Oligonucleotide Array-Based Identification of Species in the *Acinetobacter calcoaceticus*-*A. baumannii* Complex in Isolates from Blood Cultures and Antimicrobial Susceptibility Testing of the Isolates[▽]

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Acinetobacter calcoaceticus, *A. baumannii*, *Acinetobacter* genomic species (gen. sp.) 3, and *Acinetobacter* gen. sp. 13TU, which are included in the *A. calcoaceticus*-*A. baumannii* complex, are difficult to distinguish by phenotypic methods. An array with six oligonucleotide probes based on the 16S-23S rRNA gene intergenic spacer (ITS) region was developed to differentiate species in the *A. calcoaceticus*-*A. baumannii* complex. Validation of the array with a reference collection of 52 strains of the *A. calcoaceticus*-*A. baumannii* complex and 137 strains of other species resulted in an identification sensitivity and specificity of 100%. By using the array, the species distribution of 291 isolates of the *A. calcoaceticus*-*A. baumannii* complex from patients with bacteremia were determined to be *A. baumannii* (221 strains [75.9%]), *Acinetobacter* gen. sp. 3 (67 strains [23.0%]), *Acinetobacter* gen. sp. 13TU (2 strains [0.7%]), and unidentified *Acinetobacter* sp. (1 strain [0.3%]). The identification accuracy of the array for 12 randomly selected isolates from patients with bacteremia was further confirmed by sequence analyses of the ITS region and the 16S rRNA gene. Antimicrobial susceptibility testing of the 291 isolates from patients with bacteremia revealed that *A. baumannii* strains were less susceptible to antimicrobial agents than *Acinetobacter* gen. sp. 3. All *Acinetobacter* gen. sp. 3 strains were susceptible to ampicillin-sulbactam, imipenem, and meropenem; but only 67.4%, 90%, and 86% of the *A. baumannii* strains were susceptible to ampicillin-sulbactam, imipenem, and meropenem, respectively. The observed significant variations in antimicrobial susceptibility among different species in the *A. calcoaceticus*-*A. baumannii* complex emphasize that the differentiation of species within the complex is relevant from a clinical-epidemiological point of view.

The genus *Acinetobacter* currently comprises 17 validly named and 14 unnamed (genomic) species (12). Of these, *Acinetobacter calcoaceticus*, *A. baumannii*, and unnamed *Acinetobacter* genomic species (gen. sp.) 3 and *Acinetobacter* gen. sp. 13TU are phenotypically similar; and therefore, it has been proposed that these species be referred to as a group, the *A. calcoaceticus*-*A. baumannii* complex (17). Members of the complex are the most common acinetobacters among clinical isolates (2, 13). It is of note, however, that *A. calcoaceticus* is a soil organism and is rarely isolated from clinical specimens (3, 14). In recent years, isolates of the *A. calcoaceticus*-*A. baumannii* complex have become an increasing cause of concern in hospitals worldwide because they can give rise to outbreaks of infection and the strains involved are frequently resistant to commonly used antimicrobial agents, including cephalosporins, aminoglycosides, fluoroquinolones, and carbapenems

(27). Two other *Acinetobacter* gen. sp., indicated as “close to *Acinetobacter* gen. sp. 13TU” and “between *Acinetobacter* gen. sp. 1 and 3,” have been described and allocated to the *A. calcoaceticus*-*A. baumannii* complex (18); but reports on their clinical role have not been published. Grouping of species in the *A. calcoaceticus*-*A. baumannii* complex is unsatisfactory for clinical reasons because it may obscure the inherent variations in the biology and the pathology of each species. Therefore, precise identification of the species within the complex is important for elucidation of the ecology, epidemiology, and pathology of each species and may be helpful for antimicrobial therapy.

Apart from DNA-DNA hybridization, which is recognized as the “gold standard,” reliable identification of different species of the *A. calcoaceticus*-*A. baumannii* complex can be achieved by amplified fragment length polymorphism fingerprint analysis (21), ribotyping (16), and restriction analysis of the 16S rRNA genes (35, 36) of the 16S-23S ribosomal intergenic spacers (ITSs) (14) or the entire rRNA genes (15). Analysis of the sequences of the 16S rRNA gene (35), the *rpoB* gene (23), and the *gyrB* gene (39) have been shown to be promising; but more data from evaluations of multiple strains of each species are required to show whether all currently known spe-

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TABLE 1. Target and nontarget microorganisms used for sensitivity and specificity test with the oligonucleotide array

