生菌など）では，検査法の周知は今後の課題となっている。

感染症発生動向調査に基づく薬剤耐性菌の検出状況では，MRSAやESBLの頻度は高い。一方で，MDRPはMRSAの1/50程度であり，MDRAやVREの頻度はいまのところ非常に低い。但し，これらは感染症を発症したと診断された症例のみであり，保菌状態あるいは診断がされていない症例も含めれば潜在的な感染者はこれよりもかなり多いものと推測される。

治療薬としては，グラム陽性菌については近年いくつかの新薬が使用可能となっている。一方グラム陰性菌については，チゲサイクリンが近年認可されたが，MDRP治療薬として使用可能なコリスチンはまだ承認されておらず，やむを得ず感受性を示す薬剤の併用療法などがおこなわれている。

4.2 医療関連感染で問題となる病原体の保菌と感染症[1-3]

薬剤耐性菌は，感染した直後に感染症を発症することは少なく，一旦体内に保菌状態となった後に感染症を発症する。このため，耐性菌の保菌部位と感染症は密接に関連することになる。MRSAは，ヒトの皮膚，鼻前庭，上気道，直腸周囲，咽頭などに保菌され，術創部感染などの皮膚軟部組織感染症，院内肺炎，血管留置カテーテル関連血流感染の起炎菌となる。さらに，血流感染の二次感染として，心内膜炎や骨髄炎などを引き起こす。VREは元来ヒトの常在菌である腸球菌の薬剤耐性菌であり，多くは感染症を引き起こすことなく大腸内に保菌状態となる。基本的に病原性が低いため，何らかの医療処置，特に人工物に関連する感染症の原因菌となり，血管留置カテーテル関連血流感染やカテーテル関連尿路感染などが主な感染症となる。また血流感染の二次感染としての心内膜炎や術創部感染を引き起こすこともある。

薬剤耐性グラム陰性桿菌の多くは，腸内に保菌状態となり，様々な感染症の起炎菌となる。アシネトバクターは，肛門膣周囲および皮膚常在菌となり，*Klebsiella pneumoniae*では大腸に加えて鼻腔，口腔，皮膚，セラチア属菌では加えて尿路内にも保菌状態となる。主な感染症として，肺炎，創部感染，カテーテル関連尿路感染などが挙げられるが，セラチアは時にカテーテル関連血流関連の起炎菌ともなりうる。*C. difficile*は，大腸内に保菌状態となり，抗菌薬使用などのリスク因子により*C. difficile*関連腸炎を引き起こす。

これらの薬剤耐性菌が保菌状態となるリスク因子も重要である。多くの薬剤耐性菌に関連する因子として，病院環境との接触（入院）や耐性菌保菌患者との環境の共有，抗菌薬使用歴などが挙げられる。他に，免疫不全を伴うような様々な基礎疾患（糖尿病，悪性腫瘍，腎不全・透析など）は保菌状態を促進する因子となる。また，様々な侵襲的な処置（手術，カテーテル挿入など）も保菌のリスク因子となる。

4.3 医療関連感染に関連する細菌の一般的な制御法（表5）[1-3]

薬剤耐性菌や*C. difficile*は，それらの菌が検出されれば，接触予防策の対象となり，原則隔離予防策を行うこととなる。部屋の配置としては個室管理が望ましいが，わが国の医療環境では，個室の確保がしばしば困難であり，その場合には集団隔離（コホート管理）を考慮する。また，非保菌者と同室管理にする場合には，ベッドの間隔を広く取る，同室管理にする患者は免疫状態や全身状態が良好な患者のみとするなどの配慮が必要である。患者のケア時には，病原体の拡散伝播を防止するために，手袋やガウンなどの個人防護具（PPE）の着用を行い，使用後は直ちに廃棄する。標準予防策として，診療前後は十分な手指衛生を行う。手洗いについてであるが，芽胞菌（*C. difficile*, バチルス属菌など）では，アルコールに耐性であるため，アルコール含有速乾性手指消毒薬は無効である。このため，石けんと流水による手洗いが感染制御のため必要となる。

環境整備（消毒）も感染制御において重要である[8-10]。医療関連感染で問題となる微生物は，体外に放出された後も，一定期間生息しうる（表6）。特に，黄色ブドウ球菌や腸球菌などのグラム陽性菌や，アシネトバクターなどの菌は乾燥に強く，長期間病院環境に生息しうる。*C. difficle*は，生息に適さない環境下では芽胞を形成し，長時間生息する。セラチアや緑膿菌は，一般に乾燥に弱いとされるが，不潔な湿潤環境下では長期間生息可能である。薬剤耐性菌を保菌/感染した患者周辺の環境は，それらの菌によって濃厚に汚染されていると考えられる。したがって，日常的な清掃（通常1日1回）に加えて，患者やスタッフが高頻度に接触する環境（ベッドレール，ベッドサイドテーブル，ドアノブなど）については，1日数回以上適切な消毒薬を用いた清拭消毒が必要となる。また，患者に日常的に用いる医療器材（血圧計，聴診器など）は，患者専用とする。*C. difficle*は，アルコール抵抗性のため，次亜塩素酸系の消毒薬を用いて消毒を行う必要がある。レジオネラやバチルス属菌は，それぞれ水設備やリネンおよびタオル類での菌の増殖と感染症発症が関連しており，適正な環境整備が重要である[8,11]。

4.4 医療関連感染に関連する細菌の特異的な制御法（表5）

4.4.1 積極的なスクリーニング（Active screening）[3]

潜在的保菌状態にある病原体を，積極的に発見するために特別に微生物検査を行う事がある。通常，保菌され

表5．医療関連感染で問題となる細菌の制御法

	制御法	アウトブレイクに関連する主な感染症	アウトブレイクの原因となる保菌/汚染場所	検出/スクリーニングの方法
MRSA	保菌/感染症発症患者の隔離 ハイリスク患者における積極的なスクリーニング（Active surveillance） 医療従事者の教育（手指衛生，環境整備，接触予防策） 抗菌薬適正使用（特に広域β-ラクタム系抗菌薬） 除菌（特に術前）	術創部感染，血流感染	輸液製剤，医療器材，医療者の手，植え込みデバイス	鼻前庭培養（MRSAスクリーニング培地）
VRE	保菌/感染症発症患者の隔離 VRE流行地域，施設における積極的なスクリーニング（Active surveillance） 医療従事者の手指衛生遵守 抗菌薬適正使用（特に広域セフェム，抗嫌気性菌薬，バンコマイシン） 環境の徹底した消毒	新生児敗血症，膀胱炎，血流感染	新生児，手術患者，汚染された器材や環境，医療者の手	便/肛門周囲スワブ（VREスクリーニング培地）
薬剤耐性グラム陰性菌	保菌/感染症発症患者の隔離	緑膿菌：尿路感染，肺炎，創部感染	緑膿菌：水周り，人工呼吸器，自動尿測定器，内視鏡	便（多剤耐性緑膿菌検出培地）
	積極的なスクリーニング(Active surveillance)：特にカルバペネム耐性腸内細菌科細菌	大腸菌：創部感染，尿路感染，新生児敗血症/髄膜炎	大腸菌：汚染された器材や輸液製剤	
	微生物検査室におけるカルバペネム耐性腸内細菌科細菌の確実な検出	*K. pneumoniae*：尿路感染，肺炎	*K. pneumoniae*：尿道カテーテル，人工呼吸器，汚染された輸液製剤	
	水回りの環境整備（特にセラチア，緑膿菌）	Enterobacter属：尿路感染，血管留置カテーテル関連血流感染	Enterobacter属：汚染された輸液製剤，完全中心静脈栄養，手/皮膚炎	
	環境の徹底した消毒（特にアシネトバクター）	アシネトバクター：肺炎，尿路感染	アシネトバクター：汚染された器材，熱傷，手術，呼吸器関連器材，輸液療法，水周り	
	抗菌薬適正使用（特にカルバペネム）	セラチア：尿路感染，血流感染	汚染された輸液製剤，吸入療法の器材，消毒薬，EDTA入り採血管，エアコン，不潔な器材	
Clostridium difficile	CD関連腸炎患者の隔離（下痢期間中） 医療従事者の手指衛生（流水と石けん） 次亜塩素酸系消毒薬による環境消毒 抗菌薬適正使用（抗菌薬の使用量や期間をできる限り短くする）	CD関連腸炎	病院環境	スクリーニングは推奨されない
レジオネラ	水設備の適切な管理（定期的な消毒およびスクリーニング検査）	肺炎	冷却塔水，給湯設備	水設備の水の培養（レジオネラ検出培地）
バチルス	リネン，タオル類の適切な管理	血流感染	リネン，タオル類	環境培養

表6．医療関連感染で問題となる細菌の乾燥無生物体表面での生息期間

微生物	生息期間
黄色ブドウ球菌（MRSAを含む）	7日～12ヶ月以上
腸球菌（VREを含む）	5日～46ヶ月以上
百日咳菌	3～5日間
溶血性レンサ球菌	3日～6.5ヶ月
大腸菌	1.5時間～16ヶ月
クレブシエラ属菌	2時間～30ヶ月以上
セラチア	3日～2ヶ月
緑膿菌	6時間～16ヶ月
アシネトバクター	3日～11ヶ月
Clostridium difficile（芽胞菌）	5ヶ月以上
結核菌	1日～4ヶ月
インフルエンザウイルス	1～2日
ノロウイルス	8時間～2週間以上

る部位の検体を採取し，培養検査を行う。近年は，より迅速な診断のために，核酸増幅検査を用いた検出も行われている。但し，常時実施した場合には，スクリーニング培養などのコストが余分にかかるため，アウトブレイク時やハイリスク患者などに限定して通常は実施される。

