別記様式第６号の１

臨床研究等実施決定通知書

平成27年11月19日

研究責任者
荒川 宜親 殿

審 査 区 分 ： 一般審査

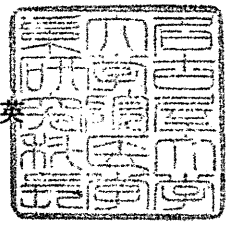
課 題 名 ： 在宅医療患者等における多剤耐性菌の分離率及び分子疫学解析
Isolation frequency and molecular epidemiology of multi-drug-resistant microbes colonizing in patients receiving healthcare services at home or nursing facilities

受 付 番 号 ： 6660

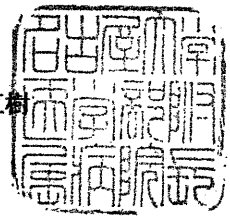
申 請 日 ： 平成27年09月08日

研 究 期 間 ： 開始：2015年11月19日
終了：2018年03月31日

名古屋大学大学院医学系研究科長
（本研究の実施に係る機関の長）
高橋 雅英



名古屋大学医学部附属病院長
石黒 直樹



あなたの申請された上記研究課題の実施について，下記のとおり決定しましたので，通知します。

記

①. 承認 （承認番号：2015-0304）

２．条件付承認

３．不承認

以上

研究の概要(説明会用メモ)　　2015年12月19日　荒川宜親

研究課題:「在宅医療患者等における多剤耐性菌の分離率及び分子疫学解析」

研究費の出所:厚生労働省(厚生労働科学研究費補助金)
研究の期間:平成27年度から平成29年度(2018年3月末)

研究の目的:急性期疾患医療施設において近年様々な多剤耐性菌が問題となっているが、在宅患者様や療養型施設等の入所者様における多剤耐性菌の実態が不明であり、行政的に調査が必要となりました。

研究の内容:在宅患者様や療養型施設等の入所者様より、承諾を頂いた上で、咽頭粘液、便、尿、褥瘡膿などより、以下の7種類の多剤耐性菌の分離を試み、その分離率や遺伝子型などを解析いたします。
1. MRSA, 2. VRE, 3. PR(I)SP, 4. MDRP, 5. MDRA, 6. ESBL産生菌, 7. CRE

倫理上の配慮:被験者になって頂く方は、無作為に選んで頂きます。連結不可能匿名化によりデータ解析を行います。
インフォームド・コンセントの手続きを踏む。
＊対象者が決まった段階で、本人または代理の人から、同意書を頂きます。本人からインフォームド・コンセントが得られていない場合も、咽頭スワブの採取など、一定の不快感を伴う材料採取については、インフォームド・アセントとして、検査の公共性(社会的役割)、予測される苦痛などについての説明をお願いします。

被験者の人数:1施設20名程度を考えていますが、施設規模や協力を申し出て頂ける方の数により、30名程度となることも想定しています。

経費:検体の採取および輸送などに必要な経費は、全て厚労省からの研究費で賄われます。

謝礼等:公的研究での調査研究のため、被験者の方々への謝礼などはお支払い致しません。

その他:この研究は、在宅、介護老人福祉施設、介護老人保健施設などにおける耐性菌分離の状況を把握するための研究であり、対象者の相当数は、インフォームド・コンセントの手続きを踏むことが困難であることが予測できます。そのような場合は、保護者等の代理による代諾でインフォームド・コンセントに代えますが、検体採取の際は、本人にも採取することの意味、行為を説明し、被検者の安心、平安、安全を保つように配慮をお願いします。

質問事項

1. 「厚生労働省の研究班による調査研究へのご協力のお願い」（院内掲示用）は、（院内掲示用）の記述を削除して、入所者家族等への「通信文」や「お知らせ」の中に、挟み込むなどして利用して良いか？
回答：構いません。ご自由にご活用下さい。

2. 検体を採取する期間はいつか？
回答：１月より順次始め、６月頃までに終えたい。その後は、分離された耐性菌株の解析を中心に研究を行います。

3. 検体を採取する回数は何回か？
回答：被験者１人あたり１回を考えている。

4. 尿が取りにくい被験者は、取らなくていいか？
回答：無理して取って頂かなくて構いません。

5. 咽頭スワブや褥瘡スワブは看護師が採取し、便や尿の採取は、介護職員にも手伝ってもらう事を考えているが、それで良いか？
回答：それで結構です。

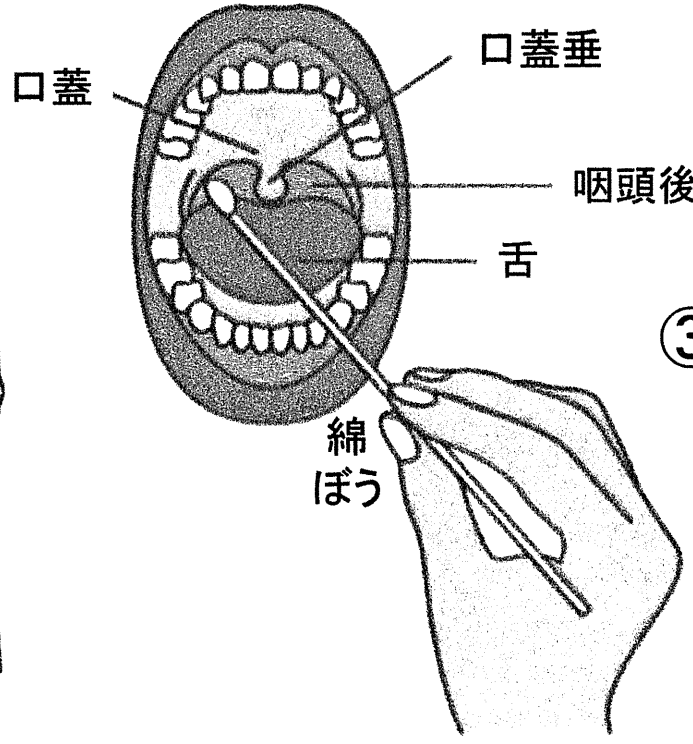
6. 検体を提出（送る）タイミングは、どうか？
回答：検体が２−３人分集まった時点でまとめて提出（送る）して頂ければと思います。
ただし、PRSPは死滅し易く、できるだけ早く検査に回した方が良いので、採取する予定が決まったら、前もって教えてください。
名古屋市内なら、直接受け取りに行くことも考えています。

7. 検体を取る具体的な方法は？
回答：現在、プロトコルを作成しているので、完成しましたら、検体採取用の資材とともに、提供させて頂きます。
検体の採取を開始していただく前に、再度、ご説明に伺う予定です。

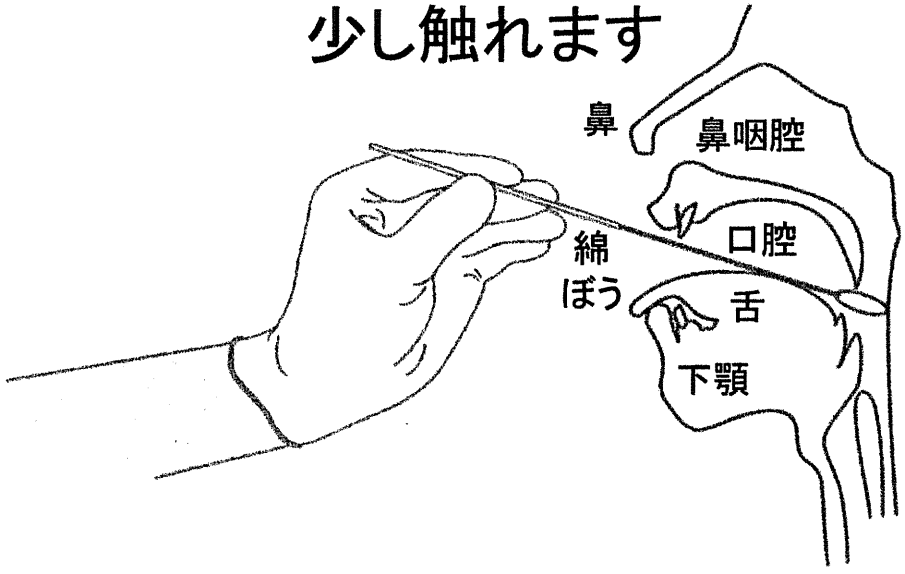
以下は、咽（のど）からの粘液を、綿ぼうで取らせて頂く手順です
（少し、“むずがゆい”かも知れませんが、痛くないです）

① 口をあけてください

口を大きくあけてください
（5秒以内におわります。）



② 綿ぼうの先で、のどの奥に
少し触れます



③ はい
おわりです
（たいへん
ありがとう
ございました）

検体リスト　　提出日（　　年　　月　　日）

被験者氏名		検体番号	年齢	性別	検体名	検体採取日	特記事項 （採取者名等）	三ヶ月以内の	
								投薬歴	入院歴

研究課題(課題番号):在宅医療患者等における多剤耐性菌の分離率及び分子疫学解析(H26－医療－一般－012)
研究期間：平成27年　6月　1日から平成30年　3月　31日
研究組織：研究代表者：荒川　宜親（名古屋大学教授）
研究分担者：飯沼　由嗣（金沢医科大学教授）、川村　久美子（名古屋大学准教授）
村上　啓雄（岐阜大学教授）＜平成28年4月1日より参加予定＞
藤本　修平（東海大学教授）＜同上＞

【A. 研究の概要】

研究の背景：・近年、急性期医療現場のみならず市中でも、薬剤耐性菌の増加が進み、国際的に大きな問題となっている。
・大規模な公的医療機関における薬剤耐性菌の実体は概ね把握されている。
・しかし、在宅医療現場、療養施設、介護施設等での薬剤耐性菌の実体については不明な点が多い。

研究の目的：・在宅医療患者、療養施設、介護施設の入所者等における薬剤耐性菌の保菌状況を把握する。
・在宅医療患者、療養施設、介護施設の入所者等から分離された薬剤耐性菌の遺伝系統や耐性遺伝子の型別を行う。
・以上の解析情報を、行政が在宅医療現場、療養施設、介護施設等における耐性菌対策を検討する際に活用できるように提供する。