Species	Reference strain ^a	No. of clinical isolates ^b	Total no. of strains
Target species (<i>A. calcoaceticus</i> - <i>A. baumannii</i> complex) (<i>n</i> = 52)			
<i>Acinetobacter calcoaceticus</i>	BCRC 11562, LMG 992, LMG 1046 ^T	2	5
<i>A. baumannii</i>	BCRC 10591 ^T , BCRC 15884, BCRC 15886, LMG 984	27	31
<i>Acinetobacter</i> gen. sp. 3	BCRC 15420, CCUG 26384, LMG 1035	7	10
<i>Acinetobacter</i> gen. sp. 13TU	BCRC 15417	5	6
Subtotal		41	52
Nontarget species (<i>n</i> = 137)			
<i>Acinetobacter haemolyticus</i>	BCRC 14852, BCRC 15887, LMG 997, LMG 1001	7	11
<i>A. junii</i>	BCRC 14854	5	6
<i>Acinetobacter</i> gen. sp. 6	BCRC 15421	4	5
<i>A. johnsonii</i>	BCRC 14853, BCRC 15888, LMG 1002	10	13
<i>A. lwoffii</i>	BCRC 14855, NCCB 83020	13	15
<i>Acinetobacter</i> gen. sp. 9	LMG 985, LMG 1300	0	2
<i>Acinetobacter</i> gen. sp. 10	BCRC 15423, LMG 10600	3	5
<i>Acinetobacter</i> gen. sp. 11	BCRC 15424, LMG 10603	4	6
<i>A. radioresistens</i>	BCRC 15425, CCUG 26388, CCUG 34434	5	8
<i>Acinetobacter</i> gen. sp. 14BJ	CCUG 14816, CCUG 34435	2	4
<i>Acinetobacter</i> gen. sp. 14TU	LMG 1235, LMG 10627	4	6
<i>Acinetobacter</i> gen. sp. 15BJ	CCUG 34436		1
<i>Acinetobacter</i> gen. sp. 15TU	CCUG 26390	4	5
<i>Acinetobacter</i> gen. sp. 16	BCRC 15883	2	3
<i>Acinetobacter</i> gen. sp. 17	CCUG 34437	1	2
<i>A. parvus</i>		3	3
<i>A. schindleri</i>	LMG 19576	2	3
<i>A. ursingii</i>	LMG 19575	2	3
<i>A. venetianus</i>	CCUG 45561	2	3
Non- <i>Acinetobacter</i> spp. (33 species) ^c			33
Subtotal		73	137

^a ATCC, American Type Culture Collection, Manassas, VA; BCRC, Bioresources Collection and Research Center, Taiwan; CCUG, Culture Collection of the University of Göteborg, Göteborg, Sweden; LMG, Belgian Coordinated Collections of Microorganisms, Brussels, Belgium; NCCB, Netherlands Culture Collection of Bacteria, The Netherlands.

^b Clinical isolates of the *A. calcoaceticus*-*A. baumannii* complex were identified to the species level by ITS sequencing (7).

^c Including *Enterococcus* (two species), *Klebsiella* (two species), *Staphylococcus* (four species), *Streptococcus* (six species), *Vibrio* (three species), non-*Acinetobacter* nonfermenting gram-negative bacilli (eight species), and other bacteria (eight species).

cies can be unambiguously identified. The ITS region separating the 16S and 23S rRNA genes has been suggested to be a good candidate for the species identification of various bacteria (6, 8, 19, 30, 34, 37). In our previous study, the feasibility of ITS sequence analysis for species differentiation of the *A. calcoaceticus*-*A. baumannii* complex was established (7).

Seifert et al. showed that acinetobacters belonging to the *A. calcoaceticus*-*A. baumannii* complex are generally more resistant to antimicrobial agents than other *Acinetobacter* species, such as *A. lwoffii* and *A. johnsonii* (32). The *A. baumannii* isolates reported, however, would represent *A. baumannii* gen. sp. 3 and gen. sp. 13TU, as the phenotypic scheme used (4) was insufficient for their discrimination (17). Due to the difficulty of differentiation of members of the *A. calcoaceticus*-*A. baumannii* complex, the actual distribution of different *Acinetobacter* gen. sp. in blood cultures and, hence, their antimicrobial susceptibility patterns are not clear.

The aims of this study were to construct and validate an oligonucleotide array for the identification of species of the *A. calcoaceticus*-*A. baumannii* complex, to apply this system to the characterization of a large set of blood isolates, and to determine the variation in antimicrobial susceptibilities among the species. The probes of the array were based on the ITS se-

quence specific for species of the *A. calcoaceticus*-*A. baumannii* complex.