Active screeningのためには，通常それぞれの耐性菌を効率的に検出するための選択培地を用いることが多い（表7）。選択培地は，目的とする菌を検出するだけではなく，培地内に抗菌薬や特別な色素などを混入し，薬剤耐性菌を検出しやすくし，発見しやすくした培地である。但し，検体採取の方法や菌の薬剤耐性機序などにより，検出感度は大きく変化するため，選択培地で検出されない場合もある。逆に，検出されないことで対応が遅れ，大規模なアウトブレイクが発生した後に初めて判明する場合もある。それぞれの培地の特性や検出限界をよく理解した上で利用する必要がある。

4.4.2 抗菌薬適正使用[12)]

薬剤耐性菌制御において抗菌薬適正使用は非常に重要である。耐性菌は抗菌薬を用いることにより選択され，また耐性因子獲得のきっかけともなる。従って，臨床的に不要となった抗菌薬はただちに中止すべきである。例えば，周術期抗菌薬では，通常術直前と手術中のみが投

表7．薬剤耐性菌スクリーニングのための選択培地

耐性菌	スクリーニング培地	製造販売
MRSA	クロモアガーMRSA	関東化学
	MDRS-K	極東製薬
	chromID MRSA	シスメックス・ビオメリュー
	BCP 不含 MS-CFX	日水製薬
	MRSA 選択培地	日本BD
VRE	VRES	極東製薬
	chromID VRE	シスメックス・ビオメリュー
	VRE 選択培地	日本BD
ESBL/MBL 産生菌	ESBL/MBL スクリーニング寒天培地	極東製薬
	chromID ESBL	シスメックス・ビオメリュー
	chromID CARBA（カルバペネム産生腸内細菌科用）	シスメックス・ビオメリュー
MDRP	クロモアガーMDRP	関東化学

与が必要であり，術後3日目以後に必要となることはまずない。さらに，使用する抗菌薬も問題となる。一般的に広域抗菌薬（グラム陽性菌ではグリコペプチド系抗菌薬，グラム陰性菌では広域セフェム，カルバペネム）が用いられた場合に，高度耐性菌が選択されやすくなる。このため，これらの抗菌薬の使用は必要最小限にとどめて，感受性検査結果を参考に狭いスペクトラムの抗菌薬に変更する（de-escalation）ことを常に心がける必要がある。また，抗嫌気性菌活性を有する抗菌薬（β-ラクタマーゼ阻害剤配合剤，カルバペネムなど）は，腸管内常在細菌叢のかく乱を引き起こすリスクが高く，腸管内保菌に関連する薬剤耐性菌や *C. difficile* のリスク因子となり，こちらも適正使用が求められる。

4.4.3 アウトブレイク対策（詳細は医療現場における感染制御　3）アウトブレイク時の抑制 参照）[1, 3]

アウトブレイクとは，通常発生しているレベル以上に感染症が増加することと定義される。薬剤耐性菌によるアウトブレイクは，同一クローン（あるいは同一耐性機序）のある特定の耐性菌が施設内に伝播蔓延することによって発生するが，アウトブレイクの早期発見のためには，まずこれらの耐性菌の施設内での発生状況を把握しておく必要がある。このような疫学調査のことを，サーベイランスと呼ぶが，各施設あるいは各部署毎の発生状況を週単位あるいは月単位で把握し，アウトブレイクの早期発見を行う必要がある。

アウトブレイクの発生が疑われた場合には，直ちに対策を始めなければならない。感染拡大を防ぐために，まずは，その病原体による感染者を正確に把握するための積極的なスクリーニングを行う。菌が検出された患者は，確実に接触予防策を実施する。次に，アウトブレイクの要因分析を行う。菌が検出された患者の共通するリスク因子を検出する疫学的な調査が必要である。医療行為の他，病院環境もしばしばアウトブレイクの原因となるため，環境要因の調査も実施する。厳密な尿量測定のために蓄尿が行われる場合があるが，貯められた尿中の耐性菌（特にグラム陰性菌）が増殖し，環境を高度に汚染し，アウトブレイクの原因となることがある。また近年蓄尿を自動的に行う事のできる自動尿測定器が開発され，導入している病院も多いが，耐性菌による汚染が発生し感染アウトブレイクの原因となっていることがあり，その取り扱いには日常的に厳重な衛生管理が必要である。

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Identification of a Novel 6′-*N*-Aminoglycoside Acetyltransferase, AAC(6′)-Iak, from a Multidrug-Resistant Clinical Isolate of *Stenotrophomonas maltophilia*

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***Stenotrophomonas maltophilia* IOMTU250 has a novel 6′-*N*-aminoglycoside acetyltransferase-encoding gene, *aac(6′)-Iak*. The encoded protein, AAC(6′)-Iak, consists of 153 amino acids and has 86.3% identity to AAC(6′)-Iz. *Escherichia coli* transformed with a plasmid containing *aac(6′)-Iak* exhibited decreased susceptibility to arbekacin, dibekacin, neomycin, netilmicin, sisomicin, and tobramycin. Thin-layer chromatography showed that AAC(6′)-Iak acetylated amikacin, arbekacin, dibekacin, isepamicin, kanamycin, neomycin, netilmicin, sisomicin, and tobramycin but not apramycin, gentamicin, or lividomycin.**

Stenotrophomonas maltophilia is a globally emerging multidrug-resistant Gram-negative pathogen that is most commonly associated with respiratory infections in humans (1) and causes an increasing number of nosocomial respiratory tract and bloodstream infections in immunocompromised patients. *S. maltophilia* exhibits resistance to a broad spectrum of antibiotics, namely, β-lactam antibiotics, macrolides, cephalosporins, fluoroquinolones, aminoglycosides, carbapenems, chloramphenicol, tetracyclines, and polymyxins (1). Several intrinsic antibiotic resistance traits in *S. maltophilia* are known; an increase in membrane permeability and the presence of chromosomally encoded multidrug resistance efflux pumps have also been observed (2).

Aminoglycoside-resistant mechanisms involve primarily aminoglycoside-modifying enzymes (3) and 16S rRNA methylases (4). The 6′-*N*-aminoglycoside acetyltransferases [AAC(6′)s] are of particular interest because they can modify a number of clinically important aminoglycosides. There are two main AAC(6′) subclasses, which differ in their activities against amikacin and gentamicin. The AAC(6′)-I-type enzymes effectively acetylate amikacin but not gentamicin, whereas the AAC(6′)-II-type enzymes effectively acetylate gentamicin but not amikacin (5). To date, 45 genes encoding AAC(6′)-I types, designated *aac(6′)-Ia* to *-Iaj*, have been cloned, and their bacteriological or biochemical properties have been characterized (5–8).

S. maltophilia IOMTU250 was isolated from the endotracheal tube of a patient in a medical ward of a hospital in Nepal in 2012. *Escherichia coli* DH5α (TaKaRa Bio, Shiga, Japan) and *Escherichia coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA) were used as hosts for recombinant plasmids and protein expression, respectively. MICs were determined using the microdilution method (9). The MICs of tested aminoglycosides for *S. maltophilia* IOMTU250 are shown in Table 1. The MICs of other antibiotics were as follows: ampicillin, >1,024 μg/ml; ampicillin-sulbactam, 128 μg/ml; aztreonam, 128 μg/ml; ceftazidime, 8 μg/ml; cephradine, 1,024 μg/ml; cefepime, 64 μg/ml; cefotaxime, 64 μg/ml; cefoxitin, 512 μg/ml; chloramphenicol, 8 μg/ml; colistin, 32 μg/ml; fosfomycin, 128 μg/ml; imipenem, 256 μg/ml; levofloxacin, 1 μg/ml; meropenem, 64 μg/ml; minocycline, ≤0.25 μg/ml; penicillin, 512 μg/ml; ticarcillin-clavulanate, 8 μg/ml; tigecycline, ≤0.25 μg/ml; and trimethoprim-sulfamethoxazole, 4 μg/ml.

Genomic DNA was extracted from *S. maltophilia* IOMTU250 using DNeasy blood and tissue kits (Qiagen, Tokyo, Japan) and sequenced with a MiSeq system (Illumina, San Diego, CA). More than 20-fold coverage was achieved. A new 6′-*N*-aminoglycoside acetyltransferase variant was designated *aac(6′)-Iak*.

A synthetic *aac(6′)-Iz* gene (462 bp) was produced by Funako-

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TABLE 1 MICs of various aminoglycosides for *S. maltophilia* IOMTU250 and *E. coli* strains transformed with *aac(6′)-Iak* and *aac(6′)-Iz*

	MIC[b] (μg/ml)											
Strain[a]	ABK	AMK	APR	DIB	GEN	ISP	KAN	LIV	NEO	NET	SIS	TOB
S. maltophilia IOMTU250	512	64	>512	512	32	64	128	>512	512	512	64	64
E. coli DH5α/pSTV28	0.5	0.25	1	0.25	0.25	0.25	1	2	0.5	0.25	0.25	0.5
E. coli DH5α/pSTV28-*aac(6′)-Iak*	2	1	1	16	0.25	0.5	2	2	4	1	2	4
E. coli DH5α/pSTV28-*aac(6′)-Iz*	4	2	1	16	0.25	0.5	2	2	4	8	2	16

[a] The MICs for *S. maltophilia* and *E. coli* strains were determined with Mueller-Hinton broth preparations and individual aminoglycosides.
[b] ABK, arbekacin; AMK, amikacin; APR, apramycin; DIB, dibekacin; GEN, gentamicin; ISP, isepamicin; KAN, kanamycin; LIV, lividomycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; TOB, tobramycin.