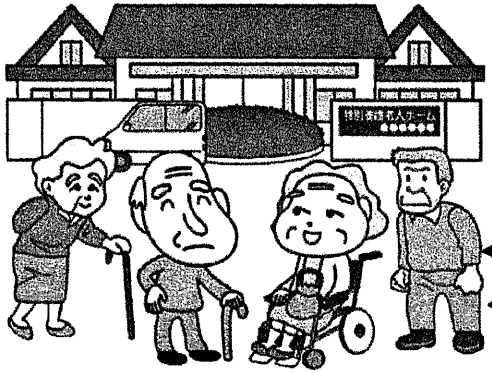
研究の方法：・研究を開始するに先立ち、その研究内容に関し、研究者が所属する機関により倫理審査を受け、承諾を得る。
・被験者は、在宅医療患者および療養施設、介護施設等に入所している方々、約200名とする。
・検査する検体は、咽頭スワブと便とし、可能な場合は、尿と褥瘡スワブを加える。
・検出する薬剤剤耐性菌は、以下の7種類とする。
①メチシリン耐性黄色ブドウ球菌(MRSA)、②バンコマイシン耐性腸球菌(VRE)、
③ペニシリン耐性肺炎球菌(PRSP)、④耐性緑膿菌(MDRP)、⑤多剤耐性アシネトバクター(MDRA)、
⑥カルバペネム耐性腸内細菌科細菌(CRE)、⑦基質特異性拡張型β-ラクタマーゼ(ESBL)産生菌

研究の成果：・在宅医療現場、療養施設、介護施設における薬剤耐性菌の分離率、分離状況を把握する。
・分離された薬剤耐性菌の細菌学的、遺伝学的特長を正確に把握する。

研究の効果：・急性期医療現場にみならず、療養施設や介護施設等における耐性菌対策の向上に資する。

【A. 研究の概要】在宅医療患者等における多剤耐性菌の分離率及び分子疫学解析

在宅医療現場、
療養施設、介護施設



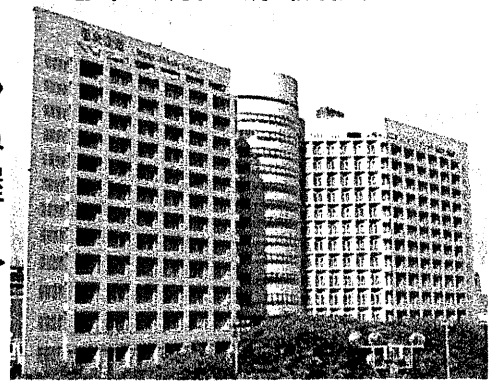
医療環境で実際に問題となっている薬剤耐性菌の実体

①メチシリン耐性黄色ブドウ球菌(MRSA)、
②バンコマイシン耐性腸球菌(VRE)、
③ペニシリン耐性肺炎球菌(PRSP)、
④多剤耐性緑膿菌(MDRP)、
⑤多剤耐性アシネトバクター(MDRA)、
⑥カルバペネム耐性腸内細菌科細菌(CRE)、
⑦基質特異性拡張型β-ラクタマーゼ(ESBL)産生菌

実体 殆ど不明

実体 概ね把握

大学附属病院
大規模公的病院等の
急性期医療機関



患者／入所者の移動に伴う薬剤耐性菌の伝播

入院や退院

日常診療の検査により
薬剤耐性菌の分離状況
などは概ね把握されている。

実態の調査

- 研究倫理審査と承認
- 説明会の開催、インフォームド・コンセントの実施
- 咽頭スワブ、便等の検体採取
- 7種類の主要な薬剤耐性菌株の分離と詳しい解析
- データの分析と整理

研究の成果

行政による

在宅医療現場、療養施設、介護施設
等における薬剤耐性菌対策の
立案と実施に資する、
学術的、科学的データ、
エビデンスの提供

研究の効果

国内における

・在宅医療現場、療養施設、介護施設
等における薬剤耐性菌の保菌率の低下

・急性期医療機関における各種
薬剤耐性菌による感染症患者の減少

を通じて、患者予後の向上

に貢献

参考資料

（健常者における ESBL 産生株保菌調査結果）

JCM
Journals.ASM.org

New PCR-Based Open Reading Frame Typing Method for Easy, Rapid, and Reliable Identification of *Acinetobacter baumannii* International Epidemic Clones without Performing Multilocus Sequence Typing

Masahiro Suzuki,[a,b] Eriko Hosoba,[c] Mari Matsui,[d] Yoshichika Arakawa[b]

Laboratory of Bacteriology, Aichi Prefectural Institute of Public Health, Nagoya, Japan[a]; Department of Bacteriology, Nagoya University Graduate School of Medicine, Nagoya, Japan[b]; Department of Infectious Diseases and Immunology, Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya, Japan[c]; Department of Bacteriology II, National Institute of Infectious Diseases, Tokyo, Japan[d]

Antimicrobial resistance issues have become a global health concern. The rapid identification of multidrug-resistant microbes, which depends on microbial genomic information, is essential for overcoming growing antimicrobial resistance challenges. However, genotyping methods, such as multilocus sequence typing (MLST), for identifying international epidemic clones of *Acinetobacter baumannii* are not easily performed as routine tests in ordinary clinical laboratories. In this study, we aimed to develop a novel genotyping method that can be performed in ordinary microbiology laboratories. Several open reading frames (ORFs) specific to certain bacterial genetic lineages or species, together with their unique distribution patterns on the chromosomes showing a good correlation with the results of MLST, were selected in *A. baumannii* and other *Acinetobacter* spp. by comparing their genomic data. The distribution patterns of the ORFs were visualized by agarose gel electrophoresis after multiplex PCR amplification and digitized. *A. baumannii* sequence types (STs) corresponding to international clones I and II were successfully discriminated from other STs and *Acinetobacter* species by detecting the distribution patterns of their ORFs using the multiplex PCR developed here. Since bacterial STs can be easily expressed as digitized numeric data with plus (+) expressed as 1 and minus (−) expressed as 0, the results of the method can be easily compared with those obtained by different tests or laboratories. This PCR-based ORF typing (POT) method can easily and rapidly identify international epidemic clones of *A. baumannii* and differentiate this microbe from other *Acinetobacter* spp. Since this POT method is easy enough to be performed even in ordinary clinical laboratories, it would also contribute to daily infection control measures and surveillance.

Antimicrobial resistance has become a global health concern. The World Health Organization has stated that weak or absent antimicrobial resistance surveillance and monitoring systems accelerate the emergence and spread of antimicrobial resistance (see http://www.who.int/drugresistance/documents/surveillancereport/en/). One of the weak points of current antimicrobial resistance surveillance and monitoring systems is the absence of genetic data for the bacterial isolates. Microbial genotyping is indispensable for a precise understanding of the genetic lineages of clinical isolates that cause nosocomial outbreaks (1).

Acinetobacter baumannii is one of the major multidrug-resistant nosocomial pathogens. In particular, *A. baumannii* epidemic clones, the so-called international clones I and II, usually show multidrug resistance, and only limited antimicrobials are efficacious for treating infections caused by them (2). On the other hand, *A. baumannii* clinical isolates other than the epidemic international clones are still susceptible to several antimicrobials. The performance of appropriate precautions that target the epidemic clones is indispensable for blocking their further nosocomial transmission. Therefore, it has become very important to rapidly discriminate the *A. baumannii* epidemic clones from other nonepidemic *A. baumannii* lineages and non-*baumannii Acinetobacter* species, such as *Acinetobacter nosocomialis* and *Acinetobacter pittii*. In this regard, multilocus sequence typing (MLST) is indeed useful for the exact identification of the epidemic clones, which are classified into several sequence types (STs), such as ST1 and ST2, by MLST performed at the Institut Pasteur. ST1 and ST2 are also assigned to clonal complex 109 (CC109) and CC92, respectively, by the MLST of Bartual et al. (19) as reported by Zarrilli et al. (3) However, MLST of *Acinetobacter* clinical isolates can be performed only in limited cases of nosocomial outbreaks even in Japan, and this results in a delay in the ability to alert for the emergence and spread of epidemic clones in hospital settings. Early identification of epidemic clones of *A. baumannii* is very important especially in the areas where they have not been prevalent yet. Therefore, the establishment of easy and rapid genotyping methods has been much awaited.

The construction of new analytical methods that make it easy to obtain genetic information of clinical isolates in ordinary clinical laboratories is desired. We consider that the most convenient way to simplify microbial genotyping would be to display the re-

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doi:10.1128/JCM.01064-14

TABLE 1 ORF candidates relating to small genomic islets (SGI) and their distributions among the *A. baumannii* genomes[a]