MATERIALS AND METHODS

Strains for validation. A total of 52 strains (11 reference strains and 41 clinical isolates) belonging to the *A. calcoaceticus*-*A. baumannii* complex were used to verify the performance of the array developed in this study (Table 1). Clinical isolates of the *A. calcoaceticus*-*A. baumannii* complex were identified to the species level by ITS sequencing (7). For assessment of the specificity of the array, a collection of 137 strains, including 104 strains of *Acinetobacter* spp. other than the *A. calcoaceticus*-*A. baumannii* complex and 33 non-*Acinetobacter* sp. strains, was used (Table 1). Reference strains were obtained from the Bioresources Collection and Research Center (BCRC; Hsinchu, Taiwan), the Culture Collection of the University of Göteborg (Göteborg, Sweden), the Belgian Coordinated Collections of Microorganisms (Brussels, Belgium), and The Netherlands Culture Collection of Bacteria. Clinical isolates were obtained from the National Cheng Kung University Hospital (Tainan, Taiwan), the Kaohsiung Chang Gung Memorial Hospital (Taipei, Taiwan), the Leiden University Medical Center (Leiden, The Netherlands), and the Ghent University Hospital (Ghent, Belgium). All reference strains and clinical isolates were subcultured at 37°C on sheep blood agar for 24 to 48 h.

Bacterial isolates from blood cultures. A total of 291 nonrepetitive isolates recovered from blood cultures over a period of 5.5 years, from June 1999 to December 2005, at the National Cheng Kung University Hospital were analyzed in this study. These isolates were presumptively identified as the *A. calcoaceticus*-*A. baumannii* complex with the API 20NE system (bioMérieux Vitek, Taipei,

TABLE 2. Oligonucleotide probes used to identify (genomic) species of the *Acinetobacter calcoaceticus*-*A. baumannii* complex

Microorganism	Probe information					
	Code	Sequence (5'-3')	Length (nt) ^a	T _m ^b (°C)	Location ^c	Gen Bank accession no.
<i>Acinetobacter</i> spp.	Aci	GAATCGAGCGTTTTGGTATATGAATTttttt ttt ^d	26	56.0	486-511	AY601823
<i>A. calcoaceticus</i> - <i>A. baumannii</i> complex	Acb	GACTGGTTGAAGTTATAGATAAAAGATttt tttt	27	48.6	144-170	AY601823
<i>A. calcoaceticus</i>	Acal	CATTGATCATGTCTTATTACTCCTTGTAG Gtttt	30	56.2	574-613	AY601820
<i>A. baumannii</i>	Abau	CGGTAATTAGTGTGATCTGACGAtttttttt	23	51.3	351-373	AY601823
<i>Acinetobacter</i> gen. sp. 3	Aun3	GATGAAGAATCGCACGGACAACAtttttttt tttt	23	58.6	560-582	AY601829
<i>Acinetobacter</i> gen. sp. 13TU	Aun13TU	GTGGGTAACGTCGACTATtttttttttt	18	41.3	586-603	AY601830
Positive control	PC ^e	TGGGGTGAAGTCGTAACAAGGTAGCCGT Attttttttt	29	65.8	1463-1491	AY616658

^a nt, number of nucleotides.^b T_m, melting temperature.^c The location of the probe is indicated by the nucleotide number in the ITS region.^d t, multiple bases of thymine were added to the 3' end of the probe.^e PC, positive control probe, which was designed from the 3' end of the 16S rRNA gene.

Taiwan) and by tests for the following: growth at 37°C, 41°C, or 44°C; oxidation of glucose; gelatin hydrolysis; and the assimilation of 11 carbon sources (4, 31).

Design of oligonucleotide probes. Four oligonucleotide probes (18- to 30-mers) based on the ITS sequences (Table 2) were designed to identify the four (genomic) species in the *A. calcoaceticus*-*A. baumannii* complex. Multiple-sequence alignment of the ITS fragments was performed by using Vector NTI software (Invitrogen Corporation, Carlsbad, CA), and areas displaying low intraspecies and high interspecies sequence divergences were used for probe synthesis. An *Acinetobacter*-specific probe and an *A. calcoaceticus*-*A. baumannii* complex-specific probe were also designed (Table 2). A positive control probe was designed from a highly conserved region in the 16S rRNA gene (GenBank accession no. AY616658) (29). The designed probes were checked for internal repeats, self-binding, secondary structures, and G+C contents by using the same software. Five to 12 additional bases of thymine were added to the 3' ends of the probes to increase the hybridization signals (5) (Table 2). In addition, a digoxigenin (DIG)-labeled fungus-specific probe (5'-DIG-GCATATCAATAAGCGG AGGA-3') (38) was spotted on the array and used as a position marker for hybridization.