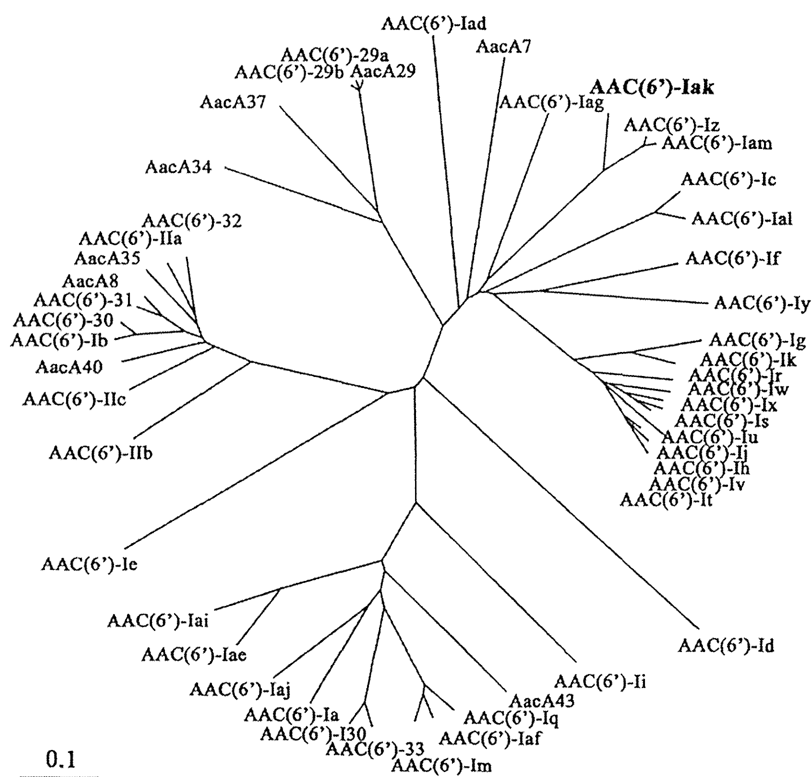

FIG 1 Dendrogram of 6'-*N*-aminoglycoside acetyltransferases [AAC(6')s] for comparison with AAC(6')-Iak. The dendrogram was calculated using the CLUSTAL W2 program. Branch lengths correspond to numbers of amino acid exchanges for the proteins. EMBL/GenBank/DDBJ accession numbers of the proteins are as follows: AAC(6')-Ia, M18967-1; AAC(6')-Ib, M23634; AAC(6')-Ic, M94066; AAC(6')-Id, X12618; AAC(6')-Ie, M13771; AAC(6')-If, X55353; AAC(6')-Ig, L09246; AAC(6')-Ih, L29044; AAC(6')-Ii, L12710-1; AAC(6')-Ij, L29045; AAC(6')-Ik, L29510; AAC(6')-Im, CAA91010; AAC(6')-Iq, AF047556-1; AAC(6')-Ir, AF031326; AAC(6')-Is, AF031327; AAC(6')-It, AF031328; AAC(6')-Iu, AF031329; AAC(6')-Iv, AF031330; AAC(6')-Iw, AF031331; AAC(6')-Ix, AF031332; AAC(6')-Iy, AF144880; AAC(6')-Iz, AF140221; AAC(6')-Iad, AB119105; AAC(6')-Iae, AB104852; AAC(6')-Iaf, AB462903; AAC(6')-Iag, AB472901; AAC(6')-Iai, EU886977; AAC(6')-Iaj, AB709942; AAC(6')-Iak, AB894482; AAC(6')-Ial, AB871481; AAC(6')-Iam, AB971834; AAC(6')-IIa, M29695; AAC(6')-IIb, L06163; AAC(6')-IIc, AF162771; AAC(6')-29a, AF263519; AAC(6')-29b, AF263519; AAC(6')-30, AJ584652; AAC(6')-31, AJ640197; AAC(6')-32, EF614235; AAC(6')-33, GQ337064; AAC(6')-I30, AY289608; AacA7, U13880; AacA8, AY444814; AacA29, AY139599; AacA34, AY553333; AacA35, AJ628983; AacA37, DQ302723; AacA40, EU912537; and AacA43, HQ247816.

shi Co. Ltd. (Tokyo, Japan). The *aac(6')-Iak* and *aac(6')-Iz* genes were cloned into the corresponding sites of pSTV28 using primers SalI-*aac(6')-Iak*-F (5'-ATGCGTCGACATGACCGGCAGCGCG GCCACGATCCGCCCG-3') and PstI-*aac(6')-Iak*-R (5'-ATCTG CAGTCACGCCGATGGCTCCAGTGGCATGCGGAAA-3') and SalI-*aac(6')-Iz*-F (5'-ATGGTCGACATGATCGCCAGCGCGCC CACGATCCGCC-3') and PstI-*aac(6')-Iz*-R (5'-ATCTGCAGTC ACGCCGATGGCTCCAGCGGCATGCGGAA-3'), respectively. *E. coli* DH5α was transformed with pSTV28-*aac(6')-Iak* or pSTV28-*aac(6')-Iz* to assess aminoglycoside resistance.

The open reading frame of AAC(6')-Iak was cloned into the pQE2 expression vector using the primers SalI-*aac(6')-Iak*-F and PstI-*aac(6')-Iak*-R for protein expression. Purification of the recombinant AAC(6')-Iak protein and thin-layer chromatography (TLC) analysis were performed as previously described (7). The kinetic activities of AAC(6')-Iak were determined as previously described (8).

AAC(6')-Iak consists of 153 amino acids. As shown by the dendrograms of AAC(6') based on amino acid sequences in Fig. 1, AAC(6')-Iak is close to AAC(6')-Iz and AAC(6')-Iam (accession no. AB971834) from *S. maltophilia* (10). Multiple sequence alignments among AAC(6') enzymes revealed that AAC(6')-Iak had 86.3% identity to AAC(6')-Iz from *S. maltophilia* (11), 84.3% identity to AAC(6')-Iam from *S. maltophilia* (10), 47.7% identity to AAC(6')-Iag from *Pseudomonas aeruginosa* (8), 43.1% identity to AAC(6')-If from *Enterobacter cloacae* (12), and 42.8% identity to AAC(6')-Iy from *Salmonella enterica* (13).

We compared the enzymatic properties of AAC(6')-Iak with those of AAC(6')-Iz because they had similar amino acid sequences and were detected in *S. maltophilia*. As shown in Table 1, *E. coli* expressing AAC(6')-Iak or AAC(6')-Iz showed decreased susceptibility to all aminoglycosides tested except for apramycin, gentamicin, and lividomycin. The MICs of netilmicin and tobramycin for *E. coli* expressing AAC(6')-Iak were significantly lower than those for *E. coli* expressing AAC(6')-Iz. The MICs of the other aminoglycosides tested were not significantly different between *E. coli* isolates expressing AAC(6')-Iak and AAC(6')-Iz (Table 1).

S. maltophilia IOMTU250 was highly resistant to all aminoglycosides tested, whereas *E. coli* expressing *aac(6')-Iak* was susceptible to all aminoglycosides tested except for dibekacin (Table 1). The discrepancy of aminoglycoside susceptibilities between *S. maltophilia* and *E. coli* could be explained by the presence of efflux pump genes specific for *S. maltophilia*. Whole-genome sequencing with the MiSeq system in this study showed that IOMTU250 had the efflux pump genes specific for *S. maltophilia*, including

TABLE 2 Kinetic parameters of AAC(6′)-Iak and AAC(6′)-Iz enzymes[a]

	AAC(6′)-Iak			AAC(6′)-Iz		
Aminoglycoside[b]	K_m (μM)[c]	k_{cat} (s^{-1})[c]	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)[c]	k_{cat} (s^{-1})[c]	k_{cat}/K_m (μM^{-1} s^{-1})
ABK	36 ± 13	0.57 ± 0.10	0.016	17 ± 6	0.57 ± 0.06	0.036
AMK	32 ± 17	0.11 ± 0.03	0.004	24 ± 5	0.26 ± 0.02	0.011
DIB	24 ± 3	0.25 ± 0.04	0.010	31 ± 5	0.46 ± 0.05	0.015
ISP	44 ± 9	0.05 ± 0.01	0.001	49 ± 8	0.11 ± 0.01	0.002
KAN	30 ± 7	0.04 ± 0.01	0.001	44 ± 9	0.05 ± 0.01	0.001
NEO	10 ± 2	0.32 ± 0.01	0.034	13 ± 2	0.29 ± 0.01	0.023
NET	70 ± 6	0.30 ± 0.02	0.004	25 ± 8	0.34 ± 0.05	0.013
SIS	4 ± 1	0.16 ± 0.01	0.038	7 ± 1	0.20 ± 0.03	0.028
TOB	16 ± 4	0.08 ± 0.01	0.006	12 ± 2	0.25 ± 0.03	0.021

[a] The proteins were initially modified by a His tag, which was removed after purification.
[b] ABK, arbekacin; AMK, amikacin; DIB, dibekacin; ISP, isepamicin; KAN, kanamycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; TOB, tobramycin.
[c] K_m and k_{cat} values represent the means of results from 3 independent experiments ± standard deviations.

smeABC, *smeDEF*, *smeZ*, *smeJK*, and the *pcm-tolCsm* operon. These genes are known to be associated with aminoglycoside resistance (14–16), although it is difficult to clarify whether or not these efflux pump genes contribute to aminoglycoside resistance together with *aac(6′)-Iak* in *S. maltophilia* IOMTU250.

As shown in Table 2, recombinanttk;1 AAC(6′)-Iak and AAC(6′)-Iz acetylated arbekacin, amikacin, dibekacin, isepamicin, kanamycin, neomycin, netilmicin, sisomicin, and tobramycin. The profile of enzymatic activities of AAC(6′)-Iak were similar to those of AAC(6′)-Iz, although AAC(6′)-Iak had higher k_{cat}/K_m ratios for neomycin and sisomicin (Table 2).

