した。

【結果と考察】分離株を血液寒天で培養したところ、扁平で内部が陥凹したタイプのコロニー(SP111)に混じり、厚みがあり中央部の陥凹がはっきりしないコロニー(SP112)を生じた。各々を血液寒天で継代すると、SP111 からは再び SP112 タイプのコロニーが約 1/100 の割合で形成されたが、SP112 からは SP111 タイプのコロニーが現れることはなかった。SP111 と SP112 はともに血清型 19F であり、複数の制限酵素を用いたパルスフィールドゲル電気泳動によっても、両者のゲノムパターンを区別することはできなかった。SP111 と SP112 では、β-ラクタム剤に対する感受性に 2-4 倍の差が見られた(SP111 [PCG, 8; ABPC, 16]; SP112 [PCG, 2; ABPC, 8])。この原因を調べる目的で、競合 PBP アッセイを行ったが、SP111、SP112 に共通して PBP1a, PBP2x, PBP2b に対する PCG の結合性の低下は見られたものの、SP111 と SP112 間で結合性に差は見られなかった。また、*pbp1a, pbp2x, pbp2b* すべてに耐性型の変異が存在したが、SP111 と SP112 間の相違は見られなかった。コロニー形態に差が見られることから、細胞壁融解酵素の遺伝子 *lytA* の発現を Northern blotting で調べたが、両菌株間で差は見られなかった。SP111 と SP112 では莢膜の厚さが異なることが観察されたが、これが感受性の差の原因であるかどうかは不明である。また、ABPC の MIC が PCG よりも高い現象は PBP の変異だけからは説明することができず、ゲノムの比較を含めた今後の解析が必要である。

分担研究課題： 臨床分離 *Proteus mirabilis* のフルオロキノロン高度耐性化機構

分担研究者：山本友子（千葉大学大学院薬学研究院）

近年、最も繁用されている抗菌薬のひとつであるフルオロキノロンに対する耐性化の現況を明らかにする目的で、2000 年 4 月~2001 年 3 月に分離された臨床由来グラム陰性桿菌の耐性調査を実施した（2003 年報告）。

フルオロキノロン耐性グラム陰性菌の比率は、*Escherichia coli* で 6 % (8 株/133 株)、*Klebsiella pneumoniae* で 2 % (2 株/100 株)、*Pseudomonas aeruginosa* で 32% (38 株/118 株) *Citrobacter koseri* で 84 %(11 株/13 株)、*Serratia marcescens* で 23 % (5 株/21 株)、*Proteus mirabilis* で 86% (12 株/14 株)であった。分離された耐性 *E. coli* に対する MIC はすべて 16 μg/ml~64 μg/ml の高値を示した。又耐性 *P. aeruginosa* の中で MIC 64 μg/ml~512 μg/ml を示す高度耐性菌は全体の 73%を占めており、かなりのスピードで高度耐性化していると考えられた。又、*P. mirabilis* における超高度耐性化（MIC 1024 μg/ml）が顕著であった。*P. mirabilis* のフルオロキノロン耐性機構については報告例が少なく、また超高度耐性については未だ報告されていないことから、本研究ではこの耐性化機構を明らかにする目的で以下の検討を行った。

まず、フルオロキノロン耐性の主要因である DNA gyrase 遺伝子 *gyrA*、*gyrB* および Topoisomerase IV 遺伝子 *parC*、*parE* 変異について解析を行った。その結果、LVFX に対する MIC が 16μg/mL 以上を示す全ての耐性菌において、GyrA の変異（Ser83Ile)、ParC の変異（Ser80Ile）、ParE の変異(Asp420Asn)が認められた。一方、MIC が 256-1024μg/mL を示す高度耐性菌においては、これらの変異に加えて GyrA の変異（Glu87Lys）と GyrB の変異（Ser462Phe）が共通に認められたことから、他の近縁菌種とは異なり、*P. mirabilis* のフルオロキノロン高度耐性においては GyrB の変異が寄与する可能性が示唆された。

次に、薬剤排出ポンプによるフルオロキノロン耐性への寄与について検討するため、LVFX の菌体内蓄積量を調べた。MIC 16-1024μg/mL を示した 10 株について比較検討した結果、LVFX の細胞内蓄積は同程度であった。このことから、本菌のフルオロキノロン超高度耐性化における薬剤ポンプの関与は低いと考えられる。以上の結果から、*P. mirabilis* のフルオロキノロン耐性においては、他の近縁菌種で報告のある *gyrA*、*parC* の変異加えて *gyrB* の変異が重要であることが示された。しかしながら、MIC 256μg/mL を示す株でこれら全ての変異を獲得していたことから、MIC 1024μg/mL の超高度耐性株は新たなフルオロキノロン耐性化機構を獲得している可能性が考えられる。PFGE による DNA 解析の結果、MIC 1024μg/mL の超高度耐性株は 2 つのタイプに分けられた。

アミノグリコシド高度耐性 *Proteus mirabilis* より同定された新規 16S rRNA methyltransferase, RmtC の解析

研究代表者：荒川　宜親[1)]
研究協力者：和知野　純一[1), 2)]、山根　一和[1)]、鈴木　里和[1)]、柴田　尚宏[1)]、
木村　幸司[1)]、池　康嘉[2)]、
国立感染症研究所細菌二部[1)]　群馬大学大学院医学系研究科細菌感染制御学[2)]

【目的】我々はアミノグリコシド耐性を付与する 16S rRNA methyltransferase 遺伝子が腸内細菌科やブドウ糖非醗酵群に属するグラム陰性桿菌の一部に存在することを見出した。現在まで *rmtA, rmtB, armA* の 3 種類の 16S rRNA methyltransferase 遺伝子が同定されている。本研究に用いた *P. mirabilis* ARS68 株はアミノグリコシドに高度耐性を示すものの、既知の 16S rRNA methyltransferase 遺伝子は保有していなかった。そこで新規の 16S rRNA methyltransferase 遺伝子の保有を疑い、その同定及び解析を行った。

【結果】*P. mirabilis* ARS68 株より抽出した plasmid を *E. coli* DH5α に形質転換したところ、アミノグリコシドに対して耐性を示すようになった。上記の結果よりアミノグリコシド耐性を付与する遺伝子は plasmid 上に存在する可能性が示唆された。アミノグリコシド耐性遺伝子をクローン化し、塩基配列を決定したところ、新規 16S rRNA methyltransferase 遺伝子(*rmtC*)が同定された。His-tag を付加した RmtC 蛋白は *E. coli* DH5α より抽出した 16S rRNA を速やかにメチル化した。*rmtC* は tranposase gene (*tnpA*)を含む IS*Ecp1*-element の下流に存在し、*rmtC* の転移はこの IS*Ecp1*-element に支配されていた。また、*rmtC* の転写開始点は IS*Ecp1*-element 内に存在した。

【考察】*rmtC* は他の 16S rRNA methyltransferase 遺伝子と同様トランスポゾンによりその転移が担われていた。既に *armA* に関しては東アジア、ヨーロッパの国々で確認されており、*armA* 同様 *rmtC* に関してもその拡散が懸念される。今後はメチル化部位の特定やこれら 16S rRNA methyltransferase 遺伝子の progenitor strain の特定を行う予定である。

VanD4の遺伝子構造とその発現および染色体D-Ala:D-Ala ligase 遺伝子について

主任研究者　池 康嘉[1, 2]
研究協力者　谷本弘一[1]、野村隆浩[2]、荒川宜親[3]
[1]群馬大学大学院医学系研究科 細菌感染制御学、[2]同・附属薬剤耐性菌実験施設
[3]国立感染研細菌第二部

【目的】患者より分離された VRE、*Enterococcus raffinosus* GV5 は日本で最初の VanD 型 VRE で、VanD 型 VRE として最初の *E. raffinosus* である。VanD 型耐性遺伝子群の構造と発現調節を明らかにするために、耐性遺伝子群の全塩基配列の決定と northern hybridization を行った。また、既報の VanD 株の *ddl* 遺伝子は突然変異によって不活化されていることが知られているので GV5 の *ddl* 遺伝子の塩基配列も決定した。

【方法】塩基配列の決定は目的の遺伝子を含む PCR 産物を直接シークエンスすることにより行った。染色体 *ddl* 遺伝子の塩基配列決定にはインバース PCR 法を用いた。*E. raffinosus* の野生型 *ddl* 遺伝子の塩基配列決定には標準株 JCM8733 を用いた。