Fabrication of oligonucleotide arrays. The array (4 by 4 mm) contained 12 dots (4 by 3 dots), including one *Acinetobacter*-specific probe (code Aci), one *A. calcoaceticus*-*A. baumannii* complex-specific probe (code Acb), four probes specific for the identification of each of the four species in the *A. calcoaceticus*-*A. baumannii* complex, one positive control probe (code PC), two negative control probes (code NC; tracking dye only), and three position markers (code M) (Fig. 1A). The oligonucleotide probes were diluted 1:1 (final concentration, 10 μM) with a tracking dye solution, drawn into the wells of 96-well microtiter plates, and spotted onto positively charged nylon membranes (Roche, Mannheim, Germany), as described previously (33). However, the final concentrations of the position marker probe and the positive control probe were 0.1 μM and 2.5 μM, respectively. The arrays were prepared with an automatic arrayer (SR-A300; Ezspot, Taipei, Taiwan) by use of a 400-μm-diameter solid pin. Once all the probes had been spotted, the nylon membrane was air dried and exposed to shortwave UV (Stratalinker 1800; Stratagen, La Jolla, CA) for 30 s to immobilize the probes on the membrane.

Amplification of ITS regions. Bacterial DNA was extracted by the boiling method (28). Bacterium-specific universal primers 2F (5'-DIG-TTGT ACACA CCGCCGTC-3') and 10R (5'-DIG-TTCGCCTTCCCTCACGGTA-3') (19) were used to amplify a DNA fragment that encompassed small portions of the 16S rRNA and 23S rRNA genes and the ITS region. Each of the two primers was labeled with a DIG molecule at its 5' end. PCR was performed with 5 μl (50 ng) of template DNA in a total reaction volume of 50 μl consisting of 75 mM Tris-HCl (pH 8.8), 20 mM ammonium sulfate, 1.5 mM MgCl₂, 0.8 mM deoxyribonucleoside triphosphates (0.2 mM each), 1 μM (each) primer, and 1 U of *Taq* DNA polymerase (Fermentas). The PCR program consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), and extension (72°C for 1.5 min) and a final extension step at 72°C for 7 min. A negative control was included with each test run by

replacing the template DNA with sterilized water in the PCR mixture. A PXE0.2 thermal cycler (Hybaid Ltd., Middlesex, United Kingdom) was used for PCR.

Species identification by array hybridization. Unbound oligonucleotides on the array were removed by four washes (2 min each) at room temperature in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate. All reagents used for hybridization except the buffers were included in the DIG nucleic acid detection kit (Roche). The procedures for prehybridization, hybridization (50°C for 90 min), and color development after hybridization with the alkaline phosphatase-conjugated anti-DIG antibodies were the same as those described previously (9). The hybridized spot, which had a diameter of 400 μm, could be easily recognized by the naked eye. The images of the hybridized arrays were captured with a scanner (Powerlook 3000; Umax, Taipei, Taiwan). A strain was identified as one of the (genomic) species of the *A. calcoaceticus*-*A. baumannii* complex when the probes specified for a species, the positive control probe (code PC), the *Acinetobacter*-specific probe (code Aci), and the *A. calcoaceticus*-*A. baumannii* complex-specific probe (code Acb), were all hybridized (Fig. 1).

Sequencing of ITS region and 16S rRNA genes. To confirm the identification results obtained by array hybridization, the identities of 10 randomly selected isolates of *Acinetobacter* gen. sp. 3 and the 2 isolates of *Acinetobacter* gen. sp. 13TU were rechecked by sequencing of the ITS and 16S rRNA genes. Bacterium-specific universal primers 8FPL (5'-GTTTGATCCTGGCTCAG-3') and 1492RPL (5'-GGYTACCTTGTTACGACTT-3') (29) were used to amplify the 16S rRNA genes. The PCR mixtures and thermocycling conditions were the same as those used for ITS region amplification. The PCR products were purified with a PCR-M cleanup kit (Viogene, Taipei, Taiwan) and sequenced in both directions by using the two primers described above and an additional primer, primer 1055r (5'-CACGAGCTGACGACAGCCAT-3'), with the BigDye Terminator cycle sequencing kit (Applied Biosystems, Taipei, Taiwan). The methods used for the amplification of the ITS region were described in the previous section, and PCR products were sequenced by the method of Chang et al. (7). The ITS region and 16S rRNA gene sequences determined were compared to reference sequences in the databases of the National Center for Biotechnology Information by use of the BLASTN algorithm.