SGI ORF candidate[b]	ORF corresponding to the SGI ORF candidates among the indicated *A. baumannii* strain (Pasteur sequence type, GenBank accession no.)[c]			
	AB0057 (ST1, CP001182)	ACICU (ST2, CP000863)	ATCC17978 (ST77slv[d], CP000521)	SDF (ST17, CU468230)
ACICU_00180*		ACICU_00180 (100)	A1S_0157 (99)	
AB57_0388*	AB57_0388 (100)			
AB57_0454*	AB57_0454 (100)		Between A1S_0376[e] and A1S_0378 (99)	Between ABSDF3133 and ABSDF3134 (98)
AB57_0526	AB57_0526 (100)			Between ABSDF 3067 and ABSDF3068 (99)
ACICU_00563*		ACICU_00563 (100)		ABSDF2963 (100)
AB57_0815*#	AB57_0815 (100)		A1S_0767 (99)	
AB57_1987	AB57_1987 (100)	ACICU_01794 (99)		ABSDF1977 (98)
AB57_2085	AB57_2085 (100)		Between A1S_1754 and A1S_1755 (96)	
ACICU_01870*#		ACICU_01870 (100)	A1S_1782 (95)	ABSDF1960 (96)
ACICU_02042 *#		ACICU_02042 (100)	A1S_1927 (99)	
AB57_2484*#	AB57_2484 (100)	ACICU_02351 (98)		
ACICU_02468		ACICU_02468 (100)	A1S_2266 (99)	ABSDF1260 (97)
ACICU_02520*	Between AB57_2751 and AB57_2752 (99)	ACICU_02520 (100)	Between A1S_2318 and A1S_2319 (99)	
ACICU_02597		ACICU_02597 (100)		
AB57_2930	AB57_2930 (100)	ACICU_02697 (99)	A1S_2485 (98)	
ACICU_02886	AB57_3056 (96)	ACICU_02886 (100)	A1S_2641 (97)	
ACICU_02966*#		ACICU_02966 (100)	Between A1S_2707 and A1S_2708 (98)	ABSDF0764 (98)
AB57_3308*#	AB57_3308 (100)			
ACICU_03137*#		ACICU_03137 (100)		ABSDF0546 (98)
AB57_3624*	AB57_3624 (100)	ACICU_03369 (99)	Between A1S_3168 and A1S_3169 (99)	
ACICU_03379*		ACICU_03379 (100)		ABSDF0314 (95)
ACICU_03418*		ACICU_03418 (100)	Between A1S_3220 and A1S_3221 (99)	ABSDF0260 (100)
A1S_3257			A1S_3257 (100)	ABSDF3356 (98)
ACICU_03581*		ACICU_03581 (100)	A1S_3381 (99)	ABSDF3529 (99)

[a] SGI, small genomic islet.
[b] ORFs showing the same distribution patterns among clonal isolates are indicated by an asterisk (*), and ORFs selected for PCR-based ORF typing are indicated by a hash tag (#).
[c] Numbers in parentheses are the percent sequence similarities over representative SGI ORFs listed in the first column.
[d] slv, single locus variant.
[e] When nucleotide sequences corresponding to an SGI ORF candidate are found in the genomes of some *A. baumannii* strains but they have not been named in the annotated genome data, the ORFs flanking the nucleotide sequence similar to the SGI ORF candidate are provided.

sults as "1" for "+" and "0" for "−", the so-called binary typing, which does not require any further handling of specimens, such as performing nucleotide sequence analyses, counting the allelic repeats, or analyzing complicated restriction enzyme digestion patterns. We previously succeeded in developing a genotyping method for *Staphylococcus aureus* by detecting the distribution patterns of its open reading frames (ORFs) using multiplex PCR that can be replaced with pulsed-field gel electrophoresis (PFGE) (4, 5). In the genotyping of *S. aureus*, the distribution patterns of small genomic islets (SGIs) showed good correlations with the clonal complex (CC) types obtained by MLST. SGIs consist of one to several ORFs (6). Therefore, we hypothesized that the CCs of *A. baumannii* and the *Acinetobacter calcoaceticus-A. baumannii* complex might be also estimated or predicted by detecting the distribution patterns of SGIs specific to each *Acinetobacter* species. The distribution patterns of SGIs can easily be visualized by agarose gel electrophoresis after multiplex PCR; therefore, clone typing of isolates can be performed in many ordinary microbiology laboratories in which equipment for only PCR and agarose gel electrophoresis is available.

In the present study, therefore, we developed a new multiplex PCR-based method for easy, rapid, and reliable discrimination of the clonal complexes of *A. baumannii*, especially the epidemic clones.

MATERIALS AND METHODS

Bacterial isolates. A total of 226 *Acinetobacter* clinical isolates collected from patients in Japan between 2001 and 2012, including 79 *A. baumannii*, 20 *A. pittii*, 77 *A. nosocomialis*, 15 *Acinetobacter* species close to *A. nosocomialis*, 24 *A. radioresistens*, three *A. ursingii*, three *A. bereziniae*, two *A. soli*, one *A. junii*, one *Acinetobacter* genomic species 13BJ, and one *Acinetobacter* genomic species 14BJ, were used. These isolates were identified using their *rpoB* gene sequence (7). Two American Type Culture Collection (ATCC) reference strains available in our laboratory (*A. baumannii* strains ATCC 19606 and ATCC BAA-1605) were also used. The 79 *A. baumannii* clinical isolates and two ATCC reference strains were analyzed by MLST. The isolates were cultured overnight on soy bean casein digest agar plates at 37°C, and chromosomal DNA was extracted with the QuickGene SP kit DNA tissue (SP-DT) (Wako Pure Chemical Industries, Osaka, Japan). MLST analysis was performed according to the protocol of the Institut Pasteur MLST databases (http://www.pasteur.fr/mlst). The clustering of related STs, which was defined as a CC, was determined with the aid of the eBURST program (http://eburst.mlst.net/).

For *Acinetobacter*-specific ORF screening, *Pseudomonas aeruginosa* strain JCM 14847, *Pseudomonas putida* strain JCM 13063, *Pseudomonas fluorescens* strain JCM 5963, *Pseudomonas stutzeri* strain JCM 5965, *Pseu-*

TABLE 2 Species-specific ORF candidates

ORF[a]	Contig no., nucleotide position[b]	No. found/no. tested for *Acinetobacter* organism: *A. baumannii*	*A. pittii*	*A. nosocomialis*	*Acinetobacter* species close to *A. nosocomialis*	Other *Acinetobacter* species
pittii-1	9, 490–1095	0/8	0/4	0/8	0/4	NT[c]
pittii-2	19, 296023–297078	0/8	3/4	0/8	0/4	NT
pittii-3	21, 113290–112808	5/8	2/4	0/8	0/4	NT
pittii-4	23, 336772–337306	5/8	3/4	0/8	0/4	NT
pittii-5	23, 435468–436845	0/8	2/4	1/8	4/4	NT
pittii-6*	25, 270084–271118	0/81	19/20	0/77	0/15	0/35
pittii-7	26, 56553–57056	0/8	2/4	0/8	0/4	NT
pittii-8	31, 96908–97756	0/8	3/4	0/8	1/4	NT
pittii-9	31, 288435–286909	0/8	0/4	0/8	1/4	NT
nosocomialis-1	6, 48398–49115	0/8	0/4	7/8	0/4	NT
nosocomialis-2	12, 64890–63445	8/8	0/4	8/8	1/4	NT
nosocomialis-3*	90, 13009–11208	0/81	0/20	76/77	0/15	0/35
Asp-1*	12, 41330–40363	0/81	0/20	0/77	15/15	0/35
Asp-2	15, 52010–54341	0/8	0/4	0/8	0/4	NT
Asp-3	15, 207828–209480	0/8	0/4	0/8	0/4	NT
Asp-4	16, 155456–156670	0/8	0/4	0/8	1/4	NT
Asp-5	21, 98536–99348	0/8	0/4	0/8	0/4	NT

[a] The ORFs selected for species identification are indicated by an asterisk.
[b] ORFs were selected from *A. pittii* D499 (pittii-1 to -9 [GenBank accession no. AGFH00000000]), *A. nosocomialis* NCTC 8102 (nosocomialis-1 to -3 [GenBank accession no. AIEJ00000000]), and *Acinetobacter* species GG2 (Asp-1 to -5 [GenBank accession no. ALOW00000000]).
[c] NT, not tested.

domonas nitroreducens strain JCM 2782, *Azotobacter vinelandii* strain JCM 21475, and *Brevundimonas diminuta* strain JCM 2788 were used as a negative control. These strains were provided by the Japan Collection of Microorganisms, Riken BioResource Center (BRC), which participates in the National BioResource Project of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT). *Achromobacter xylosoxidans* and *Escherichia coli* clinical isolates were also used as a negative control.

Searching small genomic islets from *A. baumannii* whole-genome sequences. The whole-genome DNA sequences of six *A. baumannii* strains, AB0057 (DDBJ/EMBL/GenBank accession no. CP001182), AB307-0294 (GenBank accession no. CP001172), AYE (GenBank accession no. CU459141), ACICU (GenBank accession no. CP000863), ATCC 17978 (GenBank accession no. CP000521), and SDF (GenBank accession no. CU468230), were obtained from an Internet database (PubMed [http://www.ncbi.nlm.nih.gov/sites/entrez]) and compared to each other using the MBGD website (http://mbgd.genome.ad.jp/) and blast+ (National Center for Biotechnology Information, Bethesda, MD, USA), with a tabular output option, and homologues were visualized by ACT (8). The nonconserved regions among the six strains were identified and selected as potential SGIs. Among the selected SGIs, those containing single to several ORFs without the presence of structures resembling insertion sequences, transposases, or integrases were selected for the determination of CCs. Nonconserved regions with larger structures, such as transposons, prophages, and antimicrobial resistance islands, were excluded. The distribution patterns of the SGI candidates (Table 1) were investigated by PCR using 42 *A. baumannii* representative clinical isolates and two ATCC strains.

Searching species-specific ORFs from whole-genome sequences. The whole-genome DNA sequences of four *A. pittii* (strains D499 [GenBank accession no. AGFH00000000], DSM 9306 [AIEF00000000], DSM 21653 [AIEK00000000], and SH024 [NZ_ADCH00000000]), two *A. nosocomialis* (strains NCTC 8102 [AIEJ00000000] and RUH2624 [NZ_ACQF00000000]), three *Acinetobacter calcoaceticus* (strains PHEA-2 [CP002177], DSM 30006 [NZ_APQI00000000], and RUH2202 [NZ_ACPK00000000]), one *A. bereziniae* (strain LMG 1003 [NZ_AIEI00000000]), one *Acinetobacter haemolyticus* (strain ATCC 19194 [NZ_ADMT00000000]), one *Acinetobacter johnsonii* (strain SH046 [NZ_ACPL00000000]), one *A. junii* (strain SH025 [NZ_ACPM00000000]), three *Acinetobacter lwoffii* (strains NCTC 5866 [AIEL00000000], SH145 [NZ_ACPN00000000], and WJ10621 [NZ_AFQY00000000]), one *Acinetobacter parvus* (strain DSM 16617 [AIEB00000000]), four *A. radioresistens* (strains DSM 6976 [AIDZ00000000], SH164 [NZ_ACPO00000000], SK82 [NZ_ACVR00000000], and WC-A-157 [ALIR00000000]), one *A. ursingii* (strain DSM 16037 [AIEA00000000]), one *Acinetobacter venetianus* (strain RAG-1 [AKIQ00000000]), and four *Acinetobacter* species that have not been given scientific names (GG2 [ALOW00000000], ATCC 27244 [ABYN00000000], HA [NZ_AJXD00000000], and NBRC 100985 [NZ_BAEB00000000]) and the genomic data of six *A. baumannii* isolates mentioned in the section above were obtained from PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) and compared using blast+. ORFs showing high percent sequence similarities among all *Acinetobacter* species used in the method were selected as candidates of markers specific to *Acinetobacter* species. ORFs found only in *A. pittii*, *A. nosocomialis*, or *Acinetobacter* species close to *A. nosocomialis* (corresponding to strain GG2) genomes were selected as candidates of species-specific ORFs (Table 2). The presence of species-specific ORFs was screened by PCR for representative isolates, including eight *A. baumannii*, four *A. pittii*, eight *A. nosocomialis*, and four *Acinetobacter* species close to *A. nosocomialis*.