【結果と考察】DNA 塩基配列の結果から GV5 の耐性遺伝子群は VanD4 型であることがわかった。既報の D4 株の遺伝子群と比較するとアミノ酸置換が $VanS_D$ で 2 カ所、$VanH_D$ に 1 カ所、VanD に 1 カ所見つかった。$VanY_D$ には 1 塩基の挿入が見つかった。$VanR_D$、$VanX_D$ には変化は見られなかった。$vanS_D$、$vanY_D$、*vanD* 遺伝子をプローブとして northern hybridization を行ったところ、バンコマイシンの有無にかかわらず耐性遺伝子群は転写されていた。この結果から、GV5 の耐性遺伝子群の発現は既報の VanD 型 VRE 同様に constitutive であることがわかった。既報の VanD 型 VRE においては 1 株をのぞき 2 成分系のセンサー蛋白である $VanS_D$ に変異があることが知られている。アミノ酸置換や挿入・欠失によるフレームシフトによって $VanS_D$ の脱リン酸化活性のみが損なわれ活性化された $VanR_D$（調節蛋白）が蓄積することによって常に耐性遺伝子群が発現しているとされている。$VanR_D$ へのリン酸の受け渡しを行う His 残基周辺のアミノ酸置換で脱リン酸化活性のみが損なわれることが知られているが、GV5 ではこの領域にアミノ酸置換が起こっていた。このような株の *ddl* 遺伝子(D-Ala:D-Ala ligase)は変異を持ち活性をなくしていることが知られている。GV5 と *E. raffinosus* 標準株 JCM8733 の *ddl* 遺伝子の塩基配列から GV5 は 2 つのアミノ酸置換を C 末領域に持っていたが挿入・欠失変異はなく、活性を失いかねない明らかな変異はなかった。しかしながら、GV5 における 2 つのアミノ酸置換のうち 1 つが ATP 結合に関与している領域にありこの変異により *ddl* 遺伝子が不活化されているかもしれない。

Ｖ．研究成果の刊行に関する一覧表および別刷

(別添５)

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Wachino J, Kurokawa H, Suzuki S, Yamane K, Shibata N, Kimura K, Ike Y, Arakawa Y.	Horizontal transfer of blaCMY-bearing plasmids among clinical *Escherichia coli* and *Klebsiella pneumoniae* isolates and emergence of cefepime-hydrolyzing CMY-19.	Antimicrob Agents Chemother.	50(2)	534-41	2006
Wachino J, Yamane K, Shibayama K, Kurokawa H, Shibata N, Suzuki S, Doi Y, Kimura K, Ike Y, Arakawa Y.	Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a *Proteus mirabilis* isolate demonstrating extraordinary high-level resistance against various aminoglycosides.	Antimicrob Agents Chemother.	50(1)	178-84	2006
Inoue T, Tomita H, Ike Y.	Bac 32, a Novel Bacteriocin Widely Disseminated among Clinical Isolates of *Enterococcus faecium*.	Antimicrob Agents Chemother.	50(4)	1202-12	2006
Nagano N, Nagano Y, Nakano R, Okamoto R, M. Inoue M.	Genetic diversity of the C protein β-antigen gene and its upstream regions within clonally related groups of type Ia and Ib group B streptococci.	Microbiology.	152	*in press.*	2006
Kaneko K, Sato Y, Tokunaga S, Tamaki S, Okamoto R, Inoue M.	AmpC β-lactamase-mediated cefpodoxime-resistant *Escherichia coli* isolated from faecal samples of healthy volunteers.	J Antimicrob Chemother.	57	369-71	2006
Morozumi M, Nakayama E, Iwata S, Aoki Y, Hasegawa K, Kobayashi R, Chiba N, Tajima T, Ubukata K.	Simultaneous detection of pathogens in community-acquired pneumonia by real-time PCR with pathogen-specific molecular beacon probes.	J Clinical Microb.	44(4)	1440-6	2006
Morozumi M, Ito A, Murayama SY, Hasegawa K, Kobayashi R, Iwata S, Kawamura N, Kuroki H, Nakayama E, Tajima T, Ubukata K.	Assessment of real-time PCR for diagnosis of *Mycoplasma pneumoniae* pneumonia in pediatric patients.	Canadian Journal of Microbiology	52	1-6	2006
Ishii Y, Alba J, Maehara C, et al.	Identification of atypical phenotypic *Staphylococcus aureus* clinical isolates with three automated identification systems.	J Med Microbiol.		*in press*	2006
Ishii Y, Alba J, Kimura S, Yamaguchi K.	Evaluation of Antimicrobial Activity of β-Lactam Antibiotics by Etest against Clinical Isolates from 100 Medical Centers in Japan (2004).	Diagn Microbiol Infect Dis.		*in press*	2006

Takaya A, Watanabe M. Yamamoto T.	Organization of Tn*2610* containing two transposition modules.	Antimicrob. Agents Chemother.	50:4	*in press*	2006
Yagi T, Wachino J, Kurokawa H, Suzuki S, Yamane K, Doi Y, Shibata N, Kato H, Shibayama K, Arakawa Y.	Practical methods using boronic acid compounds for identification of class C beta-lactamase-producing Klebsiella pneumoniae and *Escherichia coli*.	J Clin Microbiol.	43(6)	2551-8	2005
Yamane K, Wachino J, Doi Y, Kurokawa H, Arakawa Y.	Global spread of multiple aminoglycoside resistance genes.	Emerg Infect Dis.	11(6)	951-3	2005
Yamaguchi Y, Kuroki T, Yasuzawa H, Higashi T, Jin W, Kawanami A, Yamagata Y, Arakawa Y, Goto M, Kurosaki H.	Probing the role of Asp-120(81) of metallo-beta-lactamase (IMP-1) by site-directed mutagenesis, kinetic studies, and X-ray crystallography.	J Biol Chem.	27;280 (21)	20824-32	2005
Tomita H, Ike Y.	Genetic analysis of transfer-related regions of the vancomycin resistance Enterococcus conjugative plasmid pHTbeta: identification of *ori*T and a putative relaxase gene.	J Bacteriol.	187 (22)	7727-37	2005
Takeuchi K, Tomita H, Fujimoto S, Kudo M, Kuwano H, Ike Y.	Drug resistance of *Enterococcus faecium* clinical isolates and the conjugative transfer of gentamicin and erythromycin resistance traits.	FEMS Microbiol Lett.	15;243 (2)	347-54	2005
Tanimoto K, Nomura T, Hamatani H, Xiao YH, Ike Y.	A vancomycin-dependent VanA-type *Enterococcus faecalis* strain isolated in Japan from chicken imported from China.	Lett Appl Microbiol.	41(2)	157-62	2005
Kaneko K, Okamoto R, Nakano R, Kawakami S, Inoue M.	Gene mutations responsible for overexpression of AmpC β-lactamase in some clinical isolates of Enterobacter cloacae.	J Clin Microbiol.	43	2955-8	2005
Maeda K, Ida T, Sanbongi Y, Suzuki T, Fukushima T, Kurazono M, Yonezawa M, Ubukata K, Inoue M.	Comparison of activities of β-lactam antibiotics against *Steptcoccus pneumoniae* with recombinant penicillin-binding protein genes from a penicillin-resistant strain.	J Infect Chemotherapy.	11	107-11.	2005
Tsuji A, Kobayashi I, Oguri T, Inoue M, Yabuuchi E, Goto S.	An epidemiological study of the susceptibility and frequency of multiple-drug-resistant strains of *P. aeruginosa* isolates at medical institutes nationwide in Japan.	J Infect Chemother.	11	64-70	2005
Hosaka Y, Irinoda K, Nakano R, Tanabe S, Koizumi W, Saigenji K, Inoue M.	Use of the Restriction Enzyme *Eco*RI for Pulsed-Field Gel Electrophoretic Analysis of *Helicobacter pylori*.	J Clin Microbiol.	43	931-2	2005