To examine the acetyltransferase activity of AAC(6′)-Iak against aminoglycosides, we performed TLC using the purified recombinant AAC(6′)-Iak. Lividomycin was used as a negative control because it has a hydroxyl group instead of an amino group at the 6′ position and therefore cannot be acetylated by AAC(6′). As shown in Fig. 2, all aminoglycosides tested except for apramycin, gentamicin, and lividomycin were acetylated by AAC(6′)-Iak, and amikacin, isepamicin, kanamycin, and tobramycin were partially acetylated by AAC(6′)-Iak under the experimental conditions used. The TLC data for apramycin, gentamicin, and lividomycin were consistent with the MICs of the aminoglycosides for *E. coli* with pSTV28-*aac(6′)-Iak* and *E. coli* with the control vector (Table 1).

The substrate specificity of AAC(6′)-Iak was similar to that of AAC(6′)-Iz, although some kinetic parameters of AAC(6′)-Iak were different from those of AAC(6′)-Iz; i.e., the K_m for netilmicin and the k_{cat} for tobramycin of AAC(6′)-Iak were different from those of AAC(6′)-Iz (Table 2). The chemical structure of netilmicin is similar to that of sisomicin except for a residue at position 1 in 2-deoxystreptamine ring II (position R_2; ethylamino and amino groups, respectively). The ethylamino group at position R_2 in netilmicin, therefore, must be critical for the substrate affinity of AAC(6′)-Iz but not that of AAC(6′)-Iak. The chemical structure of tobramycin is similar to that of dibekacin, but tobramycin has a hydroxyl group at position 4′ in ring I (position R_1), whereas dibekacin does not, indicating that the hydroxyl group of tobramycin at position R_1 negatively affects the turnover rate (k_{cat}) of AAC(6′)-Iak but not that of AAC(6′)-Iz.

The structure of the genetic environment surrounding *aac(6′)-Iak* was similar to that of a region surrounding *aac(6′)-Iam* in *S. maltophilia* K279a, obtained from a patient in the United Kingdom (10) (Fig. 3). The genetic environment surrounding *aac(6′)-Iak* from nucleotides (nt) 23623 to 46040 had 92% identity to a genetic region in *S. maltophilia* K279a (accession no. AM743169) from nt 3660929 to 3683268 (10). The genetic environment surrounding *aac(6′)-Iak* in *S. maltophilia* IOMTU250 contained at least 5 housekeeping genes, including *purA*, *acnA*, *aroK*, *aroB*, and *thrA*, indicating that *aac(6′)-Iak* was located in the chromosomal genome.

All the *aac(6′)-Iz*, *aac(6′)-Iak*, and *aac(6′)-Iam* genes were detected in clinical isolates of *S. maltophilia* (10, 11, 17). These genes contributed to decreased susceptibility to 2-deoxystreptamine aminoglycoside antibiotics, such as neomycin, netilmicin, sisomicin, and tobramycin but not gentamicin (Table 1) (17). The deletion of *aac(6′)-Iz* in a clinical isolate of *S. maltophilia* resulted in the increased susceptibility of the isolate (17).

This study was approved by the Institutional Review Board of

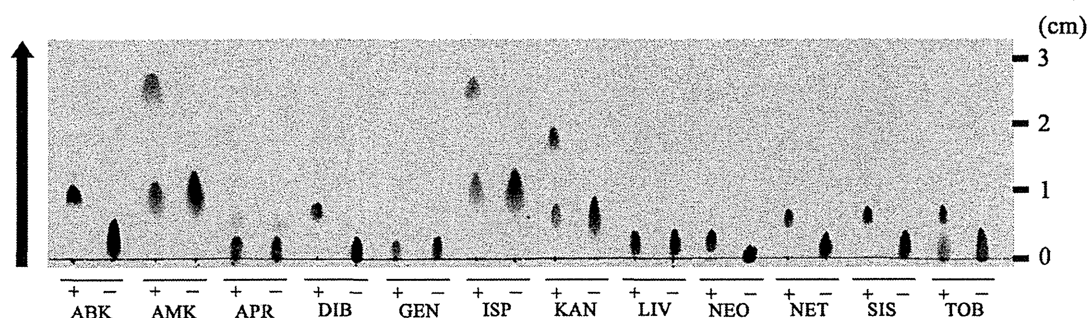

FIG 2 Analysis of acetylated aminoglycosides by thin-layer chromatography. AAC(6′)-Iak and various aminoglycosides were incubated in the presence (+) or absence (−) of acetyl coenzyme A. ABK, arbekacin; AMK, amikacin; APR, apramycin; DIB, dibekacin; GEN, gentamicin; ISP, isopamicin; KAN, kanamycin; LIV, lividomycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; TOB, tobramycin.

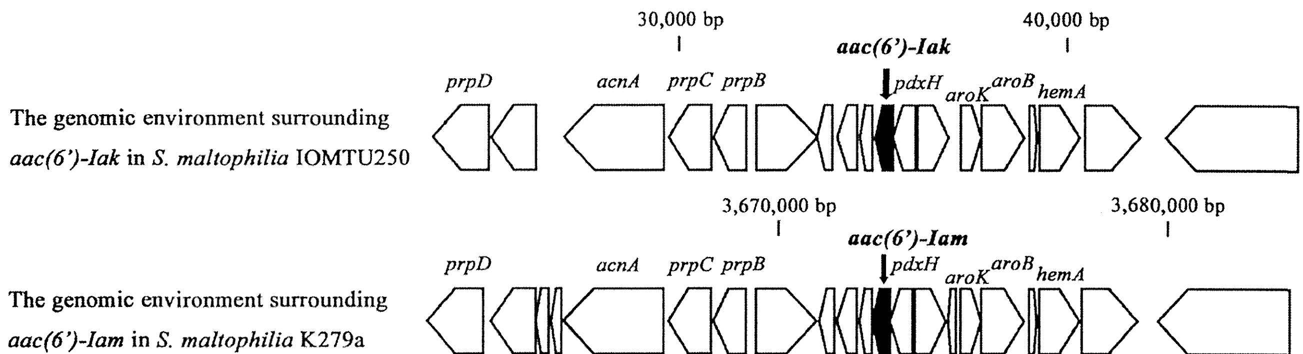

FIG 3 Genetic environments surrounding *aac(6')-Iak* in *S. maltophilia* IOMTU250 and *aac(6')-Iam* in *S. maltophilia* K279a.

the Institute of Medicine at Tribhuvan University (approval 6-11-E) and the Biosafety Committee of the Research Institute of the National Center for Global Health and Medicine (approval 25-M-038).

Nucleotide sequence accession number. The sequence for *aac(6')-Iak* and its genetic environments (76,559 bp) was deposited in GenBank under accession number AB894482.

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NDM-12, a Novel New Delhi Metallo-β-Lactamase Variant from a Carbapenem-Resistant *Escherichia coli* Clinical Isolate in Nepal

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A novel New Delhi metallo-β-lactamase variant, NDM-12, was identified in a carbapenem-resistant *Escherichia coli* clinical isolate obtained from a urine sample from a patient in Nepal. NDM-12 differed from NDM-1 by two amino acid substitutions (M154L and G222D). The enzymatic activities of NDM-12 against β-lactams were similar to those of NDM-1, although NDM-12 showed lower k_{cat}/K_m ratios for all β-lactams tested except doripenem. The bla_{NDM-12} gene was located in a plasmid of 160 kb.

Metallo-β-lactamases (MBLs) usually confer reduced susceptibility to carbapenems, cephalosporins, and penicillins but not monobactams (1). Acquired MBLs are produced by Gram-negative bacteria, including *Acinetobacer* spp., *Pseudomonas aeruginosa*, and several *Enterobacteriaceae* (1). MBLs are categorized by their amino acid sequences into various types (2–4), including AIM (5), DIM (6), FIM (7), GIM (8), IMPs (9), KHM (10), NDMs (11), SMB (12), SIM (13), SPM (14), TMBs (15), and VIMs (16). The most prevalent types of MBLs are IMP-, VIM-, and NDM-type enzymes (1, 2, 17). NDM-1 was initially isolated from *Klebsiella pneumoniae* and *Escherichia coli* in 2008 in Sweden (11). Subsequently, at least 11 NDM variants (www.lahey.org/studies) have been reported in several countries (4, 18–29).

This study was ethically reviewed and approved by the Institutional Review Board of the Institute of Medicine at Tribhuvan University (reference 6-11-E) and the Biosafety Committee at the National Center for Global Health and Medicine (approval no. 26-D-088 and 26-D-089).

E. coli IOMTU388.1 was isolated from a urine sample obtained from a patient in 2013 in a university hospital in Nepal. The isolate was phenotypically identified, and the species identification was confirmed by 16S rRNA sequencing (30). *E. coli* DH5α (TaKaRa Bio, Shiga, Japan) and *E. coli* BL21-CodonPlus(DE3)-RIP (Agilent Technologies, Santa Clara, CA) were used as hosts for recombinant plasmids and for expression of bla_{NDM-1} and bla_{NDM-12}, respectively.