Antimicrobial susceptibilities of isolates from patients with bacteremia of the *A. calcoaceticus*-*A. baumannii* complex. Twelve antimicrobial agents were used for susceptibility testing. These agents included ampicillin-sulbactam, piperacillin-tazobactam, ceftazidime, cefepime, imipenem, meropenem, amikacin, ciprofloxacin, co-trimoxazole, doxycycline, colistin sulfate, and tigecycline. The MICs of colistin sulfate and tigecycline were determined with Etest strips (AB Biodisk, Solna, Sweden) (1). Antimicrobial susceptibility testing with the other drugs was performed by the disc diffusion method recommended by the Clinical and Laboratory Standards Institute (11). The susceptibility interpretation for all drugs except tigecycline was determined by use of the recommended zone diameters or MIC breakpoints (11). The MIC breakpoint for tigecycline susceptibility was the FDA breakpoint of 2 mg/liter for members of the family *Enterobacteriaceae* (Tygacil package insert; Wyeth Pharmaceu-

FIG. 1. (A) Layout of oligonucleotide probes on the array (4 by 4 mm). The probe coded NC was the negative control (tracking dye only). The probe coded M was a DIG-labeled probe and was used as a position marker. (B) Hybridization results obtained with four reference strains and six clinical isolates of the *A. calcoaceticus*-*A. baumannii* complex. The six clinical isolates were *A. baumannii* (strains Aci98, Aci100, and Aci104), *Acinetobacter* gen. sp. 3 (strains Aci20 and Aci109), and *Acinetobacter* gen. sp. 13TU (strain Aci167). The cross-hybridization patterns of two strains (*Acinetobacter* gen. sp. 6 BCRC 15421 and *A. radioresistens* BCRC 15425) are also shown.

ticals Inc., Philadelphia, PA). For statistical analysis, Student's *t* test or the Mann-Whitney U test was used for continuous variables. All tests of significance were two tailed, and a *P* value of 0.05 or less was considered statistically significant.

RESULTS

Probe development and array performance. Four oligonucleotide probes were used to differentiate four (genomic) species in the *A. calcoaceticus*-*A. baumannii* complex. Among the strains in the collection used for validation, all 52 strains belonging to the complex hybridized to the *Acinetobacter*-specific probe (code Aci) and the *A. calcoaceticus*-*A. baumannii* complex-specific probe (code Acb). In addition to the genus- and complex-specific probes, *A. calcoaceticus*, *A. baumannii*, *Acinetobacter* gen. sp. 3, and *Acinetobacter* gen. sp. 13TU hybridized to their species-specific probes, probes Acal, Abau, Aun3, and Aun13TU, respectively (Fig. 1B). Therefore, the validation collection of the four species in the complex were well differentiated by array hybridization, resulting in a test sensitivity of 100%. In addition, all *Acinetobacter* spp. listed in Table 1 hybridized to the *Acinetobacter*-specific probe. *Acinetobacter* gen. sp. 6 BCRC 10421 and *A. radioresistens* BCRC 15425 displayed weak cross-hybridization to the probe (code Aun13TU) used to identify *Acinetobacter* gen. sp. 13TU (Fig. 1B). However, the two strains were not misidentified as *Acinetobacter* gen. sp. 13TU since the *A. calcoaceticus*-*A. baumannii* complex-specific probe (code Acb) was not hybridized by both strains; i.e., they were not identified to be part of the *A. cal-*

coaceticus-*A. baumannii* complex. Of 104 *Acinetobacter* strains (19 species) other than the *A. calcoaceticus*-*A. baumannii* complex, including 31 reference strains and 73 clinical isolates, and 33 non-*Acinetobacter* strains tested (Table 1), no strain was misidentified as a species of the *A. calcoaceticus*-*A. baumannii* complex. Therefore, the test specificity of the array was also 100%.

Species distribution of *A. calcoaceticus*-*A. baumannii* complex isolates recovered from blood cultures. By using the oligonucleotide array, 291 isolates of the *A. calcoaceticus*-*A. baumannii* complex from patients with bacteremia were identified to the species level. Of these isolates, 221 (75.9%), 67 (23.0%), and 2 (0.7%) isolates were identified as *A. baumannii*, *Acinetobacter* gen. sp. 3, and *Acinetobacter* gen. sp. 13TU, respectively. No *A. calcoaceticus* strain was detected. The remaining isolate was identified as an *Acinetobacter* sp., since only the *Acinetobacter*-specific probe (code Aci) was hybridized. The proportion of *Acinetobacter* gen. sp. 3 isolates among the complex in the first 3-year period (1999 to 2001) was similar to that in the second period (2002 to 2004) (23/115 [20.0%] and 44/175 [25.1%], respectively; *P* = 0.28). To confirm the identification results obtained with the array, the identities of 10 randomly selected isolates of *Acinetobacter* gen. sp. 3 and the 2 isolates of *Acinetobacter* gen. sp. 13TU were determined by sequencing of the ITS regions and the 16S rRNA genes (Table 3). The identities of these 12 strains, as determined by array hybridization, were confirmed by both ITS region and 16S rRNA gene sequencing.