Multiplex PCR detection of selected ORFs to identify international clones. To maximize the discriminatory power and reliability of the identification of international clones, seven ORFs in separate SGIs were selected for multiplex PCR detection in order to identify international clones with a minimum difference of two bands in the detected ORF ladder patterns of other CCs among the isolates used in this study (Table 1).

For easy execution, the selected ORFs were detected by multiplex PCR, which we call PCR-based ORF typing (POT). The primer pairs for detecting ORFs in the seven SGIs, *Acinetobacter*-specific ORF, bla_{OXA-51} (9), and three species-specific ORFs (Table 2) were designed for multiplex PCR detection (Table 3). As ORFs in SGIs were found among *A. pittii*, *A. nosocomialis*, and *Acinetobacter* spp. close to *A. nosocomialis*, as well as for *A. baumannii*, the primers were designed to adapt universally to those *Acinetobacter* species.

Template DNAs for multiplex PCR were prepared by suspending bac-

TABLE 3 Primers finally selected for multiplex PCR

Target ORF	Primer direction	Sequence (5′ to 3′)[a]	Final concn (μM)	Amplicon size (bp)
atpA	Forward	CTGAACCTAGAACAGGATTCAGT	0.2	553
	Reverse	TCACGGAAGTATTCACCCAT	0.2	
OXA-51	Forward	GCTTCGACCTTCAAAATGCT	0.2	465
	Reverse	TCCAGTTAACCAGCCTACTTGT	0.2	
pittii-6	Forward	CATGTAGGTAGTCAAATGCCTG	0.2	401
	Reverse	CCGCTGGTGATGCTTTATTC	0.2	
nosocomialis-3	Forward	GTGATCGTGGTGATAGCTGG	0.2	362
	Reverse	GTAAGTTCCTGTTGCAACTCC	0.2	
Asp-1	Forward	GGATCTTTAACTCCATGGCTC	0.2	321
	Reverse	GATTATCrTGTAATAACCACGCAC	0.2	
AB57_2484	Forward	TATGTACAAAGCCAACCGGA	0.2	271
	Reverse	GAATTTGAGCdGAAGCCATTA	0.2	
ACICU_02042	Forward 1	CCGCGTCTTTCATAATAAGCAA	0.1	234
	Forward 2	CCACGTCTCTCATAATAAGCAA	0.1	
	Reverse 1	TGGAGAAATAGATTCTTCAAAAGTTGT	0.1	
	Reverse 2	TGCAGAAATAGATTCTTCAmAAATTGT	0.1	
ACICU_02966	Forward	ACCGTAyCCCTTTTTAATAAGTTCA	0.2	189
	Reverse	GGGCAAACTTATCATAGTTATATCGAC	0.2	
ACICU_01870	Forward	GCTGCAACCCAACCAATwA	0.2	151
	Reverse	AATTGGCTTCGhTGGATATTTATG	0.2	
AB57_3308	Forward	GCAACAGTTTCAAAATTAAATGG	0.2	122
	Reverse 1	ACTGTTTGTATGGGTATTGCAG	0.1	
	Reverse 2	ACTGTTTGTATAGGCATTGCAG	0.1	
ACICU_03137	Forward	CCyGCACTGCTCTACGATAATG	0.2	102
	Reverse	TTGyTCATAATGAAAAGCCGCA	0.2	
AB57_0815	Forward	CTTTAGAmGAGGCACGTTGGTTTG	0.2	81
	Reverse	TTTCACAyGGCTCACCGT	0.2	

[a] Mixed nucleotide residues were described according to a standard code (r, A/G; d, A/G/T; m, A/C; y, C/T; w, A/T; h, A/C/T).

terial cells in 100 μl of Tris-EDTA buffer (pH 8.0) at a turbidity of McFarland standard 0.5 to 2, heating at 100°C for 10 min, and centrifugation at 14,000 rpm (approximately 15,000 × *g*) for 1 min. Next, POT was carried out with the four thermal cyclers, i.e., GeneAmp PCR system 9700 (Life Technologies Japan, Tokyo, Japan), Applied Biosystems 2720 (Life Technologies Japan), GeneAtlas 322 (Astec, Fukuoka, Japan), and the Thermal Cycler Dice Gradient (TaKaRa Bio, Otsu, Japan), to validate their compatibility on the same platforms. The primer mixture was prepared by mixing all primers listed in Table 3 to 100× the final concentration. PCR was carried out in a 20-μl mixture containing 2 μl of the heat extract template DNA, prepared as described above, PCR buffer (3 mM Mg^{2+}), 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.8 units of FastStart *Taq* DNA polymerase (Roche Diagnostics, Mannheim, Germany), and 0.2 μl of the primer mixture. The sequences and final concentrations of the primers are shown in Table 3. All four DNA preparations extracted from clinical *A. baumannii* (POT 122 [ST2] in Table 4), *A. pittii* (POT 78 in Table 4), *A. nosocomialis* (POT 105 in Table 4), and *Acinetobacter* spp. close to *A. nosocomialis* (POT 105 in Table 4) were mixed and used as the DNA template for both the positive control and the ladder marker in PCR. The thermal conditions were as follows: 95°C for 10 min, 30 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 2 min, and then at 4°C for several hours before agarose gel electrophoresis. The PCR products (2 μl) were electrophoresed on 4% agarose gels (NuSieve 3:1; Lonza, Basel, Switzerland) in 0.5× Tris-borate-EDTA (TBE) at 100 V for 50 min; the bands were then visualized with ethidium bromide.

After PCR, the seven SGI ORFs were scored in the order of their PCR amplicon size, with either "1" for "+"or "0" for "−" (binary code), depending on the presence or absence, respectively, of the band of amplicon DNA. These scores were then converted to decimal numbers, i.e., POT numbers. The results of each SGI binary were multiplied by 2^n ($n = 6 - 0$) and added. For example, the binary code of ST2 (1111010) was converted to 122 as follows: $1 \times 64 + 1 \times 32 + 1 \times 16 + 1 \times 8 + 0 \times 4 + 1 \times 2 + 0 \times 1$. Furthermore, each POT number was represented by a numerical label, ranging from a POT of 0 (0000000) to a POT of 127 (1111111).

RESULTS

A total of 24 SGI candidates (Table 1) were selected by comparing the whole-genome data of the six *A. baumannii* strains (AB0057, AB307-0294, AYE, ACICU, ATCC 17978, and SDF). Highly conserved (95 to 100% sequence similarity) DNA sequences were found in most of the SGIs of the six *A. baumannii* strains checked, and nucleotide sequence identities were also observed among

TABLE 4 Correlations between MLST and ORFs used for POT

POT no.	ST (CC or allele profile)	No. of isolates	Presence or absence of ORFs for the POT analysis by group:											
			ORFs for identification of bacterial species[a]					ORFs for calculation of POT no.[b]						
			atpA	OXA-51	pittii-6	nosocomialis-3	Asp-1	AB57_2484	ACICU_02042	ACICU_02966	ACICU_01870	AB57_3308	ACICU_03137	AB57_0815
A. baumannii														
122	ST2 (CC2)	27	+	+	−	−	−	+	+	+	+	−	+	−
69	ST1 (CC1)	3	+	+	−	−	−	+	−	−	−	+	−	+
0	ST235 (CC33)	1	+	+	−	−	−	−	−	−	−	−	−	−
8	ST33 (CC33)	18	+	+	−	−	−	−	−	−	+	−	−	−
8	ST148	1	+	+	−	−	−	−	−	−	+	−	−	−
32	CC33 (3-5-7-1-12-1-2)	2	+	+	−	−	−	−	+	−	−	−	−	−
32	ST239 (CC216)	1	+	+	−	−	−	−	+	−	−	−	−	−
44	New (1-4-2-1-42-1-4)	1	+	+	−	−	−	−	+	−	+	+	−	−
44	ST40	1	+	+	−	−	−	−	+	−	+	+	−	−
10	ST52	1	+	+	−	−	−	−	−	−	+	−	+	−
40	CC10 (1-3-2-1-4-1-4)	1	+	+	−	−	−	−	+	−	+	−	−	−
41	ST49	1	+	+	−	−	−	−	+	−	+	−	−	+
56	ST142	1	+	+	−	−	−	−	+	+	+	−	−	−
72	ST152	4	+	+	−	−	−	+	−	−	+	−	−	−
73	ST212	1	+	+	−	−	−	+	−	−	+	−	−	+
92	ST246	1	+	+	−	−	−	+	−	+	+	+	−	−
96	CC216 (3-4-2-2-7-2-2)	1	+	+	−	−	−	+	+	−	−	−	−	−
104	ST34 (CC34)	9	+	+	−	−	−	+	+	−	+	−	−	−
104	New (27-2v-2v-1-9-2-5)	3	+	+	−	−	−	+	+	−	+	−	−	−
104	ST145 (CC216)	1	+	+	−	−	−	+	+	−	+	−	−	−
106	CC109 (3-4-2-2-9-1-5)	1	+	+	−	−	−	+	+	−	+	−	+	−
108	ST133	1	+	+	−	−	−	+	+	−	+	+	−	−
A. pittii														
66	NA[c]	9	+	−	+	−	−	+	−	−	−	−	+	−
70	NA	1	+	−	+	−	−	+	−	−	−	+	+	−
74	NA	2	+	−	+	−	−	+	−	−	+	−	+	−
74	NA	1	+	−	−	−	−	+	−	−	+	−	+	−
76	NA	1	+	−	+	−	−	+	−	−	+	+	−	−
78	NA	6	+	−	+	−	−	+	−	−	+	+	+	−
A. nosocomialis														
97	NA	1	+	−	−	+	−	+	+	−	−	−	−	+
104	NA	26	+	−	−	+	−	+	+	−	+	−	−	−
105	NA	49	+	−	−	+	−	+	+	−	+	−	−	+
105	NA	1	+	−	−	−	−	+	+	−	+	−	−	+
Acinetobacter species close to *A. nosocomialis*														
41	NA	7	+	−	−	−	+	−	+	−	+	−	−	+
105	NA	4	+	−	−	−	+	+	+	−	+	−	−	+
109	NA	2	+	−	−	−	+	+	+	−	+	+	−	+
125	NA	2	+	−	−	−	+	+	+	+	+	+	−	+