MICs were determined using the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (31). The MICs of β-lactams for *E. coli* IOMTU388.1 are shown in Table 1, and the MICs of other antibiotics were as follows: amikacin, >1,024 μg/ml; arbekacin, >1,024 μg/ml; ciprofloxacin, 128 μg/ml; colistin, ≤0.125 μg/ml; fosfomycin, 8 μg/ml; gentamicin, >1,024 μg/ml; kanamycin, >1,024 μg/ml; levofloxacin, 32 μg/ml; minocycline, 8 μg/ml; tigecycline, ≤0.125 μg/ml; and tobramycin, >1,024 μg/ml. PCR analysis was performed to detect the MBL genes bla_{DIM}, bla_{GIM}, bla_{IMP}, bla_{NDM}, bla_{SIM}, bla_{SPM}, and bla_{VIM} (32, 33). The isolate was PCR positive for bla_{NDM} but negative for the other MBL genes tested. The DNA sequence of the PCR product revealed that the isolate had bla_{NDM-12}. Multilocus sequence typing (MLST) of IOMTU388.1 typed it as ST635 (*E. coli* MLST Database; http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html). bla_{NDM-1} obtained from *P. aeruginosa* IOMTU9 (29) was used as a reference gene.

The bla_{NDM-12} sequence had 2 amino acid substitutions (M154L and G222D) compared with bla_{NDM-1} (accession no. JF798502) and one substitution (G222D) compared with NDM-4 (accession no. JQ348841).

The bla_{NDM-1} and bla_{NDM-12} genes were cloned into the corresponding sites of pHSG398 (TaKaRa, Shiga, Japan) using the primer set EcoRI-NDM-F (5′-GGGAATTCATGGAATTGCCCAATATTATG-3′) and PstI-NDM-R (5′-AACTGCAGTCAGCGCAGCTTGTCGGCCAT-3′). *E. coli* DH5α was transformed with pHSG398-NDM-1 or pHSG398-NDM-12.

The open reading frames of NDM-1 and NDM-12 without signal peptide regions were cloned into the pET28a expression vector (Novagen, Inc., Madison, WI) using the primer set BamHI-TEV-NDM-F (5′-ATGGATCCGAAAACCTGTATTTCCAAGGCCAGCAAATGGAAACTGGCGAC-3′) and XhoI-NDM-R (5′-ATCTCGAGTCAGCGCAGCTTGTCGGCCATG-3′). The resulting plasmids were transformed into *E. coli* BL21-CodonPlus(DE3)-RIP (Agilent Technologies, Santa Clara, CA). Both recombinant NDM-1 and NDM-12 were purified simultaneously using Ni-nitrilotriacetic acid (NTA) agarose according to the manufacturer's instruction (Qiagen, Hilden, Germany). His tags were removed by digestion with TurboTEV protease (Accelagen, San Diego, CA) and untagged proteins were purified by an additional passage over the Ni-NTA agarose. The purities of NDM-1 and NDM-12, which were estimated by SDS-PAGE, were greater than 90%. During the purification procedure, the presence of β-lactamase activity was monitored using nitrocefin (Oxoid, Ltd., Basingstoke, United Kingdom). Initial hydrolysis rates were determined in 50 mM Tris-HCl buffer (pH 7.4) containing 0.3 M NaCl and 5 μM $Zn(NO_3)_2$ at 37°C, using a UV-visible spectrophotometer (V-530; Jasco, Tokyo, Japan). The K_m and k_{cat} values and the k_{cat}/K_m ratio were determined by analyzing β-lactam hydrolysis with a Lineweaver-Burk plot. Wavelengths and extinction coefficients for

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TABLE 1 MICs of various β-lactams for *E. coli* IOMTU388.1 and *E. coli* DH5α transformed with plasmids encoding NDM-12 or NDM-1

	MIC (μg/ml) for strain:			
Antibiotic(s)	IOMTU388.1	DH5α(pHSG398/NDM-1)	DH5α(pHSG398/NDM-12)	DH5α(pHSG398)
Ampicillin	>1,024	256	512	4
Ampicillin-sulbactam	>1,024	128	128	2
Aztreonam	64	≤0.063	≤0.063	≤0.063
Cefepime	512	0.5	1	≤0.063
Cefoselis	1,024	16	8	1
Cefotaxime	>1,024	8	16	≤0.063
Cefoxitin	>1,024	64	16	≤0.063
Cefpirome	512	2	2	≤0.063
Ceftazidime	>1,024	256	256	≤0.063
Ceftriaxone	>1,024	16	16	≤0.063
Cefradine	>1,024	512	256	16
Doripenem	32	0.063	0.063	≤0.063
Imipenem	16	0.5	0.25	≤0.063
Meropenem	64	0.25	0.125	≤0.063
Moxalactam	>1,024	16	4	0.125
Penicillin G	>1,024	256	256	32

β-lactam substrates have been reported previously (34–36). The K_m and k_{cat} values (means ± standard deviations) were obtained from three individual experiments. The enzymatic activities of NDM-1 were measured in parallel with those of NDM-12.

The plasmid harboring bla_{NDM-12} was extracted (37) and sequenced using MiSeq (Illumina, San Diego, CA). The size of the plasmid harboring bla_{NDM-12} was determined using pulsed-field gel electrophoresis (PFGE) and Southern hybridization. A probe for bla_{NDM-12} from IOMTU388.1 was amplified by PCR using the primer sets for EcoRI-NDM-F and PstI-NDM-R. Signal detection was carried out using the digoxigenin (DIG) High Prime DNA labeling and detection starter kit II (Roche Applied Science, Indianapolis, IN).

Mating-out assays between the parental strain IOMTU388.1 and the chloramphenicol-resistant *E. coli* strain BL21 were performed in LB broth using a 1:4 donor/recipient ratio for 3 h at 37°C. Transconjugants were selected on Muller-Hinton agar plates containing ceftazidime (100 μg/ml) and chloramphenicol (30 μg/ml). Selected transconjugants harboring bla_{NDM-12} were confirmed by PCR with the primer set EcoRI-NDM-F and PstI-NDM-R.

E. coli DH5α harboring bla_{NDM-1} or bla_{NDM-12} showed reduced susceptibility to moxalactam and all penicillins, cephalosporins, and carbapenems tested compared with DH5α harboring a vector control (Table 1). The MICs of the β-lactams cefoxitin and moxalactam for DH5α harboring bla_{NDM-12} were 4-fold less than those for DH5α harboring bla_{NDM-1} (Table 1).

As shown in Table 2, recombinant NDM-1 and NDM-12 hydrolyzed all β-lactams tested except for aztreonam. The profiles of enzymatic activities of NDM-12 against β-lactams tested were similar to those of NDM-1, although NDM-12 had lower k_{cat}/K_m ratios for all β-lactams tested except for doripenem. The lower k_{cat}/K_m ratios were likely to be caused by the lower k_{cat} values of NDM-12 compared with those of NDM-1, as the values of NDM-12 were 11.4 to 73.6% of those of NDM-1 (Table 2). The profiles of enzymatic activities of NDM-1 except for cefoxitin were similar to those of NDM-1 that we reported previously (29). The k_{cat}/K_m ratio for cefoxitin in Table 2 was 10-fold higher than that

TABLE 2 Kinetic parameters of the NDM-1 and NDM-12 enzymes[a]

	NDM-1[b]			NDM-12[b]		
β-Lactam	K_m (μM)	k_{cat} (s^{-1})[b]	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})[b]	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
Ampicillin	231 ± 33	249 ± 22	1.1	126 ± 4	136 ± 2	1.1
Aztreonam	NH[c]	NH	NH	NH	NH	NH
Cefepime	162 ± 7	31 ± 1	0.19	103 ± 6	11.1 ± 0.2	0.11
Cefotaxime	102 ± 16	137 ± 7	1.1	45 ± 4	38 ± 1	0.84
Cefoxitin	13 ± 1	6.7 ± 0.1	0.50	26 ± 2	0.66 ± 0.01	0.02
Ceftazidime	202 ± 7	56 ± 1	0.28	53 ± 4	5.7 ± 0.1	0.11
Cefradine	27 ± 3	72 ± 1	2.7	57 ± 4	16 ± 1	0.28
Doripenem	201 ± 27	114 ± 9	0.57	88 ± 2	53 ± 1	0.60
Imipenem	249 ± 43	44 ± 2	0.34	125 ± 22	22 ± 2	0.18
Meropenem	81 ± 10	139 ± 10	1.7	91 ± 8	53 ± 2	0.58
Moxalactam	4.5 ± 2.3	7.6 ± 0.3	2.0	67 ± 5	6.0 ± 0.2	0.09
Penicillin G	67 ± 6	104 ± 1	1.6	64 ± 8	42 ± 2	0.66

[a] The proteins were initially modified by a His tag, which was removed after purification.
[b] The K_m and k_{cat} values shown represent the means from 3 independent experiments ± standard deviations.
[c] NH, no hydrolysis was detected under conditions with substrate concentrations up to 1 mM and enzyme concentrations up to 700 nM.

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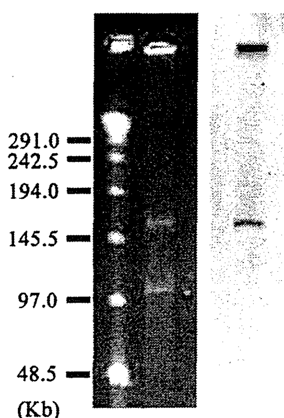

FIG 1 Localization of bla_{NDM-12} on a plasmid from *E. coli* strain IOMTU388.1 separated by PFGE. Left lane, MidRange PFG marker (New England BioLabs, Tokyo, Japan); middle lane, plasmids from *E. coli* strain IOMTU388.1; right lane, hybridization of the plasmid with a probe specific for bla_{NDM-12}.

in our previous study (see Table 2 in reference 29). The difference between the ratios may be explained by the use of different buffer solutions in the kinetics assays (Tris buffer and phosphate buffer, respectively). It was reported that phosphate ions affected the enzymatic activities of metallo-β-lactamase IMP-1 (38). Phosphate ions may affect the enzymatic activities of NDM-1 against cefoxitin.