TABLE 3. Species identification of 10 randomly selected isolates of *Acinetobacter* gen. sp. 3 and two isolates of *Acinetobacter* gen. sp. 13TU, as determined by array hybridization, by sequencing of the ITS and 16S rRNA genes

Strain	<i>Acinetobacter</i> species identification (% identity) by:			Best match
	Array hybridization	ITS sequencing	16S rRNA gene sequencing	
Aci12	<i>Acinetobacter</i> gen. sp. 3	<i>Acinetobacter</i> gen. sp. 3 (99.8)	<i>Acinetobacter</i> gen. sp. 3 (99.4)	<i>Acinetobacter</i> gen. sp. 3
Aci17	<i>Acinetobacter</i> gen. sp. 3	<i>Acinetobacter</i> gen. sp. 3 (99.5)	<i>Acinetobacter</i> gen. sp. 3 (99.3)	<i>Acinetobacter</i> gen. sp. 3
Aci18	<i>Acinetobacter</i> gen. sp. 3	<i>Acinetobacter</i> gen. sp. 3 (99.4)	<i>Acinetobacter</i> gen. sp. 3 (99.7)	<i>Acinetobacter</i> gen. sp. 3
Aci20	<i>Acinetobacter</i> gen. sp. 3	<i>Acinetobacter</i> gen. sp. 3 (99.5)	<i>Acinetobacter</i> gen. sp. 3 (99.8)	<i>Acinetobacter</i> gen. sp. 3
Aci22	<i>Acinetobacter</i> gen. sp. 3	<i>Acinetobacter</i> gen. sp. 3 (99.2)	<i>Acinetobacter</i> gen. sp. 3 (99.4)	<i>Acinetobacter</i> gen. sp. 3
Aci29	<i>Acinetobacter</i> gen. sp. 3	<i>Acinetobacter</i> gen. sp. 3 (99.5)	<i>Acinetobacter</i> gen. sp. 3 (99.7)	<i>Acinetobacter</i> gen. sp. 3
Aci30	<i>Acinetobacter</i> gen. sp. 3	<i>Acinetobacter</i> gen. sp. 3 (99.0)	<i>Acinetobacter</i> gen. sp. 3 (99.8)	<i>Acinetobacter</i> gen. sp. 3
Aci43	<i>Acinetobacter</i> gen. sp. 3	<i>Acinetobacter</i> gen. sp. 3 (99.7)	<i>Acinetobacter</i> gen. sp. 3 (99.7)	<i>Acinetobacter</i> gen. sp. 3
Aci45	<i>Acinetobacter</i> gen. sp. 3	<i>Acinetobacter</i> gen. sp. 3 (99.4)	<i>Acinetobacter</i> gen. sp. 3 (99.6)	<i>Acinetobacter</i> gen. sp. 3
Aci54	<i>Acinetobacter</i> gen. sp. 3	<i>Acinetobacter</i> gen. sp. 3 (100)	<i>Acinetobacter</i> gen. sp. 3 (99.8)	<i>Acinetobacter</i> gen. sp. 3
Aci165	<i>Acinetobacter</i> gen. sp. 13TU	<i>Acinetobacter</i> gen. sp. 13TU (99.0)	<i>Acinetobacter</i> gen. sp. 13TU (99.3)	<i>Acinetobacter</i> gen. sp. 13TU
Aci305	<i>Acinetobacter</i> gen. sp. 13TU	<i>Acinetobacter</i> gen. sp. 13TU (99.0)	<i>Acinetobacter</i> gen. sp. 13TU (99.3)	<i>Acinetobacter</i> gen. sp. 13TU

Antimicrobial susceptibility. The two isolates of *Acinetobacter* gen. sp. 13TU from patients with bacteremia were susceptible to all antimicrobial agents tested. Compared with *Acinetobacter* gen. sp. 3, the *A. baumannii* isolates were less susceptible to the 10 antimicrobial agents tested, and the differences in susceptibility were statistically significant ($P \leq 0.015$) (Table 4). Notably, all *Acinetobacter* gen. sp. 3 strains were susceptible to ampicillin-sulbactam, imipenem, and meropenem. In contrast, only 67, 90, and 86% of the *A. baumannii* strains were susceptible to ampicillin-sulbactam, imipenem, and meropenem, respectively. The colistin sulfate MICs of isolates of *A. baumannii* and *Acinetobacter* gen. sp. 3 from patients with bacteremia ranged from 0.125 to 1 $\mu\text{g/ml}$, and the distributions of the colistin sulfate MICs were similar for isolates of the two species (Fig. 2). The MIC₅₀ was 0.38 $\mu\text{g/ml}$, and the MIC₉₀ was 0.75 $\mu\text{g/ml}$, indicating that colistin is active in vitro against *A. baumannii* and *Acinetobacter* gen. sp. 3. The tigecycline MICs had a wide range, from 0.047 to 12 $\mu\text{g/ml}$. The MIC₅₀ was 0.25 $\mu\text{g/ml}$, and the MIC₉₀ was 2 $\mu\text{g/ml}$. However, there was a bimodal distribution of the tigecycline MICs for the *A. baumannii* isolates (Fig. 2). Two (3.0%) of 67 *Acinetobacter* gen. sp. 3 isolates and 14 (6.3%) of 221 *A. baumannii* isolates, both of which accounted for 16 (5.5%) of 291 isolates, were resistant to tigecycline. All 16 tigecycline-resistant isolates were susceptible to imipenem. Of 9 imipenem-

intermediate *A. baumannii* isolates and 13 imipenem-resistant *A. baumannii* isolates, all were susceptible to tigecycline.