[a] Five ORFs, i.e., *atpA*, OXA-51, pittii-6, nosocomialis-3, and Asp-1, were used in the identification of each *Acinetobacter* isolate.

[b] Seven ORFs, i.e., AB57_2484, ACICU_02042, ACICU_02966, ACICU_01870, AB57_3308, ACICU_03137, and AB57_0815, were used for calculation of the POT number of each isolate.

[c] NA, not adopted.

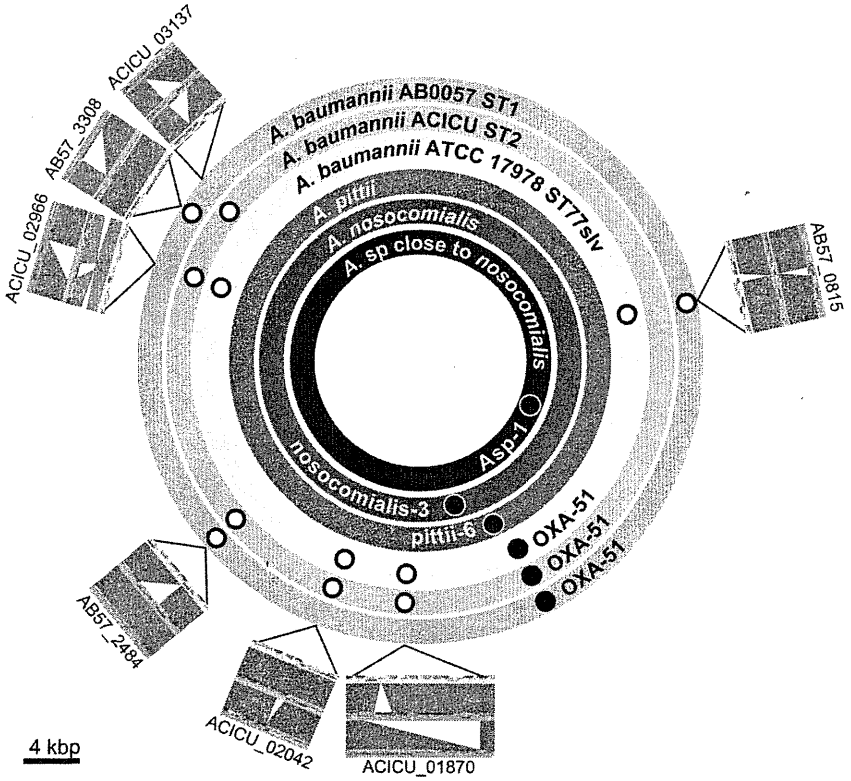

FIG 1 Distributions of small genomic islets and species-specific markers on genomes of representative strains. Open circles indicate SGIs, and closed circles indicate species-specific genetic markers. Locations of *atpA* selected as the universal marker of *Acinetobacter* species are not provided. The genome sequence data of *A. pittii*, *A. nosocomialis*, and *Acinetobacter* species close to *A. nosocomialis* are still draft data at present. The positions of each marker were decided by mapping the contigs containing markers on the basis of the genome of *A. baumannii* ACICU. Genome comparison maps illustrated by Artemis Comparison Tool (http://www.sanger.ac.uk/resources/software/act/) are arranged outside the circles, indicating genomes. The outer, second outer, and third outer rings indicate the genomes of *A. baumannii* AB0057, ACICU, and ATCC 17978, respectively. The dark-gray drawings in genome comparison maps indicate matches between the sequences, and the silver drawing in ACICU_02966 indicates an inversion match. The calibration bar indicates 4 kbp on all seven genome comparison maps. A large color version of this figure with high resolution is available in Fig. S1 in the supplemental material.

other *A. calcoaceticus-A. baumannii* complex strains, although the sequence similarities ranged from 80% to 97%.

Strains belonging to the same CC showed very similar distribution patterns in 16 of the 24 SGI ORFs (Table 1). The CC identities of strains belonging to the major CC were identified based on the distribution patterns of those 16 SGI ORFs. However, the distribution patterns of the 16 SGI ORFs in strains of Pasteur ST145 could not be distinguished from those of a novel ST (allele numbers 27-2v-2v-1-9-2-5).

SGI ORFs adopted for multiplex PCR amplification (Table 1) were selected according to the following principles. First, ORFs that were found exclusively in international clones I or II but were absent among most clones other than the epidemic ones were selected. The ORFs specific to international clone I were AB57_0815 and AB57_3308, and those specific to international clone II were ACICU_02966 and ACICU_03137. Thus, the international clones can be identified and distinguished by detecting the four ORFs AB57_0815, AB57_3308, ACICU_02966, and ACICU_03137. Second, three ORFs (AB57_2484, ACICU_02042, and ACICU_01870) were selected, because their distribution patterns in the epidemic clones were divergent in at least two alleles compared with those found in other nonepidemic lineages. These 3 ORFs were finally adopted to improve the discriminatory power of the test. The distribution patterns of the 7 SGI ORFs among the *A. baumannii* clones and *Acinetobacter* species are shown in Fig. 1 and Table 4; see also Fig. S1 in the supplemental material.

Two ORFs (*atpA* and *sucD*) were selected as the candidates for universal markers of *Acinetobacter* species by comparing whole-genome sequences. These alleles were found among all *Acinetobacter* species in the BLAST databases (whole-genome sequencing [WGS] database as of 8 May 2013) showing higher percent sequence similarities (>80%) than other orthologs. *atpA* was finally chosen as the marker specific to *Acinetobacter* species and the marker for the internal control of PCR amplification.

Genetic markers specific to *A. pittii*, *A. nosocomialis*, and *Acinetobacter* spp. close to *A. nosocomialis* were also searched for their whole-genome data. ORFs designated pittii-6, nosocomialis-3, and Asp-1 (Table 2) were finally chosen as markers specific to *A. pittii*, *A. nosocomialis*, and *Acinetobacter* spp. close to *A. nosocomialis*, respectively (Table 2). The pittii-6 marker was chosen from nine candidate markers, and its specificity and sensitivity were 100% and 95%, respectively (Table 2). As pittii-6 was also found in *A. calcoaceticus* genome sequences, with an 83% sequence similarity in its nucleotide sequence level, primers were designed for the specific detection of *A. pittii*. The nosocomialis-3 marker was chosen from 3 candidates, and its specificity and sensitivity were 100% and 99%, respectively. The Asp-1 marker was chosen from