The MBL gene bla_{NDM-12} in *E. coli* IOMTU388.1 was detected in a plasmid, pIOMTU388-NDM (accession no. AB926431), with a size of 160 kb (Fig. 1). The sequence surrounding bla_{NDM-12} was *bla_{NDM-12}-ble_{MBL}-trpF-dsbC-tnpA-sulI-qacEΔI*. This plasmid showed more than 99.9% identity at the nucleotide sequence level to the sequence located from bp 70978 to 77904 in the pGUE-NDM plasmid (accession no. JQ364967) from *E. coli* strain GUE, which was isolated in India (39), and also showed 99.9% identity at the nucleotide sequence level to the sequence located from bp 372 to 7298 in the pEC77-NDM plasmid (accession no.AB898038) from *E. coli* strain NCGM77, which was isolated in Japan (40). The plasmid harboring bla_{NDM-12} belonged to the IncF incompatibility group and was conjugated from IOMTU388.1 to *E. coli* BL21 at a conjugative frequency of 1.63×10^{-3}.

The 2 substitutions M154L and G222D in NDM-12 (compared with NDM-1) affected the activity of the enzyme (Table 2). Nordmann et al. (24) reported that a mutant containing M154L (NDM-4) possessed increased hydrolytic activity toward carbapenems and several cephalosporins compared to NDM-1. Unexpectedly, NDM-12, which contains the M154L substitution, did not show an increase in hydrolytic activities. The substitution at position 222 found in NDM-12 has been not reported in other variants, to our knowledge. Although we did not directly compare the enzymatic activity of NDM-12 with those of NDM-4, the substitution of G222D in NDM-12 may be associated with a decrease in hydrolytic activities toward these antibiotics (Table 2). Position 222 is located in loop L10 of NDM-1, which forms the active site of NDM-1 with L3 at the bottom of a shallow groove (41–44). Among all known 11 NDM-1 variants, amino acid substitutions were found at 13 amino acid positions, including positions 28, 32, 36, 69, 74, 88, 95, 130, 152, 154, 200, and 233. Positions 28, 32, and 36 were in the signal peptide region. Positions 95, 130, and 154 have been reported to affect β-lactam-hydrolyzing activities, although whether the activities are affected by the other 6 substitutions has not been reported. Residue 95 is located in α1 on the protein surface, and the amino acid substitution at position 95 affected the k_{cat} values of NDM-3 (40). The substitution at position 130 (Met to Leu) showed increased hydrolytic activity toward carbapenems and several cephalosporins compared to NDM-1 (24, 29).

This is the first report describing NDM-12-producing *E. coli* in Nepal. NDMs seem to evolve rapidly; therefore, careful monitoring of NDM-producing pathogens is required.

Nucleotide sequence accession number. The plasmid sequence including bla_{NDM-12} has been deposited in GenBank under accession no. AB926431.

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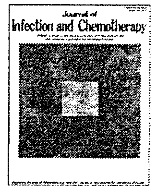

Case report

Multidrug-resistant *Acinetobactor baumannii* isolated from a traveler returned from Brunei

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ABSTRACT

We report a case of multidrug-resistant (MDR) *Acinetobactor baumannii* isolates obtained from a traveler returned from Brunei. Whole-genome sequencing analysis revealed that the isolates harbored bla_{OXA-23} and *armA*. The minimum inhibitory concentrations of antibiotics against the strain were as follows: imipenem, 32 μg/ml; meropenem, 32 μg/ml; ciprofloxacin, 16 μg/ml; amikacin, ≧1024 μg/ml; arbekacin, ≧1024 μg/ml; aztreonam, 64 μg/ml; colistin, 4 μg/ml. *A. baumannii* harboring both bla_{OXA-23} and *armA* is rarely reported in Japan, and, to the best of our knowledge, this is the second report of *A. baumannii* harboring both resistant genes in Japan.

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1. Introduction

Acinetobacter baumannii is an opportunistic pathogen of hospital-acquired infection, particularly of infections acquired in intensive care units, that is usually responsible for up to 10% of hospital-acquired infections and increases mortality up to 70% [1–4]. *A. baumannii* frequently acquires genes associated with drug resistance and pathogenicity, which gives it genetic diversity and overcomes antibiotic selection pressure [5].

The emergence of multidrug-resistant (MDR) *A. baumannii* resistant to carbapenem and aminoglycoside has become a serious problem, since carbapenem and aminoglycoside are important treatment options for *A. baumannii* infections [6]. Carbapenem-hydrolyzing class D β-lactamases (CHDLs) are considered the most prevalent cause of carbapenem resistance in *A. baumannii*; among CHDLs, OXA-23-like enzymes are the most prevalent and have been reported worldwide [6]. Acquired 16S rRNA methylase genes are responsible for high-level resistance to various aminoglycosides [7]. The 16S rRNA methylase encoding genes *armA* and *rmtC* are widely spread among various Gram-negative bacterial species, including *A. baumannii* [8]. Here, we report a case of MDR *A. baumannii* isolates harboring bla_{OXA-23} and *armA*. An clinical isolate of *A. baumannii* harboring both bla_{OXA-23} and *armA* has been reported in Japan up to now [9]. This is the second report of *A. baumannii* harboring both these resistant genes in Japan.

2. Case report

A 78-year-old Japanese man with diabetes mellitus and chronic obstructive pulmonary disease (COPD) went on a 1-month world cruise. During the previous year, he had not had any history of antimicrobial exposure. On the 15th day of his trip, he developed

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flu-like symptoms, including runny nose, sore throat, cough, joint pain, loss of appetite, and fatigue. His general condition deteriorated with prolonged fever over 38 °C, and he was therefore transferred to a hospital in Brunei on the 21st day of his trip. He was diagnosed with diffuse pneumonia and sepsis, and was admitted to the intensive care unit. Meropenem and azithromycin were administered. On the 4th day after his admission, he was intubated due to acute respiratory distress syndrome (ARDS). On day 16, he underwent tracheostomy. On day 21, MDR *A. baumannii* was isolated from the tracheostomy stoma site, and nasal, axillary, and groin areas. Intravenous colistin was started (details including doses unavailable). His clinical course was complicated with a catheter line-associated bloodstream *Candida tropicalis* infection, which was treated first with fluconazole and then with caspofungin. On day 66, a percutaneous endoscopic gastrostomy (PEG) tube was inserted for feeding. Ventilator weaning had been tried several times without success.

On day 89, the patient was transported to the National Center for Global Health and Medicine (NCGM) in Tokyo, Japan. Upon arrival, he was hemodynamically stable. He was on ventilatory support and bed bound. A chest X-ray revealed bilateral pleural effusion. Due to his prior admission to an ICU abroad, he was considered to be at risk for carrying antimicrobial-resistant organisms. He was placed in a single room on contact precaution. A culture of the tracheostomy stoma site was positive for MDR *A. baumannii*, which is resistant to all classes of antibiotics except minocycline (Table 1).

On day 11 after the patient's arrival, he developed pneumonia due to *Klebsiella pneumoniae* that was sensitive to various classes of antibiotics. Piperacillin and tazobactam were administered, and the pleural effusion was drained. His respiratory condition improved gradually, and the drain tubes were removed but ventilator weaning was never successful. The patient therefore remained in our hospital for 7 months. During his stay at our hospital, he developed ventilator-associated pneumonia due to *A. baumannii* twice, and was successfully treated with colistin and minocycline for 13 and 22 days, respectively. During the first episode of MDR *A. baumannii* infection, colistin (Colomycin® Injection) was started at a dose of 1,250,000 U, q12h, adjusted for the patient's creatinine clearance level (50 ml/min). As his renal dysfunction progressed, the colistin dose was reduced to 1,250,000 U q36h. During the second episode, the patient's renal function had further deteriorated (creatinine clearance, 40 ml/min) and his body weight had also further decreased; therefore colistin was administered at a dose of 400,000 U, q12h (20,000 U/kg/day). The patient was ultimately discharged to a nursing home with ventilatory support. During his hospitalization, he was placed in a private room. Strict infection control measures including contact precaution, emphasis on hand hygiene, and additional droplet precaution was applied when performing procedures involving respiratory droplets and secretions.

Table 1
In vitro susceptibilities of *A. baumannii* NCGM279.

Antibiotics	MIC (μg/ml)
Amikacin	≥1024
Arbekacin	≥1024
Aztreonam	64
Cefepime	64
Cefotaxime	1024
Cefoxitin	≥1024
Ceftazidime	512
Cephradine	≥1024
Ciprofloxacin	16
Colistin	4
Fosfomycin	512
Gentamicin	≥1024
Imipenem	32
Meropenem	32
Minocycline	≤2
Ofloxacin	8
Piperacillin	≥1024
Piperacillin-tazobactam	256
Tigecycline	0.25

We examined the minimum inhibitory concentrations (MICs) of various drugs against the MDR *A. baumannii* isolate (NCGM279) obtained from the patient's sputum. The MICs of amikacin, cefoxitin, ceftazidime, cephradine, ciprofloxacin, colistin, piperacillin, tigecycline (Sigma–Aldrich, St. Louis, MO, USA), arbekacin, fosfomycin (Meiji Seika Pharma, Tokyo, Japan), aztreonam (Eisai, Tokyo, Japan), cefepime (Bristol-Myers Squibb, New York, NY, USA), cefotaxime (Chugai Pharmaceutical, Tokyo, Japan), gentamicin (Nacalai Tesque, Kyoto, Japan), imipenem (Banyu Pharmaceutical, Tokyo, Japan), meropenem (Sumitomo Pharma, Osaka, Japan), minocycline (Pfizer Pharmaceutical Co., Tokyo, Japan), ofloxacin (Daiichi Sankyo Pharmaceutical Co., Tokyo, Japan), and piperacillin-tazobactam (Toyama Pure Chemical Industries, Tokyo, Japan) were determined using the microdilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [10]. The MICs of these drugs against NCGM279 are shown in the Table 1. *A baumannii* NCGM279 was found to be resistant to all antibiotics tested except for minocycline and tigecycline. The CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) have not yet determined a breakpoint for tigecylcine to *A. baumannii*. An epidemiological cut-off value for tigecycline to *A. baumannii* was 1 mg/L as defined by EUCAST. NCGM279 was not likely to be resistant to tigecycline.

