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Application of a Microsphere-Based Array for Rapid Identification of *Acinetobacter* spp. with Distinct Antimicrobial Susceptibilities▽†

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Acinetobacter* spp. have emerged as important nosocomial and multidrug-resistant pathogens in the last decade. *A. calcoaceticus*, *A. baumannii*, *Acinetobacter* genospecies 3, and *Acinetobacter* genospecies 13TU are genetically closely related and are referred to as the *A. calcoaceticus-A. baumannii* complex (ACB complex). Distinct *Acinetobacter* spp. may be associated with differences in antimicrobial susceptibility, so it is important to identify *Acinetobacter* spp. at the species level. We developed a microsphere-based array that combines an allele-specific primer extension assay and microsphere hybridization for the identification of *Acinetobacter* spp. This assay can discriminate the 13 different *Acinetobacter* spp. in less than 8.5 h, and it has high specificity without causing cross-reactivity with 14 other common nosocomial bacterial species. The sensitivity of this assay was 100 *A. baumannii* cells per ml of blood, and it could discriminate multiple species in various mixture ratios. The developed assay could differentiate clinical *Acinetobacter* spp. isolates with a 90% identification rate. The antimicrobial susceptibility test showed that *A. baumannii* isolates were resistant to most antimicrobial agents other than imipenem, while the genospecies 3 and 13TU isolates were more susceptible to most antimicrobial agents, especially ciprofloxacin and ampicillin-sulbactam. These results supported the idea that this assay possibly could be applied to clinical samples and provide accurate species identification, which might be helpful for clinicians when they are treating infections caused by *Acinetobacter* spp.

During the last decade, *Acinetobacter* spp. have become the major cause of nosocomial infections. The emergence and spread of multidrug-resistant (MDR) *Acinetobacter* spp. pose an even greater threat to hospitals. The resistance of MDR *Acinetobacter* spp. to many commonly used antibiotics, such as aminoglycosides, fluoroquinolones, cephalosporins, β-lactams, and carbapenems, has been increasingly reported worldwide (7, 12, 15, 22, 24). MDR *Acinetobacter* spp. causing nosocomial infections were first reported in Taiwan in 1998 (12). Furthermore, a significant 3.6-fold increase in nosocomial bloodstream infections and a 2.1-fold increase in all nosocomial infections caused by *Acinetobacter* spp. were noted from 1991 to 2003 (11). MDR *Acinetobacter* spp. have become endemic and constitute a therapeutic problem in hospitals in Taiwan (11, 12, 34).

At least 32 named and unnamed *Acinetobacter* spp. have been described (31). Genospecies 1 (*A. calcoaceticus*), genospecies 2 (*A. baumannii*), genospecies 3, and genospecies 13TU are genetically closely related and are referred to as the *A. calcoaceticus-A. baumannii* complex (ACB complex). Among the members of the ACB complex, *A. baumannii*, genospecies 3, and genospecies 13TU have been implicated in nosocomial infection outbreaks (2). The proper identification of *Acinetobacter* spp. at the species level is important for the application of the appropriate therapy to infections, because differences in antimicrobial efficacy against strains belonging to different species have been demonstrated (26, 28).

To identify *Acinetobacter* spp., many clinical microbiological laboratories routinely use commercial phenotypic methods, but they are unreliable when clinicians are identifying *Acinetobacter* spp. to the species level (9). Therefore, to substitute for phenotypic methods, several molecular methods have been developed for *Acinetobacter* species identification, including amplified 16S ribosomal DNA restriction analysis (30); ribotyping (8); randomly amplified polymorphic DNA; the sequencing of various genes, such as the 16S-23S rRNA gene intergenic spacer (ITS) region (3), the *recA* gene (16), and the *rpoB* gene (18); and amplified fragment length polymorphism fingerprinting (14). However, these methods usually are labor-intensive, time-consuming, or of low reproducibility. Furthermore, they usually need multiple-tube PCR, thus requiring more DNA.

A novel method with convenient, rapid, and multiplexed properties is desirable to overcome these limitations. The microsphere-based array provides the capacity for conducting up to 100 biological reactions simultaneously in a single reaction vessel, and it combines the specificity and reliability of oligonucleotide hybridization analysis with the speed and sensitivity of a flow cytometer (36). Furthermore, this method has been applied reliably to species identification (23), the genotyping (6) of pathogens, and mutation detection (29). In this study, we developed a microsphere-based array for the identification of *Acinetobacter* spp. The antimicrobial susceptibilities of the clinical *Acinetobacter* species isolates also were analyzed.

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TABLE 1. *Acinetobacter* reference strains used in this study

Species	BCRC strain no.[a]	ATCC strain no.	ITS size (bp)	Accession no.
A. calcoaceticus	11562	14987	637	AY601820
A. baumannii	10591	19606	607	AY601823
Genospecies 3	15420	17922	619	AY601829
A. haemolyticus	14852	17906	614	AY601831
A. junii	14854	17908	706	AY601832
Genospecies 6	15421	17979	636	AY601833
A. johnsonii	14853	17909	703	AY601834
A. lwoffii	14855	15309	629	AY601835
Genospecies 10	15423	17942	613	AY601837
Genospecies 11	15424	11171	593	AY601838
A. radioresistens	15425	43998	632	AY601839
Genospecies 13TU	15417	17903	615	AY601830
Genospecies 16	15883	17988	595	AY601844

[a] BCRC, Bioresources Collection and Research Center, Hsichu, Taiwan.