Okitsu N, Kaieda S, Yano H, Nakano R, Hosaka Y, Okamoto R, Kobayashi T, Inoue M.	Characterization of ermB Gene Transposition by Tn*1545* and Tn*917* in Macrolide-Resistant *Streptococcus pneumoniae* Isolates.	J Clin Microbiology.	43	168-73	2005
Saito R, Misawa Y, Moriya K, Koike K, Ubukata K, Okamura N.	Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Mycoplasma pneumoniae.*	J Medical Microb.	54	1037-41	2005
Chiba N, Kobayashi R, Hasegawa K, Morozumi M, Nakayama E, Tajima T, Iwata S, Ubukata K.	Antibiotic susceptibility according to genotype of penicillin-binding protein and macrolide resistance genes, and serotype of *Streptococcus pneumoniae* isolates from community-acquired pneumonia in children.	J Antimicrob Chemother.	156	756-60	2005
Morozumi M, Hasegawa K, Kobayashi R, Inoue N, Iwata S, Kurok H, Kawamura N, Nakayama E, Tajima T, Shimizu K, Ubukata K.	Emargence of macrolides-resistant *Mycoplasma pneumoniae* with a 23S rRNA gene mutation.	Antimicrob Agents Chemother.	49(6)	2302-6	2005
Kurosaki H, Yamaguchi Y, Higashi T, Soga K, Matsueda S, Yumoto H, Misumi S, Yamagata Y, Arakawa Y, Goto M.	Irreversible Inhibition of Metallo-β-lactamase (IMP-1) by 3-(3-Mercaptopropionylsulfanyl)propionic Acid Pentafluorophenyl Ester.	Angew Chem, Int Ed Engl.	44	3861-3864	2005
Yamaguchi Y, Kuroki T, Yasuzawa H, Higashi T, Jin W, Kawanami A, Yamagata Y, Arakawa Y, Goto M, Kurosaki H.	Probing the Role of Asp120(81) of Metallo-β-lactamase (IMP-1) by Site-directed Mutagenesis, Kinetic Studies, and X-Ray Crystallography.	J Biol Chem.	280	20824-20832	2005
Yoneda K, Chikumi H, Murata T, Gotoh N, Yamamoto H, Fujiwara H, Nishino T, Shimizu E.	Measurement of *Pseudomonas aeruginosa* Multidrug Efflux Pumps by Quantitative Real-time Polymerase Chain Reaction.	FEMS Microbiol Lett.	243	125-131	2005
Chuanchuen R, Murata T, Gotoh N, Schweizer HP.	Substrate-dependent utilization of OprM or OpmH by the *Pseudomonas aeruginosa* MexJK efflux pump.	Antimicrob Agents Chemother.	49 .	2133-2136	2005
Niga T, Ito H, Oyamada Y, Yamagishi J, Kadono M, Gotoh N, Nishino T, Inoue M.	Cooperation between Alternation of the DNA gyrase genes and Overexpression of MexB and MexX Confers High-level Fluoroquinolone Resistance in clinical strains of *Pseudomonas aeruginosa.*	Microbiol.Immunol.	49	443-446	2005

Alba J, Ishii Y, Thomson K, Moland ES and Yamaguchi K.	Kinetics study of KPC-3, a plasmid-encoded class A carbapenem-hydrolyzing β-lactamase.	Antimicrob Agents Chemother.	49	4760-2	2005
Ishii Y, Alba J, Kimura S, Shiroto K and Yamaguchi K.	Evaluation of antimicrobial activity of β-lactam antibiotics using Etest against clinical isolates from 60 medical centres in Japan.	Int J Antimicrob Agents.	25	296-301	2005
Ishii Y, Kimura S, Alba J, et al.	Extended-spectrum β-lactamase-producing Shiga toxin gene (Stx1)-positive *Escherichia coli* O26:H11: a new concern.	J Clin Microbiol.	43	1072-5	2005
Kimura S, Alba J, Shiroto K, et al.	Clonal diversity of metallo-β-lactamase-possessing *Pseudomonas aeruginosa* in geographically diverse regions of Japan.	J Clin Microbiol.	43	458-61	2005
Shiroto K, Ishii Y, Kimura S, et al.	Metallo-β-lactamase IMP-1 in *Providencia rettgeri* from two different hospitals in Japan.	J Med Microbiol.	54	1065-70	2005
Kimura S, Ishii Y and Yamaguchi K.	Evaluation of dipicolinic acid for detection of IMP- or VIM- type metallo-β-lactamase-producing *Pseudomonas aeruginosa* clinical isolates.	Diagn Microbiol Infect Dis.	53	241-4	2005
Hirose K, Terajima J, Izumiya H, Tamura K, Takai N, Watanabe H.	Antimicrobial susceptibility of *Shigella sonnei* in Japan and molecular analysis of *S. sonnei* isolates with reduced susceptibility to fluoroquinolone.	Antimicrob Agent Chemother.	49	1203-5	2005
Adachi T, Sagara H, Hirose K, Watanabe H.	Fluoroquinolone resistant *Salmonella Paratyphi* A.	Emerg Infect Diseas.	11	172-4	2005
Nagano N, Nagano Y, Cordevant C, Shibata N, Arakawa Y.	Nosocomial transmission of CTX-M-2 beta-lactamase-producing *Acinetobacter baumannii* in a neurosurgery ward.	J Clin Microbiol.	42(9)	3978-84	2004
Yamane K, Doi Y, Yokoyama K, Yagi T, Kurokawa H, Shibata N, Shibayama K, Kato H, Arakawa Y.	Genetic environments of the *rmtA* gene in *Pseudomonas aeruginosa* clinical isolates.	Antimicrob Agents Chemother.	48(6)	2069-74	2004
Doi Y, Yokoyama K, Yamane K, Wachino J, Shibata N, Yagi T, Shibayama K, Kato H, Arakawa Y.	Plasmid-mediated 16S rRNA methylase in *Serratia marcescens* conferring high-level resistance to aminoglycosides.	Antimicrob Agents Chemother.	48(2)	491-6	2004
Shiraki Y, Shibata N, Doi Y, Arakawa Y.	*Escherichia coli* producing CTX-M-2 beta-lactamase in cattle, Japan.	Emerg Infect Dis.	10(1)	69-75	2004
Tomita H, Ike Y.	Tissue-specific adherent *Enterococcus faecalis* strains that show highly efficient adhesion to human bladder carcinoma T24 cells also adhere to extracellular matrix proteins.	Infect Immun.	72(10)	5877-85	2004
Arakawa Y, Ike Y, Nagasawa M.	Where has vancomycin-heterogeneously resistant *Staphylococcus aureus* gone?	Lancet.	24;363 (9418)	1401	2004

Inoue M, Kaneko K, Nakano R, Sato Y, Arai S.	Molecular analytical evaluation of macrolide- and ketolide-resistant *Streptococcus pneumoniae.*	Jpn J Antibiot.	57	425-37	2004
Saika T, Kobayashi I, Inoue M.	A comparison of the microbiological characteristcs of *Neisseria gonorrhoeae* isolated from male and femalepatienta with gonorrhea.	Chemotherapy.	50(2)	92-7	2004
Xiong Z, Zhu D, Wang F, Zhang Y, Okamoto R, Inoue M.	A *Klebsiella pneumoniae* producing three kinds of class A beta-lactamases encoded by one single plasmid isolated from a patient in Huashan Hospital, Shanghai, China.	Int J Antimicrob Agents.	23(3)	262-7	2004
Nakano R, Okamoto R, Nakano Y, Kaneko K, Okitsu N, Hosaka Y, Inoue M.	CFE-1, a Novel Plasmid-Encoded AmpC β-Lactamase with an *ampR* Gene Originating from *Citrobacter freundii.*	Antimicrob Agents Chemother.	48	1151-8	2004
Morozumi M, Hasegawa K, Chiba N, Iwata S, Kawamura N, Kuroki H, Tajima T, Ubukata K.	Application of PCR for *Mycoplasma pneumonaie* detection in children with community-acquired pneumonia.	J Infect Chemother.	10	274-279	2004
Ubukata K, Hasegawa K, Chiba N, Kobayashi R, Iwata S, Sunakawa K.	Anitbiotic susceptibility in relation to penicillin-binding protein genes and serotype distribution of *Streptococcus pneumoniae* strains responsible for meningitis in Japan, 1999-2002.	Antimicrob Agents Chemother.	48	1488-94	2004
Hasegawa K, Chiba N, Kobayashi R, Murayama SY, Iwata S, Sunakawa K, Ubukata K.	Rapidly increasing prevalence of β-lactamase-nonproducing, ampicillin-resistant *Haemophilus influenzae* type b in meningitis.	Antimicrob Agents Chemother.	48	1509-14	2004
Kuroki H, Morozumi M, Chiba N, Ubukata K.	Characterization of children with *Mycoplasma pneumoniae* infection detected by rapid polymerase chain reaction technique.	J Infect Chemother.	10	65-7	2004
Jin W, Arakawa Y, Yasuzawa H, Taki T, Hashiguchi R, Mitsutani K, Shoga A, Yamaguchi Y, Kurosaki H, Shibata N, Ohta M, Goto M.	Comparative Study of the Inhibition of Metallo-β-lactamases (IMP-1 and VIM-2) by Thiol Compounds That Contain a Hydrophobic Group.	Biol Pharm Bull.	27	851-856	2004
Aoki S, Hirakata Y, Kondoh A, Gotoh N, Yanagihara K, Miyazaki Y, Tomono K, Yamada Y, Kohno S, Kamihira S.	Virulence of metallo-β-lactamase-producing *Pseudomonas aeruginosa in vitro* and *in vivo.*	Antimicrob Agents Chemother.	48	1876-1878	2004
Kimura S, Ishiguro M, Ishii Y, Alba J and Yamaguchi K.	Role of a mutation at position 167 of CTX-M-19 in ceftazidime hydrolysis.	Antimicrob Agents Chemother.	48	1454-60	2004