The entire genome of NCGM279 was sequenced by Illumina GAIIx (Illumina, San Diego, CA, USA). We obtained 1,455,378 reads and 4,257,140 bp from 989 contigs in NCGM279. The multilocus sequence types (MLSTs) according to the *A. baumannii* MLST Database website (http://pubmlst.org/abaumannii/), the β-lactamase genes and the aminoglycoside-resistance genes were determined using the entire genome data. The NCGM279 belonged to ST208. Whole-genome analysis revealed that NCGM279 had β-lactamase genes bla_{TEM-1}, bla_{ADC}, bla_{OXA-23}, and bla_{OXA-66}; and aminoglycoside-resistance genes *aac(6′)-Ib*, *aadA1* and *armA*. NCGM279 had point mutations causing amino acid substitutions of Ser-83 to Leu in *gyr*A and Ser-80 to Leu in *par*C, but had no point mutation in *gyr*B, whose mutations were associated with resistance to fluoroquinolnes [11–14].

3. Discussion

The optimum therapeutic choices for MDR *A. baumannii* infections still require clarification by future research; we used a combination therapy of minocycline and colistin in this patient [15].

The patient in this case had traveled overseas and received medical care in Brunei. He had never received medical care in any other foreign country prior to this episode. Thus, it is likely that this patient acquired MDR *A. baumannii* during his stay in Brunei. Receiving medical care abroad has been suggested as a potential route of transmission of MDR organisms [16,17]. The sequence type of *A. baumannii* isolated in this study was ST208, which often carries bla_{OXA-23} and appears to be an emerging lineage mediating the spread of carbapenem resistance [18]. Gram-negative pathogens harboring both bla_{OXA-23} and *armA* have been reported in Bulgaria [19], France [20], India [21], Korea [22], Norway [23] and the United States of America [24]. Patients who have been hospitalized abroad have a potential risk of carrying MDR bacterial

species. Screening for drug-resistant organisms should be performed upon the hospital admission of patients transferred from foreign countries.

Conflict of interest

None to declare.

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Evaluation of an Automated Rapid Diagnostic Test for Detection of *Clostridium difficile*

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Abstract

The Verigene *Clostridium difficile* Nucleic Acid Test (Verigene CDF Test) (Nanosphere, Northbrook, IL, USA) is a new multiplex qualitative polymerase chain reaction (PCR) test used to detect *C. difficile* toxin genes in fecal specimens. To evaluate the performance of the new method, we tested 69 fecal samples from patients with suspected *C. difficile* infection using the Verigene CDF test, an enzyme immunoassay (EIA) and PCR following anaerobic fecal culture. The sensitivity, specificity, and accuracy of the Verigene CDF test were 96.7% (29/30), 97.4% (38/39), and 97.1% (67/69) respectively, using PCR following fecal culture as a reference method. We also analyzed the potential clinical impact of the Verigene CDF test using chart reviews of the 69 patients with suspected *C. difficile* infection and found that 11 of the 69 patients were incorrectly diagnosed, and the Verigene CDF test would have led to them receiving more appropriate management including practice of treatment and contact precaution, although, of the 69 patients, there are two whose samples were incorrectly identified with the Verigene CDF test. The Verigene CDF test will have a positive impact on patient care.

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Introduction

Clostridium difficile (*C. difficile*) infection is a serious problem in healthcare, with high incidence, mortality, and healthcare costs [1,2]. Accurate diagnosis is crucial to the overall management of this infection, including administration of appropriate treatment and the use of contact precautions to prevent nosocomial spread. For the diagnosis of *C. difficile* infection, various testing methods are available including enzyme immunoassays (EIAs), anaerobic fecal culture, cell culture cytotoxicity neutralization assays, and polymerase chain reaction (PCR). Although an EIA for *C. difficile* toxins A and B is easy to use and rapid, it is no longer recommended as a primary, stand-alone test due to its poor sensitivity [3,4].

Clinical practice guidelines for *C. difficile* suggested a two-step method that uses the EIA detection of glutamate dehydrogenase (GDH) as an initial screening followed by a cell cytotoxicity assay or toxigenic culture [3,4]. Anaerobic fecal culture is the most sensitive test and is essential for epidemiological studies, but it is not clinically practical because it does not provide timely results and has not been standardized. In Japan, a cell cytotoxicity assay, toxigenic culture, and PCR have not been introduced into routine laboratory procedures, and thus *C. difficile* infection is diagnosed mostly on the basis of EIA results and clinical symptoms.

There are now several FDA-approved assays of *C. difficile* available in the United States [5–12]. The Verigene *Clostridium difficile* Nucleic Acid Test (Verigene CDF Test) (Nanosphere, Northbrook, IL, USA) is a new molecular, qualitative multiplexed *in vitro* diagnostic sample-to-result test for the rapid detection of toxin A (*tcdA*) and toxin B (*tcdB*) gene sequences of toxigenic *C. difficile* from unformed (liquid or soft) fecal specimens collected from patients with suspected *C. difficile* infection. The Verigene CDF test also detects binary toxin gene sequences and the single base pair deletion at the *tcdC* gene for a presumptive identification of the epidemic *C. difficile* strain ribotype 027.

One recent study reported the comparative results regarding the performance of the Verigene CDF test with a cell culture cytotoxicity neutralization assay [12]. However, comparisons to other testing methods, such as EIAs and anaerobic fecal cultures, were not conducted, and PCR was performed only for the detection of the strain ribotype 027. The clinical impact of the Verigene CDF test was not analyzed. Here we evaluated the performance of the Verigene CDF test in comparison to those methods and its potential clinical impact, by conducting a retrospective chart review.

Materials and Methods

Study design and data collection

From August to October 2013 and May to June 2014, fecal samples were collected from patients with suspected *C. difficile* infection at the National Center for Global Health and Medicine

Table 1. Comparison of the results of an EIA and PCR following fecal culture for detecting *C. difficile* in fecal specimens.

			PCR following fecal culture[c]	
			tcdA+, tcdB+	*tcdA-, tcdB-*[d]
EIA[a]	GDH+ (toxin+)	31 (13)[b]	28[e] (13)[f]	3 (0)
	GDH-	38	2	36
Total		69	30	39

[a]The EIA detects *C. difficile* antigen (GDH) and *C. difficile* toxin (*tcdA* and/or *tcdB*).
[b]Of the 31 GDH-positive samples, 13 were toxin-positive in the EIA.
[c]According to the results of the EIA, the numbers of toxin-positive or -negative samples with PCR following fecal culture are shown.
[d]Numbers of samples negative with either fecal culture or PCR.
[e]Of the 31 GDH-positive samples, 28 were *tcdA*- and *tcdB*-positive with PCR following fecal culture.
[f]All 13 samples that were GDH- and toxin-positive with EIA were *tcdA*- and *tcdB*-positive with PCR following fecal culture.
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(NCGM), which also serves as a tertiary hospital with 801 inpatient beds. When fecal samples were collected, all of them were tested using the EIA assay "C. Diff Quik Chek Complete" kit (TechLab, Blacksburg, VA), which detects *C. difficile* antigen (GDH) and *C. difficile* toxins (*tcdA* and/or *tcdB*).

This study included all GDH-positive and four GDH-negative samples from August 1st to October 22th 2013 (defined as the first period), and all samples submitted from May 20th to June 5th 2014 (defined as the second period), without knowledge of the patients' clinical information. A total of 400 samples were submitted during the first period. The samples analyzed during the first period were 27 GDH-positive and four GDH-negative samples, and the samples submitted and analyzed during the second period were 38 samples. The EIA-results of the 38 samples were four GDH-positive and 34 GDH-negative. The total of 69 samples were tested in the study. Of the 69 samples, 62 samples were tested within 24 hours of sample collection, and the other seven samples were tested within 4 days. All specimens were kept at 4°C until tested. A single fecal sample per patient was included in the study.

All samples were cultured anaerobically on cycloserine-cefoxitin-mannitol agar (Nissui Pharmaceutical Co., Tokyo, Japan) for the isolation of *C. difficile*. Cycloserine-cefoxitin mannitol broth with taurocholate lysozyme cysteine (CCMB-TAL broth, Anaerobe Systems, Morgan Hill, CA) for enrichment and cycloserine-cefoxitin fructose agar with horse blood and taurocholate (CCFA-HT, Anaerobe Systems) for subculture were also used during the second period. If *C. difficile* was isolated, the presence of the *tcdA*, *tcdB* and binary toxin gene was examined by PCR as described [13,14]. PCR following the fecal culture mentioned above was performed as a reference method. The laboratory staff performing the Verigene CDF test were not aware of the results of the PCR following the fecal culture at the time of testing. Information about the administration of therapeutic antibiotics (i.e., metronidazole or oral vancomycin), the use of contact precautions and previous history of *C. difficile* infection was retrieved from patient records after these assays were performed.

Verigene system

The Verigene system is a bench-top sample-to result platform molecular diagnostics workstation consisting of two modules: the Verigene Processor *SP* and the Verigene Reader. The user loads a specimen into the Verigene Processor *SP*, and the Verigene Processor *SP* automates the sample analysis steps including DNA extraction, PCR-based amplification, and hybridization. After the procedure, the result of the test is reported in the Verigene Reader.