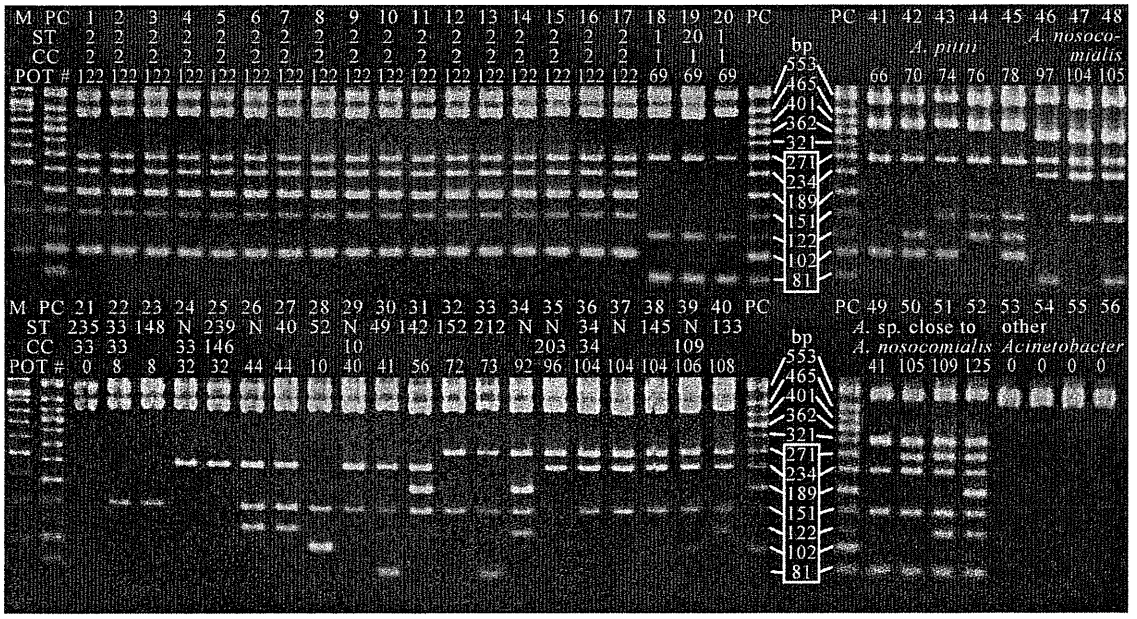

FIG 2 Agarose gel electrophoresis patterns of PCR-based ORF typing using 12-plex PCR. Lane M, 50-bp ladder marker; lane PC, positive control; lanes 1 to 40, *A. baumannii*; lanes 41 to 45, *A. pittii*; lanes 46 to 48, *A. nosocomialis*; lanes 49 to 52, *Acinetobacter* species close to *A. nosocomialis*; lane 53, *A. radioresistens*; lane 54, *A. ursingii*; lane 55, *A. bereziniae*; lane 56, *A. soli*. International clones II (lanes 1 to 17) and I (lanes 18 to 20) showed unique patterns and can be distinguished from other *A. baumannii* clones (lanes 21 to 40) and other *Acinetobacter* species (lanes 41 to 56). The POT numbers of both *A. baumannii* ST49 (lane 30) and an *Acinetobacter* species close to *A. nosocomialis* (lane 49) become 41, but these isolates can apparently be discriminated from each other by the ladder patterns of the upper 5 fragments from bp 321 to bp 553. ST, sequence type; CC, clonal complex. "N" in ST lines indicates a novel ST. The ladder used for the binary digitization of the genotype of each isolate is shown in the white box. The binary numbers corresponding to each band were 1 for 81 bp, 2 for 102 bp, 4 for 122 bp, 8 for 151 bp, 16 for 189 bp, 32 for 234 bp, and 64 for 271 bp, from the bottom of the ladder in the box. The remaining 5 bands from 321 bp to 553 bp were used for the identification of *Acinetobacter* spp.

5 candidates, and its specificity and sensitivity were 100% and 100%, respectively.

The ladder patterns of the PCR amplicons described above were clearly distinguishable by the 12-plex PCR established as the POT in the present study (Fig. 2). The same results were obtained by all four thermal cyclers we evaluated. To substantiate the 12-plex PCR, 44 *A. baumannii* strains used for SGI ORF screening were tested by both monoplex PCR and POT, and complete data agreement was observed between the two methods.

A total of 81 *A. baumannii* strains, which have been classified into 18 CCs by MLST, were analyzed by the POT method. International clones I and II were distinguished from other genetic lineages with more than two differences in the bands of their ladder patterns. According to the ladder patterns of seven ORFs, *A. baumannii* strains were classified into 17 POT types, 11 of which exhibited one-to-one correspondence to the CCs. Moreover, clinically isolated *Acinetobacter* species other than *A. baumannii* can be classified into three to five POT types at present (Table 4).

DISCUSSION

In the present study, we first showed that the newly established POT is capable of rapidly identifying *A. baumannii* international clones in ordinary clinical laboratories without performing nucleotide sequencing analyses of multiple genes as with MLST. To increase the feasibility of the test, the number of SGI ORFs adopted for POT was optimized. International clones I and II were fully distinguished by this method from other clones or lineages of *Acinetobacter* species. Moreover, the CCs of *A. baumannii* can be estimated by POT. The discriminatory power of POT can be controlled by optimizing the number of ORFs and loci selected for analysis. Such a newly developed POT method that compares the distribution patterns of ORFs and/or SGIs in each clinical isolate may well promise to be an easy and rapid genotyping method for identifying bacterial genetic lineages and molecular epidemiology, which is feasible in ordinary clinical microbiology laboratories.

Indeed, several methods to identify international clones using PCR have been reported (10, 11). However, they cannot identify newly emerging multidrug-resistant epidemic clones that might spread in the future. In fact, multidrug-resistant isolates other than the *A. baumannii* international clones have been reported (12–18). The POT method constructed in the present study is applicable to the identification of new CCs of *A. baumannii* or *A. calcoaceticus-A. baumannii* complex species, including *A. nosocomialis* and *A. pittii*, in the future.

Although SGIs were first reported from *Salmonella enterica* in 2001 by whole-genome analysis (6), little attention has been paid to them so far. Using genomic comparisons of *S. aureus* strains, we found that the distribution patterns of SGI ORFs correlate with the clonal complex in *S. aureus* (5). In the present study, it was also proven that the distribution patterns of ORFs in SGIs correlated well with the CCs in *A. baumannii*. This finding indicates that a very similar concept can be applicable even to various bacterial genera, and that close correlations between the distribution patterns of SGIs and CCs may be a general phenomenon in the microbial world. In fact, the CCs of *P. aeruginosa* were successfully predicted with a strategy and protocol similar to those of the POT constructed in the present study (M. Suzuki and Y. Iinuma, unpublished data).

To obtain the genetic information of clinical isolates from antimicrobial resistance surveillance data, digitized numeric data provided by new genotyping methods, like the POT, contribute to easy and feasible genotyping. Since the POT is very simple and requires equipment only for PCR and agarose gel electrophoresis, this can become a routine performance method in many ordinary clinical microbiology laboratories in various countries and regions, including developing countries around the world. If many clinical microbiologists and researchers would employ the POT for genotyping of clinical isolates, they could report the genotype of each clinical isolate as a digitized numeric number, and this would make it very easy to quickly compare the genotypes of clinical isolates with those of other clinical isolates recovered in different continents or areas. Therefore, the POT would enable us to identify newly emerging genetic lineages in the very early stage of their outbreak. Present weak antimicrobial resistance surveillance and monitoring systems depending mainly on the antimicrobial resistance phenotypes of clinical isolates can be reinforced from the genetic viewpoint by using the POT instead of MLST.

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Long-Term Colonization by bla_{CTX-M}-Harboring *Escherichia coli* in Healthy Japanese People Engaged in Food Handling

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The actual state of intestinal long-term colonization by extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* in healthy Japanese people remains unclear. Therefore, a total of 4,314 fecal samples were collected from 2,563 food handlers from January 2010 to December 2011. Approximately 0.1 g of each fecal sample was inoculated onto a MacConkey agar plate containing cefotaxime (1 μg/ml). The bacterial colonies that grew on each plate were checked for ESBL production by the double-disk synergy test, as recommended by the Clinical and Laboratory Standards Institute. The bacterial serotype, antimicrobial susceptibility, pulsotype, sequence type (ST), and ESBL genotype were checked, and the replicon types of plasmids harboring the ESBL gene were also determined after conjugation experiments. ESBL producers were recovered from 70 (3.1%) of 2,230 participants who were checked only once. On the other hand, ESBL producers were isolated at least once from 52 (15.6%) of 333 participants who were checked more than twice, and 13 of the 52 participants carried ESBL producers for from more than 3 months to up to 2 years. Fluoroquinolone (FQ)-resistant *E. coli* strains harboring bla_{CTX-M} were repeatedly recovered from 11 of the 13 carriers of bla_{CTX-M}-harboring *E. coli*. A genetically related FQ-resistant *E. coli* O25b:H4-ST131 isolate harboring $bla_{CTX-M-27}$ was recovered from 4 of the 13 carriers for more than 6 months. Three FQ-resistant *E. coli* O1:H6-ST648 isolates that harbored $bla_{CTX-M-15}$ or $bla_{CTX-M-14}$ were recovered from 3 carriers. Moreover, multiple CTX-M-14- or CTX-M-15-producing *E. coli* isolates with different serotypes were recovered from 2 respective carriers. These findings predict a provable further spread of ESBL producers in both community and clinical settings.

The production of extended-spectrum β-lactamases (ESBLs) is one of the most common cephalosporin resistance mechanisms acquired by bacteria belonging to the members of the family *Enterobacteriaceae* (1). In particular, the incidence of CTX-M-type-ESBL-producing *Escherichia coli* isolates among clinical isolates recovered from community-acquired infections, such as urinary tract infections and bacteremia, has been increasing (2). The increasing rates of isolation of these microbes alert us to the probable growing risk to public health in the future (3). The exact reason for the rapid spread of ESBL-producing *E. coli* isolates among healthy individuals remains unclear, although alterations of the bacterial phenotypic and genetic properties that permit easier colonization in the human intestines, as well as the considerable contamination of foods with ESBL producers, have been speculated to underlie the phenomenon (4).

The prevalence of intestinal carriage of ESBL-producing bacteria belonging to the family *Enterobacteriaceae*, in particular, the CTX-M-15-producing *E. coli* O25b-sequence type 131 (ST131) clone, has recently been regarded to be a growing public health concern worldwide (5), because CTX-M-type-ESBL-producing *E. coli* O25b ST131 clones usually show coresistance to fluoroquinolones (FQs) and sometimes cause community-acquired infections, such as urinary tract infections, as well as nosocomial infections. Some investigators have reported that the rate of enteric carriage of ESBL producers among healthy individuals, including travelers and soldiers, is considerable (6–10). However, although most studies have so far focused on hospitalized patients, the augmented prevalence of cephalosporin-resistant *Enterobacteriaceae* in clinical settings at present would also be greatly affected by the ESBL producers increasingly colonizing the intestines of healthy people in the community. Woerther et al. have recently reported on the trends in the fecal carriage of ESBL producers in the community, as well as their regional specificities, their dissemination routes, and the way to control the bacterial populations in members of the community colonized with ESBL producers (5). Moreover, some studies on the duration of fecal carriage of ESBL-producing bacteria in patients have been conducted in Thailand and Sweden (11–13). Titelman et al. have noted that the fecal carriage of ESBL-producing *Enterobacteriaceae* in patients was common for 12 months after the time of initial infection (14). Information on the duration of fecal carriage of ESBL producers is important to determine how to deal with healthy carriers of ESBL producers at the time of their admission to hospitals.

The aim of our study, therefore, was to elucidate the states of colonization by ESBL-producing *E. coli* in the intestines of 2,563 Japanese food handlers.