Alba J, Bauvois C, Ishii Y, et al.	A detailed kinetic study of Mox-1, a plasmid-encoded class C β-lactamase.	FEMS Microbiol Lett.	225	183-8	2004
Maki H, McCallum N, Bischoff M, Wada A, Berger-Bachi B.	*tcaA* inactivation; a step to vancomycin intermediate resistance in *Staphylococus aureus* (VISA) clinical isolates.	Antimicrob Agents Chemother.	48	1953-9	2004
McCallum N, Bischoff, Maki H, Wada A, Berger-Bachi B.	TcaR, a putative MarR-like regulator of *sarS* expression.	J Bacteriol.	186	2966-72	2004
Nagano N, Shibata N, Saitou Y, Nagano Y, Arakawa Y.	Nosocomial outbreak of infections by *Proteus mirabilis* that produces extended-spectrum CTX-M-2 type beta-lactamase.	J Clin Microbiol.	41(12)	5530-6	2003
Shibata N, Doi Y, Yamane K, Yagi T, Kurokawa H, Shibayama K, Kato H, Kai K, Arakawa Y.	PCR typing of genetic determinants for metallo-beta-lactamases and integrases carried by gram-negative bacteria isolated in Japan, with focus on the class 3 integron.	J Clin Microbiol.	41(12)	5407-13	2003
Nagano N, Shibata N, Saitou Y, Nagano Y, Arakawa Y.	Nosocomial outbreak of infections by *Proteus mirabilis* that produces extended-spectrum CTX-M-2 type beta-lactamase.	J Clin Microbiol.	41(12)	5530-6	2003
Yokoyama K, Doi Y, Yamane K, Kurokawa H, Shibata N, Shibayama K, Yagi T, Kato H, Arakawa Y.	Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa.*	Lancet.	6;362 (9399)	1888-93	2003
Tomita H, Tanimoto K, Hayakawa S, Morinaga K, Ezaki K, Oshima H, Ike Y.	Highly conjugative pMG1-like plasmids carrying Tn*1546*-like transposons that encode vancomycin resistance in *Enterococcus faecium.*	J Bacteriol.	185(23)	7024-8	2003
Kaieda S, Okitsu N, Yano H, Hosaka Y, Nakano R, Okamoto R, Takahashi H, Inoue M.	Induction of telithromycin resistance in *Streptococcus pneumoniae.*	J Antimicrob Chemother.	50	736-37	2003
Lee K, Lee WH, Young D, Yum JH, Chong Y, Okamoto R, Inoue M.	In vitro actibities of panipenem against clinical isolates of aerobic and anaerobic bacteria.	Infect Chemother	35	423-433	2003
Matsuoka M, Inoue M, Endo Y, Nakajima Y.	Characteristic expression of three genes, *msr*(A), *mph*(C) and *erm*(Y) that confer resistance to macrolide antibiotics on *Staphylococcus aureus.*	FEMS microbial Lett	220	287-93	2003
Matic V, Bozdogan B, Jacobs MR, Appelbaum PC, Ubukata K.	Contribution of β lactamase and PBP mutations to amoxicillin/clavulanate resistance in β lactamase positive amoxicillin/clavulanate resistant (BLPACR) *Haemophilus influenzae.*	J Antimicrob Chemother.	52	1018-21 IF: 3.08	2003

Ubukata K.	Problems associated with high prevalence of multidrug-resistant bacteria in patients with community-acquired infections.	J Infect Chemoteher.	9	285-91	2003
Hasegawa K, Yamamoto K, Chiba N, Kobayashi R, Nagai K, Jacobs MR, Appelbaum PC, Sunakawa K, Ubukata K.	Diversity of ampicillin-resistance genes in *Haemophilus influenzae* in Japan and the United States.	Microbial Drug Resistance	9(1)	39-46 IF: 2.6	2003
Hacquet D, Vogne C, Garch FE, Vejux A, Gotoh N, Lee A, Lomovskata O, Plesiat P.	MexXY-OprM efflux pump is necessary for adaptive resistance of *Pseudomonas aeruginosa* to aminoglycosides.	Antimicrob Agents Chemother.	47	1371-1375	2003
Gotoh N, Murata T, Kimura T, Ozaki T, Kondo A, Nishino T.	Intrinsic Resistance of *Escherichia coli* to mureidomycin A and C due to expression of the multidrug efflux system AcrAB-TolC: comparison with the efflux systems of mureidomycin-susceptible *Pseudomonas aeruginosa.*	J Infect Chemother.	9	101-103	2003
Morita Y, Murata T, Mima T, Shiota S, Kuroda T, Mizushima T, Gotoh N, Nishino T, Tuchiya T.	Induction of mexCD-oprJ operon for a multidrug efflux pump by disinfectants in wild-type *Pseudomonas aeruginosa* PAO1.	J Antimicrob Chemother.	51	991-994	2003
Yamaguchi Y, Tomoyasu T, Takaya A, Morioka M, Yamamoto T.	Effects of disruption of heat shock genes on susceptibility of *Escherichia coli* to fluoroquinolones.	BMC Microbiology.	3	1-8	2003
Rossi J, Bischoff M, Wada A, Berger-Bachi B.	MsrR, a putative cell envelope-associated element involved in *Staphylococcus aureus sarA* attenuation.	Antimicrob Agents Chemother.	47	:2558-64	2003
Hirose K, Itoh K, Arawawa A, Tamura K, Watanabe H.	DNA-based diagnosis method for typhoid and paratyphoid fever, and the screenng method for *Salmonella enterica* serovar *Typhi* and serovar *Paratyphi* A with decreased susceptibility to fluoroquinolones by PCR-restriction fragment length polymorphism(RFLP).	Res Adv in Microbiology.	3	109-21	2003
Hirose K, Tamura K, Watanabe H.	Screening method for *Salmonella enterica* serovar Typhi and Paratyphi A with reduced susceptibility to fluoroquinolone by PCR-rstriction fragment length polymorphism.	Microbiol Immunol.	47	161-5	2003

JOURNAL OF CLINICAL MICROBIOLOGY, June 2005, p. 2551–2558 Vol. 43, No. 6
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.6.2551–2558.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

Practical Methods Using Boronic Acid Compounds for Identification of Class C β-Lactamase-Producing *Klebsiella pneumoniae* and *Escherichia coli*

Tetsuya Yagi,† Jun-ichi Wachino, Hiroshi Kurokawa, Satowa Suzuki, Kunikazu Yamane, Yohei Doi, Naohiro Shibata, Haru Kato, Keigo Shibayama, and Yoshichika Arakawa*

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo, Japan

Received 12 October 2004/Returned for modification 24 January 2005/Accepted 24 February 2005

Detection of the resistance mediated by class C β-lactamases remains a challenging issue, considering that transferable plasmid-mediated class C β-lactamases are of worldwide concern. Methods for the identification of strains that produce extended-spectrum β-lactamases (ESBLs) or metallo-β-lactamases (MBLs) have been developed and applied for routine use in clinical microbiology laboratories, but no practical methods for identification of plasmid-mediated class C producers have been established to date. We therefore developed three simple methods for clinical microbiology laboratories that allow identification of plasmid-mediated class C β-lactamase-producing bacteria using a boronic acid derivative, 3-aminophenylboronic acid (APB), one of the specific inhibitors of class C β-lactamases. Detection by the disk potentiation test was based on the enlargement of the growth-inhibitory zone diameter (by greater than or equal to 5 mm) around a Kirby-Bauer disk containing a ceftazidime (CAZ) or a cefotaxime (CTX) disk in combination with APB. In a double-disk synergy test, the discernible expansion of the growth-inhibitory zone around the CAZ or the CTX disk toward a disk containing APB was indicative of class C β-lactamase production. A greater than or equal to eightfold decrease in the MIC of CAZ or CTX in the presence of APB was the criterion for detection in the microdilution test. By using these methods, *Escherichia coli* and *Klebsiella pneumoniae* isolates producing plasmid-mediated class C β-lactamases, ACT-1, CMY-2, CMY-9, FOX-5, LAT-1, and MOX-1, were successfully distinguished from those producing other classes of β-lactamases, such as ESBLs and MBLs. These methods will provide useful information needed for targeted antimicrobial therapy and better infection control.