DISCUSSION

The species of the *A. calcoaceticus*-*A. baumannii* complex are difficult to differentiate with routinely used commercial kits, such as the API 20NE system and the Vitek GNI card (BioMérieux, Marcy l'Etoile, France), notwithstanding the fact that the complex includes both the most frequently isolated species (*A. baumannii*) and an environmental species (*A. calcoaceticus*). While the importance of accurate identification for the management of patients may be questionable, it is essential for epidemiological purposes. Therefore, the development of an accurate method for the delineation of different species in the *A. calcoaceticus*-*A. baumannii* complex is imperative. In this study, an oligonucleotide array was successfully developed for this purpose. The accuracy of the array for identifying blood isolates was confirmed by sequence analyses of the ITS regions and the 16S rRNA genes of 12 randomly selected isolates of the complex. With a sensitivity of 100% and a specificity of 100%, the current method provides a rapid and accurate way to identify species of the *A. calcoaceticus*-*A. baumannii* complex. The whole hybridization procedure can be finished within approximately 8 h from the time that colonies are isolated.

In this study, the ITS regions of some strains in the *A. calcoaceticus*-*A. baumannii* complex had a mismatch with the complex-specific probe (probe Acb) at the ninth base (i.e., a G nucleotide) (Table 2). For example, *Acinetobacter* gen. sp. 13TU Aci167 had an A nucleotide at the position (data not shown). Some sequences retrieved from the GenBank database also have this mismatch with the Acb probe, such as *A. baumannii* (GenBank accession no. EU030657), *Acinetobacter* gen. sp. 3 (GenBank accession nos. EU030647 and EU030650), and *Acinetobacter* gen. sp. 13TU (GenBank accession nos. EU030649, EU030653, EU030654, EU030656, AY510070, and AY510071). Since the complex-specific probe had a length of 27 bases and the mismatch was at the ninth base, the remaining 18 bases still had a sufficient hybridization capability with "mismatched" strains. However, this mismatch really caused a decrease in the hybridization signal compared with that obtained

TABLE 4. Antimicrobial susceptibility testing results for clinical blood isolates of *A. baumannii* and *Acinetobacter* gen. sp. 3 for 10 antimicrobial agents

Antimicrobial agent	No. (%) of susceptible isolates		P value
	<i>A. baumannii</i> (n = 221)	<i>Acinetobacter</i> gen. sp. 3 (n = 67)	
Amikacin	113 (51.1)	61 (91)	<0.001
Ampicillin-sulbactam	149 (67.4)	67 (100)	<0.001
Cefepime	126 (57)	59 (88.1)	<0.001
Ceftazidime	105 (47.5)	61 (91)	<0.001
Ciprofloxacin	98 (44.3)	54 (80.6)	<0.001
Co-trimoxazole	68 (30.8)	55 (82.1)	<0.001
Doxycycline	112 (50.7)	63 (94)	<0.001
Imipenem	199 (90)	67 (100)	0.015
Meropenem	190 (86)	67 (100)	0.003
Piperacillin-tazobactam	91 (41.2)	58 (86.6)	<0.001

FIG. 2. Distributions of tigecycline (Tig) and colistin sulfate (Col) MICs among isolates of *Acinetobacter baumannii* ($n = 221$) and *Acinetobacter* gen. sp. 3 ($n = 67$) from patients with bacteremia. The distribution of the colistin sulfate MICs was similar for isolates of both species. However, there was a bimodal distribution of the tigecycline MICs among the *A. baumannii* isolates. Isolate no., number of isolates.

with the complete matched strains. For example, *Acinetobacter* gen. sp. 13TU BCRC 10417 (a strain with a complete match) had a stronger hybridization signal than *Acinetobacter* gen. sp. 13TU Aci167 (a strain with a mismatch) with the complex-specific probe (probe Acb) (lower right corner of the array in Fig. 1). However, all 52 strains (Table 1) of the *A. calcoaceticus*-*A. baumannii* complex successfully hybridized with the complex-specific probe.