MATERIALS AND METHODS

Sample collection and bacterial isolation. The investigation was performed with 2,563 different participants at the Okazaki City Public Health Center in Japan from January 2010 to December 2011. Among the 2,563 participants, 2,230 were checked for colonization with ESBL producers

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only once and 333 participants were checked more than twice during the period of investigation (see Fig. S1 in the supplemental material). In accordance with the requirements of the Ministry of Health, Labour and Welfare of Japan, workers who handle food are expected to be checked every month and, at a minimum, at least twice a year at regional health centers or appointed private microbiology laboratories to determine whether they carry any pathogenic bacteria, including enterohemorrhagic *E. coli*, *Shigella* spp., or *Salmonella* spp., in their intestinal tracts, although the periodic checking of the feces of food handlers is not strictly enforced in Japan. Therefore, we considered that it would be suitable to follow up healthy people with ESBL-producing microbes in their intestines for a long period through the use of this evaluation system. The study was conducted with the approval of the ethics committee for epidemiological studies of the Nagoya University Graduate School of Medicine. All people were informed of the ethical points, including the purpose of the study and the study protocol, prior to the investigation, and those who consented to join the study on their own were enrolled as participants.

A total of 4,314 stool specimens were collected from 2,563 different participants (1,050 males and 1,513 females; age range, 18 to 74 years for males and 18 to 79 years for female; average age ± standard deviation, 48.9 ± 14.4 years for males and 48.8 ± 14.0 years for females). The stool specimen (approximately 0.1 g) from each participant was directly inoculated by use of a sterilized cotton swab onto MacConkey agar plates (Eiken Chemical Co., Ltd., Tokyo, Japan) supplemented with 1 μg of cefotaxime (CTX) per ml (CTX-MacConkey). When the colonies that uniformly grew on an agar plate showed very similar features, three colonies were picked from the plate and the bacterial species was identified by using conventional biochemical tests and an API 20E system (Sysmex bioMérieux Co., Ltd., Tokyo, Japan). Four to 10 colonies were picked and subjected to further testing when multiple colonies with different features appeared on an agar plate.

Screening for ESBL producers and genetic identification. Screening of probable ESBL producers was performed by the double-disk synergy test using commercially available ceftazidime, cefotaxime, and amoxicillin-clavulanic acid disks purchased from Eiken Chemical Co., Ltd. (15). PCR analyses were performed using 6 sets of PCR primers for the detection of genes for CTX-M group 1, CTX-M group 2, CTX-M group 8, CTX-M group 9, TEM-type, and SHV-type ESBLs, and their genotypes were further determined by nucleotide sequencing analyses of the PCR amplicons (16, 17).

Serotyping of *E. coli* by antisera and detection of serotype O25b by PCR. The serotype was determined using *E. coli* antisera, Seiken set 1 (Denka Seiken Co., Ltd., Tokyo, Japan) for the O antigen and Seiken set 2 (Denka Seiken) for the H antigen, according to the manufacturer's instructions. Serotypes that could not be determined by this method were designated O antigen untypeable (OUT) or H antigen untypeable (HUT). In addition, genetic O serotyping by PCR was performed as previously described only for the *E. coli* isolates determined to be serogroup O25 by the *E. coli* antisera (18). This is because ESBL-producing and FQ-resistant *E. coli* isolates are usually identified to be serotype O25b:H4, a serological variant of serotype O25:H4, but serotype O25b:H4 cannot be discriminated from serotype O25:H4 by commercially available antisera.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed using a control strain, *E. coli* ATCC 25922, and the agar dilution method, which was done as recommended by the Clinical and Laboratory Standards Institute (CLSI) (19). Interpretation of the MIC results was done in accordance with the CLSI criteria in document M7-A9 (19). The MICs of the following antimicrobials were purchased from the indicated sources: piperacillin, cefotaxime, ceftazidime, imipenem, aztreonam, gentamicin, minocycline, fosfomycin, ciprofloxacin, and levofloxacin were from Wako Pure Chemical Co., Inc., Tokyo, Japan; cefmetazole, amikacin, and chloramphenicol were from Sigma-Aldrich Japan, Tokyo, Japan; and flomoxef was provided by Shionogi & Co., Ltd., Tokyo, Japan.

PFGE and MLST. Pulsed-field gel electrophoresis (PFGE) analysis of each ESBL-producing isolate was performed as described elsewhere (20). In brief, a plug containing whole-genomic DNA was digested with XbaI (TaKaRa Bio. Inc., Tokyo, Japan), and electrophoresis was performed using a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA, USA) with pulse times ranging from 2.2 to 54.2 s and at a voltage of 6 V/cm at 14°C for 19 h. Strain H9812 of *Salmonella enterica* serotype Braenderup was used as the control strain. A dendrogram showing the genetic relatedness among the isolates was prepared with Fingerprinting II software (Bio-Rad Laboratories). The isolates obtained from each participant were regarded to have the same genetic background when they possessed a pulsotype with ≥85% similarity, and one representative isolate of each pulsotype was selected and used for further study. When a genetic similarity of less than 85% was observed between two isolates, they were considered genetically different, and both isolates were separately characterized in the present study, as performed previously (21).

Multilocus sequence typing (MLST) of the bla_{CTX-M}-harboring *E. coli* isolates repeatedly recovered from the same participant was performed by analysis of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) according to a protocol provided by a website for MLST of *E. coli* (http://mlst.ucc.ie/mlst/dbs/Ecoli). The sequence types (STs) were compared with the population structure of the species using the eBURST program (http://eburst.mlst.net/).

Conjugation study, plasmid replicon typing, and PCR detection of bla_{CTX-M} genes. All the bla_{CTX-M}-harboring donor *E. coli* isolates that colonized the participants for a long period were uniformly susceptible to rifampin. Thus, rifampin-resistant *E. coli* CSH-2 (*metB* F^-, resistant to nalidixic acid and rifampin) was used as the recipient in the conjugation experiments, performed by the broth mating method (22). Transconjugants were selected on Luria-Bertani (LB) agar plates supplemented with CTX (20 μg/ml) and rifampin (100 μg/ml) (Wako Pure Chemical Co., Inc.). The pulsotype of each transconjugant was compared to that of the recipient strain to avoid the acquisition of rifampin resistance by the donor strain. In addition, the acquisition of bla_{CTX-M} by the transconjugant was also confirmed by PCR using positive-control strains for each ESBL gene (16, 17). For the resultant bla_{CTX-M}-harboring transconjugants, as well as their parent isolates repeatedly recovered from the same participant, the replicon types of the plasmids were checked by PCR-based replicon typing (PBRT) using 18 pairs of primers as described previously (23).

Statistical analysis. Comparisons of the proportions of participants by age and gender were made by a continuity-adjusted χ^2 test with SPSS software (version 20.0 for Windows; SPSS Inc., Chicago, IL, USA). A P value of <0.05 was considered to denote a statistically significant difference.

RESULTS

Isolation of ESBL producers from fecal samples and their characteristics. ESBL producers were recovered from 197 of the 4,314 fecal specimens evaluated in the present study. More than 2 ESBL producers were recovered at the same time from each of 10 of the 197 fecal specimens. Two ESBL producers were recovered from 9 of these 10 fecal specimens, and 3 ESBL producers were from 1 of the 10 fecal specimens. When the shapes of the colonies that grew on a plate were apparently the same, three colonies were picked up and subjected to typing of the ESBL by PCR. If the shapes of the colonies that grew on a plate were different in size and/or color, 4 to 10 colonies with a distinct appearance were fished out and tested. As a result, from 1 to 3 ESBL producers with different genotypes were isolated from each participant, and 208 ESBL producers were finally obtained from 122 participants. If the ESBL producers recovered from the same participant in each test showed very similar PFGE profiles and the same ESBL type, only 1 isolate was selected and kept for further analyses. Thus, 59 of the

TABLE 1 Characteristics of nonduplicate 149 ESBL producers obtained from 122 participants

Species	No. of isolates in each of the following CTX-M groups:					
	CTX-M-1	CTX-M-2	CTX-M-8	CTX-M-9	Non-CTX-M[a]	Total
E. coli	30	19	7[b]	82	7	145
K. pneumoniae		2[c]			1	3
A. hydrophila				1		1
Total	30	21	7	83	8	149

[a] Isolates harboring the bla_{SHV} gene.
[b] One of seven isolates harbored both the $bla_{CTX-M-8}$ and bla_{TEM-52} genes.
[c] These isolates harbored both the $bla_{CTX-M-2}$ and bla_{SHV-1} genes.

208 ESBL producers were excluded. Consequently, 149 ESBL producers possessing different genetic backgrounds were recovered from 122 (4.8%; 62 males and 60 females) of the 2,563 participants (1,050 males and 1,513 females) and evaluated in this study (see Fig. S1 in the supplemental material). Neither age nor gender bias in the rate of detection of the 149 ESBL producers from the 122 participants was observed ($P \geq 0.05$). Of these 149 isolates, 145 were identified to be *E. coli*, 3 were identified to be *Klebsiella pneumoniae*, and 1 was identified to be *Aeromonas hydrophila* (Table 1). Two ESBL-producing isolates, one *E. coli* isolate and one *K. pneumoniae* isolate, were coisolated from 2 participants, and an ESBL-producing *K. pneumoniae* isolate alone was recovered from 1 participant.

One hundred thirty-eight (95.2%) of the 145 ESBL-producing *E. coli* isolates from 120 participants harbored a bla_{CTX-M} gene, and the remaining 7 isolates harbored bla_{SHV-5}-group genes, such as bla_{SHV-12}, as shown in Table 2. The types of bla_{CTX-M} genes were mainly $bla_{CTX-M-14}$ (43.5%), followed by $bla_{CTX-M-15}$ (17.9%), $bla_{CTX-M-27}$ (13.1%), and $bla_{CTX-M-2}$ (13.1%). The most frequent O serogroup of the bla_{CTX-M}-harboring *E. coli* isolates was O25 (27 isolates, 18.6%), followed by O1 (11 isolates, 7.6%) and O153 (8 isolates, 5.5%). Evaluation of the relationship between bla_{CTX-M} genes and O serogroups showed that the serogroups of the isolates harboring $bla_{CTX-M-14}$, $bla_{CTX-M-2}$, or $bla_{CTX-M-15}$ were diverse, i.e., O1, O74, and O153; however, 16 (84.2%) of the 19 isolates harboring $bla_{CTX-M-27}$ were O25b.