The production of β-lactamases is the major mechanism of resistance to β-lactams, which are most frequently used for the treatment of various infectious diseases. Class C β-lactamases, which belong to group 1 according to the classification of Bush et al. (7), are cephalosporinases, which are poorly inhibited by β-lactamase inhibitors, such as clavulanic acid (CLA) and sulbactam. Class C β-lactamases are clinically important because they usually confer resistance to a variety of β-lactams, including oxyiminocephalosporins and some cephamycins, as well as penicillins and monobactam, when they are produced in large amounts (14, 21, 32). They are usually chromosomally encoded AmpC enzymes in several bacterial species belonging to the family *Enterobacteriaceae*, including *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Morganella morganii*, *Serratia marcescens*, and *Escherichia coli*, which are frequently encountered as nosocomial pathogens. Moreover, since the first report of transferable plasmid-mediated class C β-lactamases, such as MIR-1, in the late 1980s (3, 30), their increasing presence worldwide is becoming of great concern (9, 13, 22, 24). In Japan, MOX-1 (15), CMY-9 (10), and CMY-2 and DHA-1 (unpublished data) have been identified so far. Although simple methods for the identification of extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs) have been established and are already in laboratory use (1, 29), detection of the resistance mediated by class C β-lactamases still remains a challenging issue. Several methods that use the Kirby-Bauer (KB) disk potentiation method (20, 21, 34, 35, 45) with some β-lactamase inhibitors (2, 5) or the three-dimensional method (9, 22, 39) have been developed; and a cefoxitin agar medium-based assay that uses preparations of bacterial cell extracts has been reported (26). However, these methods are technically intricate, and interpretation of their results is not sufficiently simple for routine use in clinical microbiology laboratories. PCR or multiplex PCR analyses are able to provide satisfactory results in the identification and classification of genes for β-lactamases (25, 31, 38, 44), but equipment availability is limited to medical institutions, such as university hospitals. They are also costly and require time-consuming techniques. An enzyme-linked immunosorbent assay has also been developed and has known sensitivity and specificity for the detection of certain class C β-lactamases. This technique is less costly than genetic methods, but it is not sensitive for the detection of class C β-lactamases that possess less than 70% homology to CMY-2 (16). Thus, practical and simple methods for detection of the resistance mediated by plasmid-mediated class C β-lactamases are urgently needed for enhanced infection control.

In 1982, boronic acids were reported as reversible inhibitors of AmpC enzymes belonging to the class C β-lactamases (4). Serial studies revealed the structure-based mechanism of inhibition of AmpC β-lactamases by boronic acids (34, 37, 41), and novel compounds that inhibit AmpC β-lactamases with nano-

* Corresponding author. Mailing address: Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-Murayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771. Fax: 81-42-44-8518. E-mail: yarakawa@nih.go.jp.

† Present address: Division of Respiratory Medicine, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8511, Japan.

TABLE 1. Bacterial strains used in this study and MICs of CAZ and CTX with or without β-lactamase inhibitors

Strain	β-Lactamase	MIC (μg/ml)								Reference
		CAZ	CAZ + CLA	CAZ + SMA	CAZ + APB	CTX	CTX + CLA	CTX + SMA	CTX + APB	
E. coli NS12	CMY-2	128	64	128	4	64	32	64	2	This study
E. coli HKY515	CMY-2	256	128	256	4	64	32	64	4	This study
E. coli HKY701	CMY-2	128	128	128	2	32	32	64	4	This study
E. coli MRY041197	CMY-2	>256	256	>256	8	128	128	128	8	This study
E. coli HKY581	CMY-2	256	128	256	8	128	128	256	4	This study
E. coli C502	CMY-2	256	256	256	16	16	32	32	2	This study
E. coli KG2	CMY-2	128	128	64	4	64	32	64	8	This study
E. coli MRY041243	CMY-8	256	256	>256	4	64	32	64	0.5	This study
E. coli M68	CMY-9	256	256	256	1	>256	>256	>256	16	10
E. coli Coral Gables 66040	FOX-5	>128	>128	>128	4	64	64	64	1	G. A. Jacoby
E. coli Coral J53 (a transformant)	ACT-1	8	4	4	≤0.25	2	2	2	≤0.25	G. A. Jacoby
E. coli HKY28	Mutant AmpC	64	32	64	4	16	4	8	1	11
K. pneumoniae NU2936	MOX-1	64	32	32	0.5	256	256	256	8	15
K. pneumoniae HKY-L1	MOX-1	32	32	32	1	128	128	128	4	This study
K. pneumoniae KPW142	CMY-8	32	32	64	1	128	128	128	4	This study
K. pneumoniae HKY209	CMY-9	>128	>128	>128	2	>128	>128	>128	2	This study
K. pneumoniae HKY327	CMY-19	>256	>256	>256	16	64	64	64	1	This study
K. pneumoniae 5064	FOX-5	64	64	64	2	8	16	16	0.5	36
K. pneumoniae Bronx Lebanon 18	ACT-1	64	64	128	64	8	16	8	0.5	G. A. Jacoby
K. pneumoniae P20	LAT-1	64	64	64	2	32	32	32	1	40
Hafnia alvei EE47[a]	AmpC	64	128	64	2	32	64	32	2	This study
E. coli NCB03522[b]	CMY-2 + CTX-M-9	64	16	64	16	256	8	256	256	This study
K. pneumoniae NCB02189[a]	DHA-1 + CTX-M-9	16	128	16	1	32	8	32	2	This study
E. coli AYW-1	TEM-26	>128	0.5	>128	>128	2	<0.06	2	2	44
E. coli HKY322	TEM-91	128	0.5	128	>128	1	<0.06	1	1	18
E. coli MRY041435	TEM-132	64	1	64	64	8	≤0.25	4	4	This study
E. coli HKY453	SHV-24	>128	2	>128	>128	2	0.13	2	2	17
E. coli NCB03515	CTX-M-3	32	≤0.25	16	16	>256	≤0.25	256	256	This study
E. coli MRY04718	CTX-M-3	64	1	128	32	>256	≤0.25	>256	>256	This study
E. coli AYW-2	CTX-M-2	8	0.13	8	4	>128	<0.06	>128	>128	This study
E. coli NCB03490	CTX-M-2	4	≤0.25	4	1	128	≤0.25	256	64	This study
E. coli NCB03520	CTX-M-14	2	0.25	4	1	128	0.13	>128	128	This study
E. coli AYW-3	CTX-M-9	0.5	<0.06	0.5	0.25	32	<0.06	64	16	This study
K. pneumoniae HKY402	SHV-12	>128	1	>128	>128	32	<0.06	32	32	44
K. pneumoniae MRY041410	TEM-132	64	1	64	64	4	≤0.25	4	8	This study
K. pneumoniae K108	CTX-M-1	2	0.25	1	2	64	<0.06	64	64	This study
K. pneumoniae MRY04332	CTX-M-3	16	1	8	8	128	≤0.25	128	128	This study
K. pneumoniae HKY495	CTX-M-2	16	1	16	16	128	0.13	>128	>128	This study
K. pneumoniae MRY04504	CTX-M-2	2	≤0.25	2	4	64	≤0.25	128	64	This study
K. pneumoniae NCB03502	CTX-M-9	0.5	0.06	1	1	32	<0.06	64	32	This study
K. pneumoniae NCB03081	CTX-M-9	4	≤0.25	4	4	32	≤0.25	32	32	This study
K. pneumoniae KG525	GES-3J	>128	>128	>128	>128	64	8	64	32	42
K. pneumoniae KG502	GES-4J	>128	>128	>128	>128	32	16	16	16	43
E. coli NCB03426	IMP-1	64	64	≤0.25	64	16	16	≤0.25	16	This study
E. coli NCB02465	IMP-1	128	128	≤0.25	128	32	64	≤0.25	64	This study
K. pneumoniae KP115	IMP-1	>128	128	1	>128	64	64	0.25	64	This study
K. pneumoniae NCB03034	IMP-1	64	64	≤0.25	64	128	128	≤0.25	128	This study
E. coli EE61	OXA-30	2	2	2	2	4	4	4	4	This study

[a] Production of AmpC or DHA-1 might be augmented in the presence of clavulanic acid.
[b] *E. coli* strain NCB03522 also produces the TEM-1 penicillinase.

molar K_i values were prepared by stereoselective organic synthesis (23). However, there are only a few reports of studies that applied boronic acids to the identification of class C β-lactamase-producing bacteria (19, 34). In the present study we used one of the boronic acids, 3-aminophenylboronic acid (APB), and here we propose simple and practical methods for the identification of class C β-lactamase-producing bacteria showing resistance to broad-spectrum β-lactams, including cephamycins. The methods constructed in the present study promise to be very helpful for the screening of plasmid-mediated class C β-lactamase-producing bacteria in clinical microbiology laboratories.