In a previous study, Lagatolla et al. (22) developed two oligonucleotides from the ITS regions to identify *A. baumannii* and *Acinetobacter* gen. sp. 3, respectively. However, cross-reactions with the probe used to identify *Acinetobacter* gen. sp. 3 were found at a hybridization temperature of 45°C. For this reason, the four species-specific probes were redeveloped in this study. In addition, the *Acinetobacter*-specific and the *A. calcoaceticus*-*A. baumannii* complex-specific probes were included on the array to eliminate cross-reacting strains that may happen to hybridize to the species-specific probes. Recently, Lin et al. (26) developed a microsphere-based array that combines an allele-specific primer extension assay and microsphere hybridization for the identification of 13 *Acinetobacter* species. However, cross-reaction was a big problem with their array. For example, of 13 strains identified as *Acinetobacter* gen. sp. 3 by the microsphere hybridization assay, four strains (30.8%) were actually *Acinetobacter* gen. sp. 13TU, and a high percentage (15.8%) of *Acinetobacter* gen. sp. 3 strains were misidentified as *Acinetobacter* gen. sp. 13TU by the microsphere assay.

So far, reports on the species distribution of the *A. calcoaceticus*-*A. baumannii* complex isolated from blood cultures are very limited. Significant differences between countries in the distribution among the gen. sp. of isolates obtained from blood cultures and other superficial carriage sites have been noted. For example, *A. baumannii* and *Acinetobacter* gen. sp. 3, identified by amplified 16S rRNA gene restriction analysis, accounted for 19.8% and 40.7% of blood culture isolates in a Hong Kong hospital, respectively (10), while we found the proportions of the two species to be 75.9% and 23.0%, respec-

tively. Apart from prevalence rates which may be biased by the occurrence of epidemic strains, the susceptibility patterns of acinetobacters found in many studies may not be comparable. The difference in antimicrobial susceptibility patterns might partly be caused by the lack of a reliable and practical method that can differentiate members in the *A. calcoaceticus*-*A. baumannii* complex. Our results highlight the importance of delineating gen. sp. in the complex when susceptibility data from different studies are compared.

Most of the clinical study of species identification of *Acinetobacter* isolates found a predominance of *A. baumannii* (13, 20, 25). The other common species of the *A. calcoaceticus*-*A. baumannii* complex were either *Acinetobacter* gen. sp. 3 or *Acinetobacter* gen. sp. 13TU. *Acinetobacter* gen. sp. 13TU has been associated with respiratory tract infections and human carriage and has been recovered from the immediate clinical environment (20). In a recent study, it was the second most common isolate among clinical *Acinetobacter* strains (25.9%) and had the highest rate of resistance to imipenem, partly due to the presence of the VIM-2 metallo-beta-lactamase (24). In the present study, however, *Acinetobacter* gen. sp. 13TU was found to be a minor species causing bacteremia, and notably, the two isolates of *Acinetobacter* gen. sp. 13TU were susceptible to all 12 drugs tested.

The clinical significance of identification of species of the *A. calcoaceticus*-*A. baumannii* complex isolates has recently been demonstrated because of variations in antimicrobial susceptibility (20, 25). In this study, the blood isolates of *A. baumannii* were, in general, more resistant to many of the commonly prescribed antimicrobial agents, including carbapenems, than those of *Acinetobacter* gen. sp. 3. For *A. baumannii* isolates, there was a bimodal distribution of tigecycline MICs, which was not found among the *Acinetobacter* gen. sp. 3 isolates. It indicated that there was a subpopulation (38.8% [113/291]) of *A. baumannii* isolates with decreased susceptibility to tigecycline (MICs ≥ 1 μ g/ml). These results suggest the presence of species variations at least in terms of the antimicrobial resis-

tance of different gen. sp. of the *A. calcoaceticus*-*A. baumannii* complex. However, the major reason for species differentiation is to establish whether there are significant differences in clinical manifestations and outcomes among patients with invasive infections caused by different species of the complex.

The MICs of colistin sulfate for either *A. baumannii* or *Acinetobacter* gen. sp. 3 were less than 2 µg/ml and indicated the promise of the in vitro antibacterial activity of colistin against multidrug-resistant *A. baumannii* isolates. Furthermore, there was no concurrent resistance to imipenem and tigecycline among the 291 isolates from patients with bacteremia. Therefore, no clinical isolate recovered from a patient with bacteremia in our institution during the study period (1999 to 2005) was pandrug resistant, defined as resistance to all currently available antimicrobial agents.

In conclusion, clinical isolates of the *A. calcoaceticus*-*A. baumannii* complex can be quickly and accurately identified with the current array. *A. baumannii* (75.9%) was the predominant species among patients with monomicrobial bacteremia caused by members of the complex and was, in general, more resistant to commercially available antimicrobial agents than *Acinetobacter* gen. sp. 3 and *Acinetobacter* gen. sp. 13TU.

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