As shown in Table 3, most ESBL-producing *E. coli* isolates were susceptible to cefmetazole, flomoxef, imipenem, amikacin, and fosfomycin. However, these isolates tended to show a phenotype of multidrug resistance to aztreonam, minocycline, and FQs. The isolates harboring the $bla_{CTX-M-27}$ gene, especially the serotype O25b isolates, usually showed resistance to FQs, such as ciprofloxacin and levofloxacin, but no isolate showing resistance to gentamicin and amikacin was found among the 19 isolates harboring the $bla_{CTX-M-27}$ gene, although 23 of 63 isolates harboring the $bla_{CTX-M-14}$ gene showed resistance to gentamicin and/or amikacin. The FQ MICs for serotype O25b:H4 isolates harboring the $bla_{CTX-M-27}$ gene were significantly higher than those for the isolates of the other O serotypes harboring any of the other bla_{CTX-M} genes ($P < 0.05$) (see Fig. S2 in the supplemental material). The MIC values of aztreonam and ceftazidime for the isolates harboring the $bla_{CTX-M-15}$ gene were significantly higher than those for the isolates harboring any of the other bla_{CTX-M} genes regardless of their O serotypes ($P < 0.05$) (see Fig. S3 in the supplemental material). On the other hand, no significant differences in the MIC values of minocycline, amikacin, and fosfomycin

TABLE 2 O serogroups and CTX-M types of nonduplicate 145 ESBL-producing *E. coli* isolates

Serogroup	No. (%) of isolates of each CTX-M type									
	CTX-M-1 group				CTX-M-2 group, CTX-M-2	CTX-M-8 group, CTX-M-8	CTX-M-9 group		Non-CTX-M[a]	Total
	CTX-M-1	CTX-M-15	CTX-M-3	CTX-M-55			CTX-M-14	CTX-M-27		
O1		3				2[b]	5	1		11 (7.6)
O8		3					1		1	5 (3.4)
O15							2		1	3 (2.1)
O18							1			1 (0.7)
O25		1	1		1	1	7	16		27 (18.6)
O27							1			1 (0.7)
O29					1		1			2 (1.4)
O74		1					4			5 (3.4)
O78		3				1				4 (2.8)
O86a							1			1 (0.7)
O125		1					2		1	4 (2.8)
O128					1		1			2 (1.4)
O142					3					3 (2.1)
O148							1			1 (0.7)
O153		1			1		5	1		8 (5.5)
O157					1[c]					1 (0.7)
O166		1								1 (0.7)
O169		1					1			2 (1.4)
OUT	2	11		1	11	3	30	1	4	63 (43.4)
Total	2 (1.4)	26 (17.9)	1 (0.7)	1 (0.7)	19 (13.1)	7 (4.8)	63 (43.5)	19 (13.1)	7 (4.8)	145 (100)

[a] Isolates harboring a bla_{SHV-5}-group gene, such as bla_{SHV-12}.
[b] One of the two isolates harbored both the $bla_{CTX-M-8}$ and bla_{TEM-52} genes.
[c] This isolate did not harbor genes for Shiga-like toxins.

TABLE 3 Antimicrobial resistance profiles of 145 nonduplicate ESBL-producing *E. coli* isolates

Antimicrobial agent[b]	No. of resistant isolates of each CTX-M type[a]									
	CTX-M-1 group						CTX-M-9 group			
	CTX-M-1 ($n = 2$)	CTX-M-15 ($n = 26$)	CTX-M-3 ($n = 1$)	CTX-M-55 ($n = 1$)	CTX-M-2 group, CTX-M-2 ($n = 19$)	CTX-M-8 group, CTX-M-8 ($n = 7$)	CTX-M-14 ($n = 63$)	CTX-M-27 ($n = 19$)	Non-CTX-M[c] ($n = 7$)	Total ($n = 145$)
Ceftazidime	0	17	0	1	0	0	1	1	5	25
Aztreonam	2	25	0	1	10	0	11	3	7	59
Gentamicin	0	6	1	0	4	0	23	0	0	34
Amikacin	0	0	0	0	0	0	1	0	0	1
Minocycline	2	17	1	1	15	3	37	15	5	96
Chloramphenicol	0	7	0	0	1	0	14	0	1	23
Fosfomycin	0	1	1	0	1	0	5	1	0	9
Ciprofloxacin	0	16	1	1	1	0	28	18[d]	2	67
Levofloxacin	0	16	1	1	0	0	28	18[d]	2	66

[a] Interpretation of MIC results was done in accordance with the CLSI criteria in document M7-A9 (19).
[b] All ESBL-producing *E. coli* isolates were resistant to piperacillin and cefotaxime but were susceptible to cefmetazole, flomoxef, and imipenem.
[c] Isolates harboring a bla_{SHV-5}-group gene, such as bla_{SHV-12}.
[d] Sixteen of 18 *E. coli* isolates harboring the $bla_{CTX-M-27}$ gene were serogroup O25.

($P \geq 0.05$) were found among the groups harboring different bla_{CTX-M} genes.

PFGE analysis. As shown in Fig. 1, the O25b:H4-ST131 group (including 19 isolates of O25b:H4-ST131, 2 isolates of O25b:HNM-ST131 [where NM indicates nonmotile], and 1 isolate of O25b:HUT-ST131) and the OUT:H5-ST131 group (including 12 isolates of OUT:H5-ST131 and 1 isolate of O25:H5-ST131) constituted 2 large clusters which were defined at the 71% and the 80% similarity levels, respectively, but the isolates belonging to the O1 serogroup showed a very wide genetic diversity.

Long-term colonization by bla_{CTX-M}-harboring *E. coli*. Among the 122 carriers of ESBL producers, 52 carriers could be checked at least 2 times, and bla_{CTX-M}-harboring *E. coli* isolates were recovered more than twice from 13 (25%) of the 52 carriers during the study period (Table 4; see also Table S1 and Fig. S1 in the supplemental material). Since some of the bla_{CTX-M}-harboring *E. coli* isolates from each of the 13 participants showed the same PFGE profile, we defined the 13 participants to be long-term carriers. The periods of detection of the bla_{CTX-M}-harboring *E. coli* isolates in each participant ranged from 3 months to up to 2 years. Neither age nor gender bias was observed ($P \geq 0.05$) among the 13 long-term carriers. In two of the long-term carriers (participants 60 and 66), bla_{CTX-M}-harboring *E. coli* O1:H6-ST648 isolates were repeatedly recovered for more than 1 year. Interestingly, $bla_{CTX-M-14}$-harboring *E. coli* O1:H6-ST648 isolates were repeatedly recovered for up to 2 years in a long-term carrier (participant 66). In three long-term carriers (participants 64, 69, and 106), bla_{CTX-M}-harboring *E. coli* isolates were intermittently recovered but the participants were negative over different periods. In four cases (participants 12, 26, 29, and 87), the durations of colonization of bla_{CTX-M}-harboring *E. coli* in 2011 were uncertain because they were not checked in 2011. It was confirmed from the questionnaires submitted by all the participants at the time of submission of each fecal sample that all but one carrier (participant 69) received any obvious antimicrobial treatment during the period of investigation. Participant 69 received antimicrobial treatment for pneumonia in March 2010, but the name of the antimicrobial and its period of administration were unidentified. An ESBL-producing *E. coli* O25b:H4-ST131 isolate harboring $bla_{CTX-M-27}$ was first isolated from participant 69 in January 2010, prior to antimicrobial administration in March 2010, and the same ESBL producers were repeatedly recovered from this participant for 5 months even after antimicrobial treatments were stopped, as shown in Table 4. Two carriers (participants 50 and 88) traveled abroad during the investigation period, and ESBL-producing *E. coli* OUT:H4-ST3407 harboring $bla_{CTX-M-15}$ disappeared from one carrier (participant 50) just after overseas travel, while *E. coli* OUT:H5-ST131 harboring $bla_{CTX-M-14}$ was repeatedly recovered from participant 88 for 5 months after the foreign travel.

Serotypes and multilocus sequence types of bla_{CTX-M}-harboring *E. coli* isolates from long-term carriers. As shown in Table 4, the most frequent serotype of bla_{CTX-M}-harboring *E. coli* isolates that colonized any of the participants for long periods was O25b:H4 (4 carriers), followed by O1:H6 (3 carriers) and OUT:H5 (2 carriers). The serotypes of the remaining isolates were diverse, i.e., O15, O74, and O78.

MLST analysis identified 6 different STs, and the most predominant one was ST131 (6 isolates), followed by ST648 (3 isolates). The remaining STs were ST354, ST38, ST23, and ST3407, and the 6 STs were scattered evenly among the ST population structure of *E. coli*, as illustrated by eBURST analysis (Fig. 2). No apparent genetic relationship between ST648 and ST131 according to the nucleotide sequences of the seven housekeeping genes for which they were tested was found.

Characteristics of bla_{CTX-M}-harboring *E. coli* isolates from long-term carriers. As shown in Table 4, 6 (46%), 4 (31%), and 3 (23%) individuals were long-term carriers of *E. coli* isolates harboring $bla_{CTX-M-14}$, $bla_{CTX-M-27}$, and $bla_{CTX-M-15}$, respectively. Most bla_{CTX-M}-harboring *E. coli* isolates colonizing participants for long periods tended to show multidrug resistance profiles, and the bla_{CTX-M}-harboring *E. coli* isolates recovered from 11 of 13 long-term carriers showed coresistance to FQs. Interestingly, the ceftazidime MICs for $bla_{CTX-M-27}$-harboring *E. coli* isolates recovered from four long-term carriers (participants 12, 64, 69, and 106) were about 2 or 4 μg/ml, although CTX-M-27 generally hydrolyzes ceftazidime as well as cefotaxime.

Replicon types of bla_{CTX-M}-carrying plasmids. The conjugation experiment and replicon typing of plasmids were performed with the 17 bla_{CTX-M}-harboring *E. coli* isolates recovered from the 13 long-term carriers. As shown in Table 4, the conjugal transfer of