MATERIALS AND METHODS

Bacterial strains, chemicals, and antibiotics. The bacterial strains used in this study and the β-lactamases that they produce are shown in Table 1. The types of β-lactamase genes were previously confirmed by PCR analyses, cloning and sequencing experiments, as well as isoelectric focusing, as described elsewhere (6, 10, 15, 25, 36, 40, 42, 43, 44). APB, 3-nitrophenylboronic acid (NPB), and 2-thiopheneboronic acid (TPB) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Mueller-Hinton (MH) agar and MH broth were obtained from Becton Dickinson and Company (Paramus, N.J.). KB disks were commercially supplied by Eiken Chemical Co., Ltd. (Tokyo, Japan).

Susceptibility test. The MICs of ceftazidime (CAZ) and cefotaxime (CTX) with or without β-lactamase inhibitors were determined by the agar dilution method with MH agar, according to the recommendations of CLSI (formerly the National Committee for Clinical Laboratory Standards) in document M2-A8 (28). Clavulanic acid (GlaxoSmithKline K.K., Tokyo, Japan) was added at a concentration of 4 μg/ml, and both sodium mercaptoacetic acid (SMA) and APB were added at a concentration of 300 μg/ml. The MIC of APB was generally above 2,400 μg/ml, so the concentration of APB employed in this study did not show any detectable effect on bacterial growth or susceptibilities to antimicrobial agents.

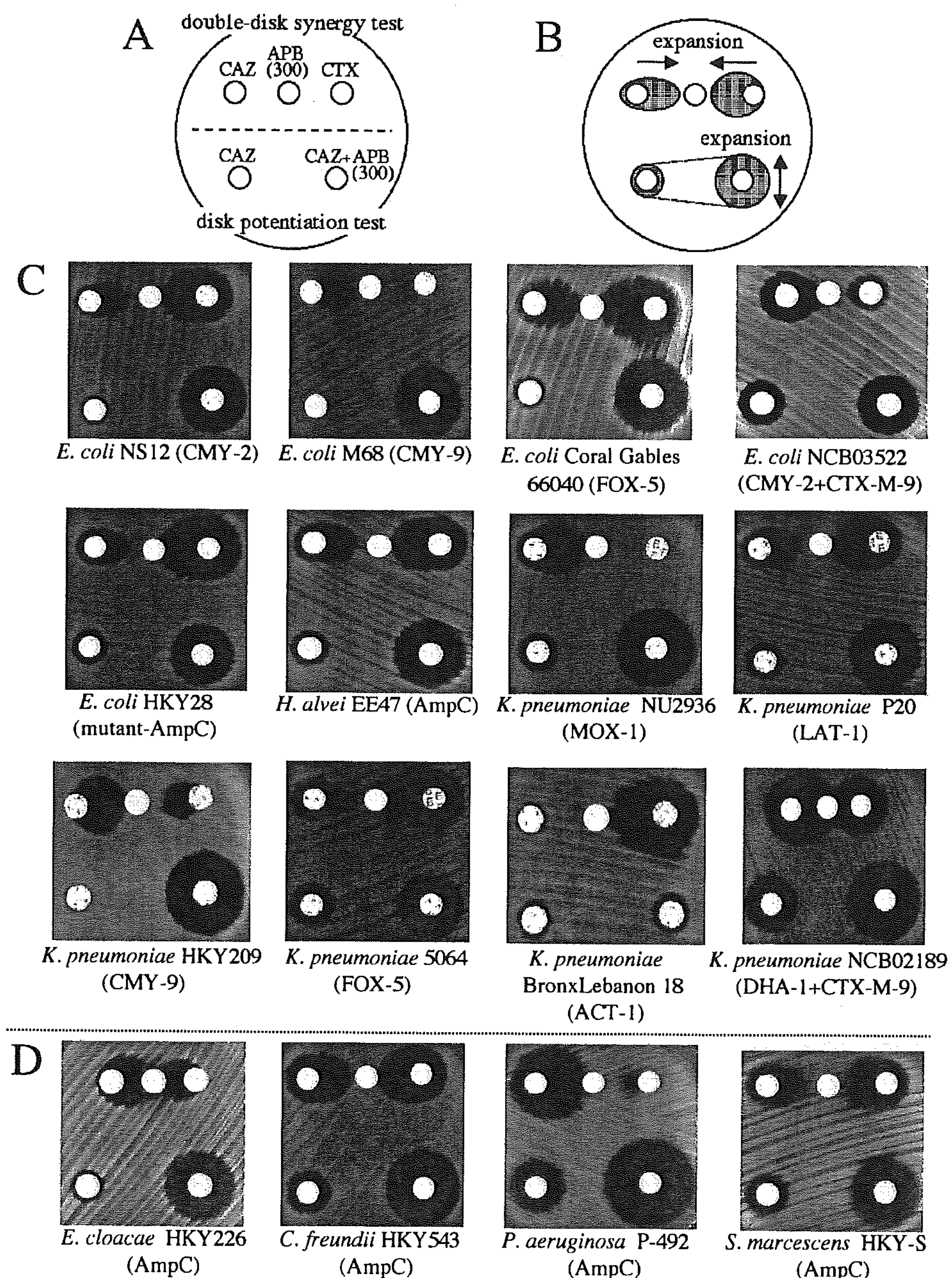

FIG. 1. DDST and disk potentiation test for class C β-lactamase producers. (A) Scheme of disk arrangement for the two tests. The upper three disks are for DDST, and the lower two are for the disk potentiation test. The amount of APB added to the disk was 300 μg. (B) Typical observations of the growth-inhibitory zones among class C β-lactamase producers. The growth-inhibitory zones are expanded toward the APB disk in DDST. In the disk potentiating test, the diameter of the growth-inhibitory zone is expanded around the disk containing both CAZ and APB compared with that around the disk containing solely CAZ. (C) Practical changes in the morphologies or the diameters of the growth-inhibitory zones among the class C β-lactamase-producing strains. Expansion of the growth-inhibitory zone toward the APB disk is observed around the disks containing CAZ or CTX in DDST (upper) among the class C β-lactamase producers. In the disk potentiation test (lower), enlargement of the diameter of the growth-inhibitory zone of greater than or equal to 5 mm is seen among all class C β-lactamase producers except *K. pneumoniae* BronxLebanon 18. (D) DDST and disk potentiation test against chromosomal AmpC producers. Similar findings are observed among gram-negative rods that produce chromosomally encoded inducible AmpC type β-lactamases, suggesting that the new identification method described in the present study can also be applied to chromosomal AmpC producers, as well as plasmid-mediated class C β-lactamase producers.

Detection of class C β-lactamase production. Class C β-lactamase production was determined by the following three methods. Clinical isolates of *Klebsiella pneumoniae* or *E. coli* producing the following plasmid-mediated class C β-lactamases were used as positive controls: ACT-1 (6), CMY-2 and CMY-9 (10), FOX-5 (36), LAT-1 (40), and MOX-1 (15). Because of the similarity of its chromosomal enzyme to one of the plasmid-mediated β-lactamases, ACC-1, an isolate of *Hafnia alvei* was added to the positive controls (24). As negative controls, we used clinical isolates of *K. pneumoniae* or *E. coli* producing other plasmid-mediated β-lactamases: TEM-26 (44); TEM-91 (17); SHV-12 (44); SHV-24 (18); CTX-M-1, CTX-M-2, CTX-M-9, and GES-3 (42); GES-4 (43); and

IMP-1 (Table 1). The boronic acids APB, NPB, and TPB were dissolved in DMSO at a concentration of 100 mg/ml and used for the following tests.