Statistical analysis

The sensitivity, specificity, and accuracy of the Verigene CDF test were examined, and the 95% confidence intervals (CIs) of them were calculated using R Software (http://www.r-project.org/). We performed a post hoc analysis of our sample size and calculated the kappa coefficient between the Verigene CDF test and the reference method, using modified R software programs [15].

Ethical considerations

The study protocol was carefully reviewed and approved by the NCGM Ethics Committee (No. 1425). Individual informed consent was waived by the ethics committee because this study used currently existing samples collected during the course of routine medical care and did not pose any additional risks to the patients.

The tests except EIA in the study including the Verigene CDF test are not currently approved for standard clinical procedure by Japanese government and not ethically permitted for clinical diagnosis. Thus, we did not inform the clinicians of the results obtained from the methods except EIA.

Results

Comparison of EIA and PCR following fecal culture

Of the 31 GDH-positive samples, 13 were shown to be toxin-positive by the EIA. The PCR following fecal culture showed that, of the 31 samples, 28 were both *tcdA*- and *tcdB*-positive (Table 1). Of the 38 GDH-negative samples, there were two *tcdA*- and *tcdB*-positive samples by PCR following fecal culture (Table 1). Finally, of all the 69 samples, *tcdA*- and *tcdB*-positive and *tcdA*- and *tcdB*-negative *C. difficile* samples were 30 and 39, respectively (Table 1). As expected, the PCR following fecal culture was more sensitive than the EIA for detecting toxigenic *C. difficile* isolates.

Comparison of PCR following fecal culture and the Verigene CDF test

Next, we compared the results of the Verigene CDF test with those obtained with PCR following fecal culture (i.e., the reference method). Of the 30 *tcdA*- and *tcdB*-positive samples, 29 were correctly detected with the Verigene CDF test and counted as true positive. The remaining one sample was not detected with the Verigene CDF test and counted as false negative. Similarly, Of the 39 *tcdA*- and *tcdB*-negative samples, 38 and one were recognized as true negative and false positive with the Verigene CDF test, respectively. The sensitivity, specificity, and accuracy of the

Table 2. Performance Characteristics of the Verigene CDF test compared with the PCR following fecal culture.

	True Positive	False Negative	True Negative	False Positive	Sensitivity (95% CI[a])	Specificity (95% CI[a])	Accuracy (95% CI[a])
Verigene CDF test	29	1	38	1	96.7% (82.8–99.9)	97.4% (86.5–99.9)	97.1% (89.9–99.6)

[a]CI: confidence interval.

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Verigene CDF test were 96.7% (29/30), 97.4% (38/39), and 97.1% (67/69), respectively (Table 2). A binary toxin-positive sample was detected by the PCR following fecal culture but not by the Verigene CDF test, and this result was counted as false negative. A post hoc power analysis using our sample size showed that the statistical power (1 – type II error rate) was high enough (>80%) to generate 95% CI width of 10% for an expected accuracy 97%. The kappa coefficient was calculated to assess the diagnostic accuracy of the CDF test compared with the PCR following fecal culture as the reference method. The score was 0.94, and this result indicated almost perfect agreement between these two methods [16].

Evaluation of clinical utility

To evaluate the potential clinical significance of the Verigene CDF test, we performed a retrospective chart review of the 69 patients whose samples were tested in the study. According to the institution's routine practices, the management of the 69 patients was determined based on the results of EIA testing and the pretest probability of *C. difficile* infection. According to the results of the Verigene CDF test and PCR following fecal culture (Table 2), 30 of the 69 patients were infected with *C. difficile* harboring *tcdA*+ *tcdB*+, and 39 patients were not. The chart reviews revealed that contact precaution was used for 20 of the 30 infected patients and for one of the 39 non-infected patients. Treatments for *C. difficile* infection (metronidazole or oral vancomycin) were administered for these 20 of the 30 infected and one of the 39 non-infected patients. The former 20 patients included all 13 toxin-positive patients by EIA shown in Table 1. The EIA-result of the latter one patient was GDH-negative and toxin-negative. Of the 30 infected patients, 10 seemed not to be recognized as having *C. difficile* infection or being a carrier, while, of the 39 non-infected patients, one seemed to be recognized as having *C. difficile* infection (Table 3). None of the former 10 patients had been diagnosed previously with a *C. difficile* infection. The 10 patients included two who had been accommodated in a private room before the EIA but were not started on contact precaution after the EIA. Whereas, there are two patients whose samples were recognized as false positive or negative with the Verigene CDF test. Both of them received appropriate management independently of the false results with the Verigene CDF test.

Discussion

In this study, we evaluated the performance of the Verigene CDF test compared with other methods and we analyzed the potential clinical impact of the test by chart reviews. As reported [4], EIA was highly sensitive for detecting *C. difficile* antigen (GDH), but insensitive for detecting *C. difficile* toxins.

The Verigene CDF test showed high sensitivity, specificity, and accuracy, using the PCR following fecal culture as a reference method. A recent study showed that the concordance rates between the Verigene CDF test and a direct culture method or the Verigene CDF test and an enriched culture method were 88.4% and 92.3%, respectively [12]. The better performance of the Verigene CDF test observed in our study may be due to differences between the reference methods and/or the relatively small number of patients in the study, though the range of 95% CI in our accuracy data was less than 10% and contained the results shown in the previous report. We are now planning a large-scale prospective clinical evaluation of the Verigene CDF test.

Retrospective chart reviews revealed that 20 of the 30 infected patients and one of the 39 non-infected patients did not receive appropriate management. The exact reasons were unclear, but the

Table 3. The number of case identified or not identified as *C. difficile* infection.

	Idetntified as *C. difficile* infection	Not idetntified as *C. difficile* infection	Total
tcdA+, tcdB+	20[a]	10	30
tcdA-, tcdB-	1	38	39

[a]The 20 cases included 10 cases that were toxin-positive by the EIA (Table 1).
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patient management may have been decided based on the results of an EIA and individual situations. The management of patients with suspected *C. difficile* infection depends mainly on the results of an EIA and each physician's judgment in a country like Japan, where other testing methods are not available or are time-consuming.

Because most of the GDH-positive patients in our study had toxigenic *C. difficile* isolates, a GDH-positive but toxin-negative patient should be followed up with additional testing for the appropriate management of *C. difficile* infection, including appropriate treatment and contact precaution. While *C. difficile* infection can spontaneously resolve solely by discontinuing antibiotics [17], this patient population may include asymptomatic carriers. It is important that contact precaution is used for these patients at the optimal timing, because they serve as a potential reservoir for environmental contamination to other hospitalized patients [18].

Several methods to recover *C. difficile* from stool samples was evaluated to determine sensitive method [19]. During the second period, we also used CCMB-TAL for enrichment and CCFA-HT for subculture, which were more sensitive than CCMA in recovering *C. difficile* from stool samples. Of all samples submitted during the second period, one sample was isolated with only CCMB-TAL and CCFA-HT, not with CCMA (data not shown). During the first period, three samples of five *tcdA*- and *tcdB*-negative were culture-negative with CCMA, and we cannot deny that some of them might have been culture-positive with CCMB-TAL and CCFA-HT.

PCR following fecal culture as our reference method was time-consuming and used mainly for confirmation of the diagnosis of *C. difficile*, and thus it may be difficult to evaluate exactly the potential clinical impact of the Verigene CDF test compared to our reference method. However, the Verigene CDF test has some strengths, such as its simple and quick procedures which do not require trained laboratory personnel. This test could be an appropriate alternative to PCR for detecting *C. difficile*, especially in countries where PCR has not been introduced into routine laboratory procedures.

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Author Contributions

Conceived and designed the experiments: MT KH NO. Performed the experiments: MT MN. Analyzed the data: MT KH NO. Contributed reagents/materials/analysis tools: KM TK. Wrote the paper: MT TK NO.

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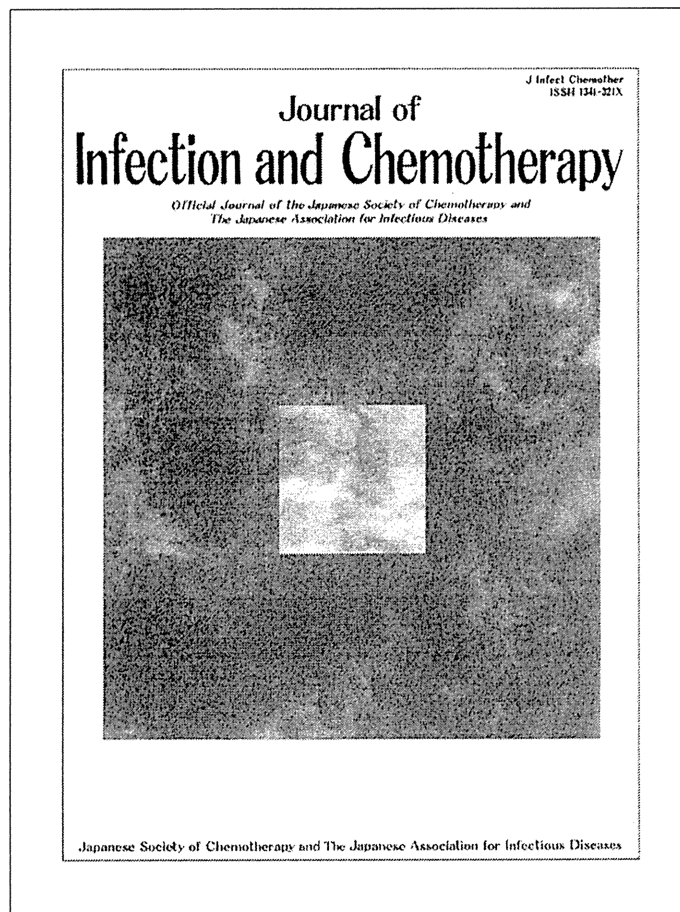

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