Disk potentiation test. A colony of a test strain which was suspected of being a class C β-lactamase producer was suspended in and diluted with MH broth medium to 10^8 CFU/ml and spread on an MH agar plate with a cotton swab, according to the protocol recommended by CLSI in document M2-A8 (28). Three hundred micrograms of one of the boronic acids, APB, NPB, or TPB, was added to a commercially available KB disk containing 30 μg of CAZ or CTX. These disks were placed on the MH agar plate described above in pairs with a KB disk containing 30 μg of CAZ or CTX with a center-to-center distance of 30 mm (Fig. 1A). The agar plates were incubated at 37°C overnight. The diameter of the growth-inhibitory zone around a CAZ disk with APB was compared with that around a CAZ disk without APB for the detection of class C β-lactamase production.

Double-disk synergy test (DDST). Three hundred micrograms of APB was added to a disk that contained no antibiotics and that was the same size as the KB disk. This APB-containing disk was placed on an MH agar plate on which the bacterial suspension to be examined had been spread. Two other KB disks containing 30 μg of CAZ and CTX were also placed on the MH agar plate, with a center-to-center distance to the boronic acid-containing disk of 18 mm (Fig. 1A). The plate was incubated at 37°C overnight, and the change in the shape of the growth-inhibitory zone around the CAZ or the CTX disk through the interaction with the boronic acid-containing disk was observed for the detection of class C β-lactamase production (Fig. 1B).

Microdilution test. MH broth containing serial dilutions of CAZ or CTX at concentrations ranging from 0.125 to 256 μg/ml and containing 300 μg (1.9 mM) of APB with the same serial dilution of CAZ or CTX was prepared and placed in a 96-well plate. A bacterial suspension was inoculated into each well, according to the recommendation of CLSI in document M7-A6 (27). The plate was incubated at 37°C overnight. The decrease in the MIC of CAZ or CTX in combination with APB was used for the identification of a class C β-lactamase producer.

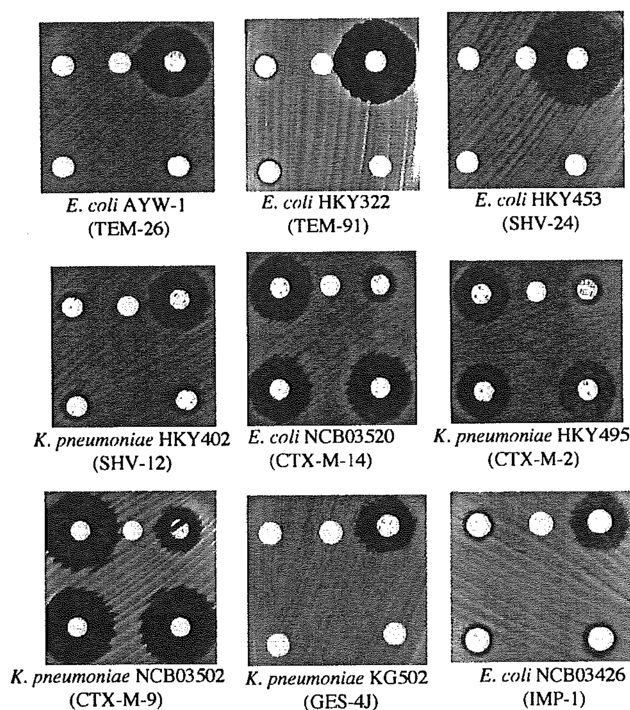

FIG. 2. DDST (upper) and disk potentiation test (lower) for non-class C β-lactamase producers. No apparent changes in the shapes or the diameters of the growth-inhibitory zones around the disks containing CAZ or CTX are observed in the presence of APB (300 μg per disk). The arrangement of the disks was as described for Fig. 1.

RESULTS AND DISCUSSION

Plasmid-mediated class C β-lactamases have been identified worldwide since the late 1980s, and they are emerging threats to antibiotic therapy for various infectious diseases because they confer to pathogenic bacteria, especially *E. coli* and *K. pneumoniae*, resistance to broad-spectrum β-lactams (9, 10, 13, 15, 22, 24, 32). Boronic acids have been recognized as specific inhibitors of AmpC β-lactamases since 1982 (4, 8, 34, 37, 41). Using three commercially available boronic acids, APB, NPB, and TPB, in the present study, we evaluated three different methods for the identification of bacteria producing class C β-lactamases which would be simple enough for routine use in a clinical microbiology laboratory.

First, we developed the disk potentiation test, which is similar to the confirmation test for ESBL production recommended by CLSI in document M100-S14 (29). We selected three commercially available boronic acids, APB, NPB, and TPB, as the specific inhibitors of class C β-lactamases and observed the enlargement of the growth-inhibitory zone diameter around the disk containing CAZ in combination with these inhibitors. The boronic acids were dissolved in DMSO and added to the KB disk containing 30 μg of CAZ. DMSO itself had no apparent effect on the growth of the isolates tested when it was added to the disk at a volume up to 10 μl (data not shown). Both NPB and TPB were found to have antibacterial activity by themselves at concentrations of about 300 μg/ml, leading to a misinterpretation of the changes in the diameter of the growth-inhibitory zone (data not shown). Therefore, we chose APB as the most practical candidate among the specific inhibitors of class C β-lactamases for further examination. Among the four drugs that we tested, CAZ, CTX, cefmetazole, and moxalactam, CAZ showed the best performance in combination with APB. When a cutoff value of a 5-mm enlargement of the growth-inhibitory zone diameter or greater was set, all *K. pneumoniae* and *E. coli* isolates producing the plasmid-mediated class C β-lactamases, except for ACT-1-producing *K. pneumoniae* BronxLebanon 18, could be detected (Fig. 1C); and the specificity of the test was nearly 100% for the negative controls of producers of other classes of β-lactamases (Fig. 2). The exception, *K. pneumoniae* BronxLebanon 18, was less inhibited by APB when CAZ was used. However, a successful test result was obtained with the combination of CTX and APB (data not shown). This strain was supposed to produce another ESBL or to have an alteration in the permeability of the outer membrane, and the test reported by Pitout et al. (33) might be useful for this kind of strain. *H. alvei* was also found to be positive as an AmpC β-lactamase producer. Also, this method could detect *E. coli* HKY28, a mutant AmpC producer which was moderately susceptible to β-lactamase inhibitors such as tazobactam and sulbactam (11). Two well-characterized isolates, *E. coli* NCB03522 and *K. pneumoniae* NCB02189, which produce plasmid-mediated CMY-2 and DHA-1, respectively, together with CTX-M-9, were examined with this disk potentiation test. Using the drug-inhibitor combinations of CAZ plus APB and CTX plus clavulanic acid, we could detect class C β-lactamases and CTX-M-9 separately, with no apparent interaction of these two different classes of β-lactamases.

Second, we applied DDST to the identification of class C β-lactamase producers. Powers et al. (34) first described the potentiation effect of a boronic acid, benzo(*b*)thiophene-2-

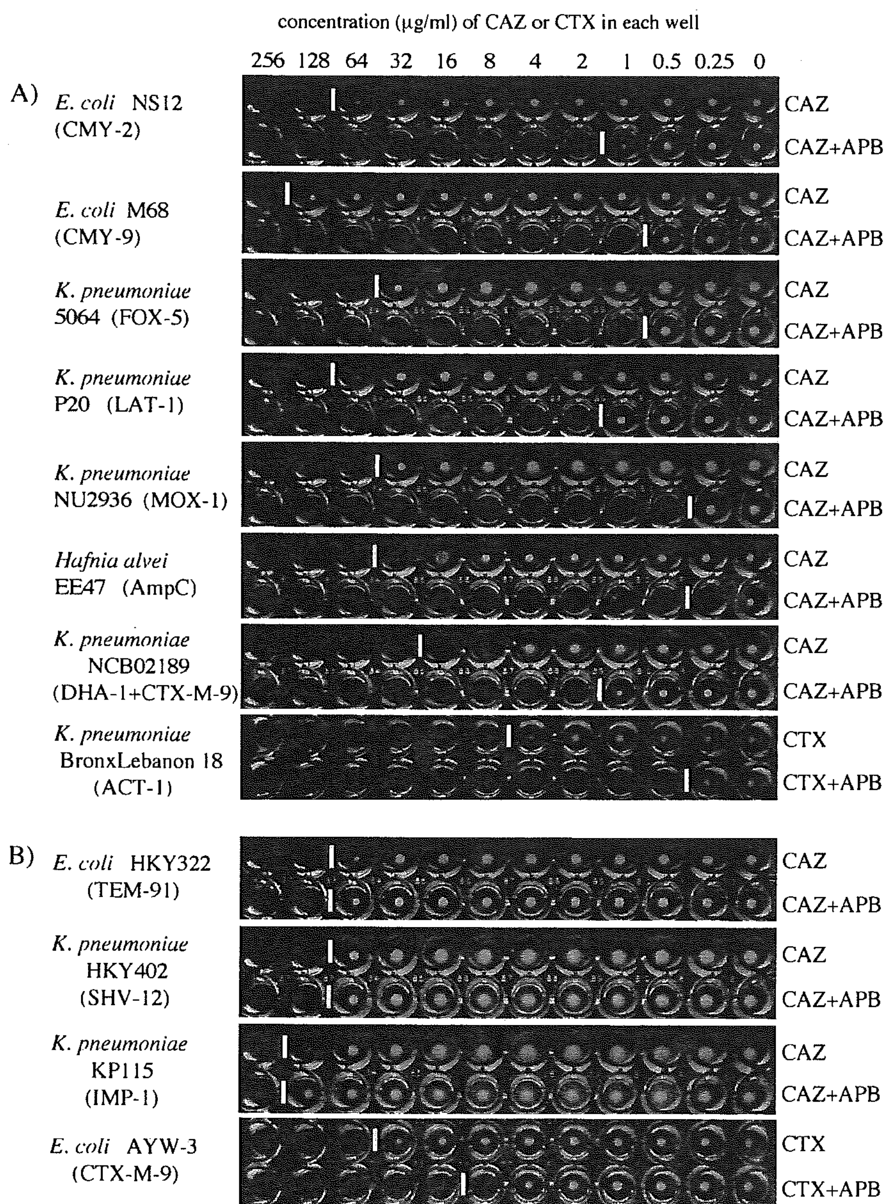

FIG. 3. Microdilution test with APB for detection of class C β-lactamases. APB was added to serial dilutions of CAZ or CTX, and the concentration of ABP in each well is 300 μg/ml. (A) Detection of plasmid-mediated class C β-lactamases in representative *E. coli* and *K. pneumoniae* isolates and chromosomal AmpC β-lactamase in *H. alvei* EE47. An eightfold or greater decrease in the MIC of CAZ or CTX with the addition of APB is indicative of the production of class C β-lactamases. (B) Negative results of microdilution test by using APB for *E. coli* and *K. pneumoniae* isolates producing class A ESBLs or a class B MBL, IMP-1. Among the strains tested, the level of resistance to cefotaxime was reduced in the presence of APB in a few strains, such as CTX-M-9-producing *E. coli* AYW-3; and the coproduction of chromosomal AmpC was suspected in this strain. It may even be possible to distinguish strains that chiefly produce a class A or a class B enzyme, together with a small amount of a class C enzyme, from those that mainly produce class C enzymes when the breakpoint was set at a decrease in the MIC of greater than or equal to eightfold (three tubes) in the presence of APB. White vertical bars between the wells indicate the upper limit of bacterial growth in each line.

boronic acid, to the antimicrobial activity of CAZ; and Liebana et al. (19) used this synergism test for confirmation of the presence of an AmpC-like enzyme. This method, similar to the simple test which we described earlier (1) for the detection of metallo-β-lactamases by the use of thiol compounds, was based on the interpretation of the change in morphology in the growth-inhibitory zone in order to detect class C β-lactamases.

An APB-containing disk and a disk containing a test drug, CAZ or CTX, were placed on an MH agar plate which had been inoculated with a test isolate, with the center-to-center distance of 18 mm. After overnight incubation, expansion of the growth-inhibitory zone toward the APB-containing disk was interpreted to be a positive result for class C β-lactamase production. With the combination of APB and CAZ or CTX,

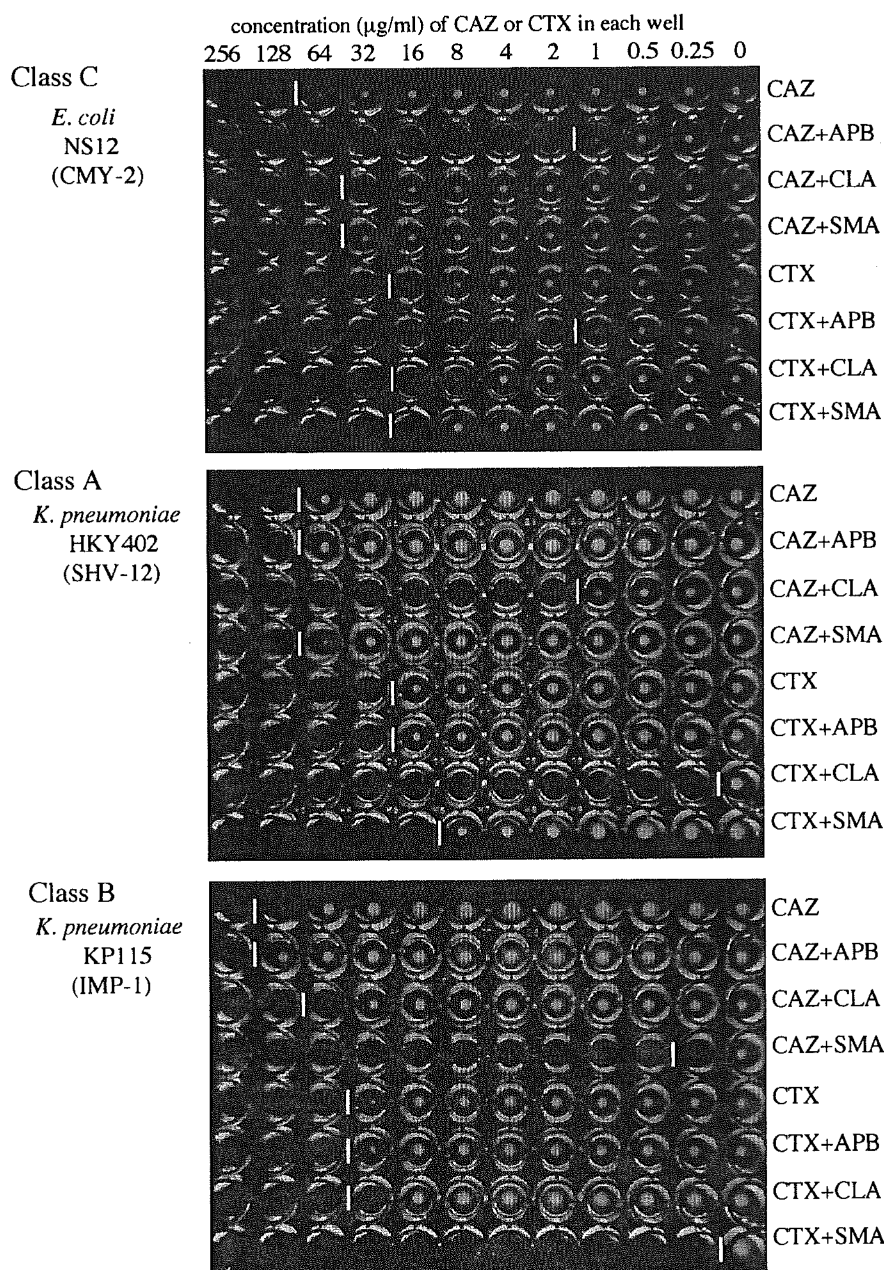

FIG. 4. Microdilution test using three inhibitors for detection of presumptive β-lactamase types. Three inhibitors, APB (300 μg/ml), CLA (4 μg/ml), and SMA (300 μg/ml), were added in each line of the wells. The inhibition patterns of each inhibitor for strains producing class A, class B, and class C β-lactamases are demonstrated by using cefotaxime and ceftazidime as indicators. White vertical bars between the wells indicate the upper limit of bacterial growth in each line.

all plasmid-mediated class C β-lactamases of the positive controls were detected (Fig. 1C), and no apparent changes in the morphology of the growth-inhibitory zone were observed for the negative controls producing other classes of β-lactamases (Fig. 2). For *E. coli* NCB03522 and *K. pneumoniae* NCB02189, which produce plasmid-mediated CMY-2 or DHA-1 together with CTX-M-9, the center-to-center distance between the CAZ and the APB disks should be shortened to 12 mm in order to detect a more discernible expansion of the growth-inhibitory zone around the CAZ disk toward the APB disk.

The microdilution method is one of the most familiar methods for the determination of MICs in clinical laboratories due to the recent introduction of rapid automated bacterial identification and antimicrobial susceptibility test systems. Three hundred micrograms of APB was added to the serial dilution of CAZ, and the MICs of CAZ determined with and without APB were compared according to the methods recommended by CLSI (27). The MICs appeared to be similar to those shown in Table 1, which were determined by the agar dilution method, according to the recommendations of CLSI (28). More