MATERIALS AND METHODS

Bacterial strains. A total of 163 isolates, including 105 *Acinetobacter* species and 58 non-*Acinetobacter* species isolates, were used. Of the 105 *Acinetobacter* species strains, 13 were reference strains, including 4 strains of the ACB complex and 9 strains of other named or unnamed *Acinetobacter* spp. (Table 1), and 92 strains were clinical isolates identified as *A. baumannii* using the API 20NE system. Of the 92 *Acinetobacter* species clinical isolates, each was from a different patient, and no two isolates were from the same outbreak according to clinical history and pulsed-field gel electrophoresis genotyping (data not shown). Fifty-eight non-*Acinetobacter* species clinical isolates were used to detect the specificity. They belonged to 14 species, including *Enterobacter aerogenes* ($n = 3$ strains), *Enterobacter cloacae* ($n = 6$), *Enterococcus faecalis* ($n = 6$), *Enterococcus faecium* ($n = 6$), *Escherichia coli* ($n = 3$), *Klebsiella pneumoniae* ($n = 3$), *Klebsiella oxytoca* ($n = 5$), *Proteus mirabilis* ($n = 4$), *Pseudomonas aeruginosa* ($n = 5$), *Staphylococcus aureus* ($n = 5$), *Staphylococcus capitis* ($n = 2$), *Staphylococcus epidermidis* ($n = 3$), *Staphylococcus haemolyticus* ($n = 1$), and *Stenotrophomonas maltophilia* ($n = 6$). Reference strains were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan), and all clinical isolates were obtained from the National Taiwan University Hospital (Taipei, Taiwan). All isolates were cultured on tryptic soy agar plates at 37°C for 20 h.

Species identification on the microsphere-based array. An overview of the microsphere-based array is given in Fig. 1.

(i) PCR. The total genomic DNA of the strain was extracted as described previously (35). The bacterium-specific universal primers used were the following: forward primer, 5′-GTCGTAACAAGGTAGCCGTA-3′; reverse primer, 5′-GGGTTYCCCCRTTCRGAAAT-3′ (where Y is C or T and R is A or G). The primers were used to amplify a DNA fragment encompassing part of the 16S rRNA gene region, the ITS, and part of the 23S rRNA gene region as previously described (3).

To remove unincorporated deoxynucleotide triphosphates and primers, we added 1 μl of shrimp alkaline phosphatase (1 U/μl; USB) and 1 μl of exonuclease I (10 U/μl; USB) to each 20 μl of PCR product. The samples were incubated at 37°C for 30 min and then inactivated at 80°C for 15 min. The purified PCR products then were used as templates for the primer extension reactions.

(ii) Multiplex ASPE. The primers for multiplex allele-specific primer extension (ASPE) and their target species are shown in Table 2. All of the extension primers were designed to possess a melting point of 50 to 57°C, and each primer was appended at the 5′ end with a 25-mer ZipCode oligonucleotide (4, 13). Reverse complements of the 25-mer ZipCode (cZipCode) oligonucleotides were attached to the given bead sets as described above. An *Acinetobacter* genus-specific extension primer (UniA) was added to each reaction mixture to serve as a measurement of PCR amplification and primer extension success for each sample.

***(a)* Coupling cZipCode oligonucleotides to microspheres.** A total of 2.5×10^6 carboxylated beads (Luminex, TX) per assay were pelleted, resuspended in 50 μl 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 4.5 (Sigma), and mixed with 1 mM cZipCode oligonucleotide, which contained a 5′ amino 12-carbon linker as previously described (4). A 3-μl aliquot of fresh 1-ethyl-3-3(3-3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) solution (10 mg/ml) (Pierce Biotechnology), with amine-modified cZipCodes attached to the carboxylated beads, was added to the bead-cZipCode mixture and incubated at room temperature in the dark for 30 min. Another 3-μl aliquot of fresh EDC solution was added and incubated at room temperature in the dark for 30 min. After incubation with EDC, 0.5 ml of 0.02% Tween 20 was added; the beads then were vortexed and centrifuged at 8,000 rpm for 2 min. The supernatant was aspirated, and 0.5 ml of 0.1% sodium dodecyl sulfate was added. The beads were vortexed and then centrifuged again at 8,000 rpm for 2 min. The supernatant was aspirated, and the beads were resuspended in 50 μl Tris-EDTA. The prepared beads were stored in the dark at 4°C until use.

***(b)* Primer extension.** The primer extension reactions were carried out in 20-μl volumes of ASPE buffer (2 mM Tris-HCl, pH 8.4, 5 mM KCl) containing 2.0 mM $MgCl_2$; 0.75 U *Tsp* DNA polymerase (Invitrogen, Carlsbad, CA); 5 μM (each) dATP, dTTP, and dGTP; 5 μM biotin-dCTP (Invitrogen); and 25 nM of each extension primer. The primer extension reaction steps were the following: preheating at 96°C for 2 min, and then 35 cycles consisting of 96°C for 30 s, 55°C for 1 min, and extension at 72°C for 2 min. The samples then were held at 4°C until hybridization took place.

(iii) Hybridization. The bead sets were diluted using 1.5× tetramethylammonium chloride (TMAC) solution (Sigma, St. Louis, MO) that contained 4.5 M TMAC, 0.15% Sarkosyl, 75 mM Tris-HCl (pH 8.0), and 6 mM EDTA (pH 8.0), such that a total of 33 μl of 1.5× TMAC solution contained 5,000 beads. The bead solution was added to 17 μl of the extension products and mixed by being pipetted up and down five times, and then it was incubated in the dark at an

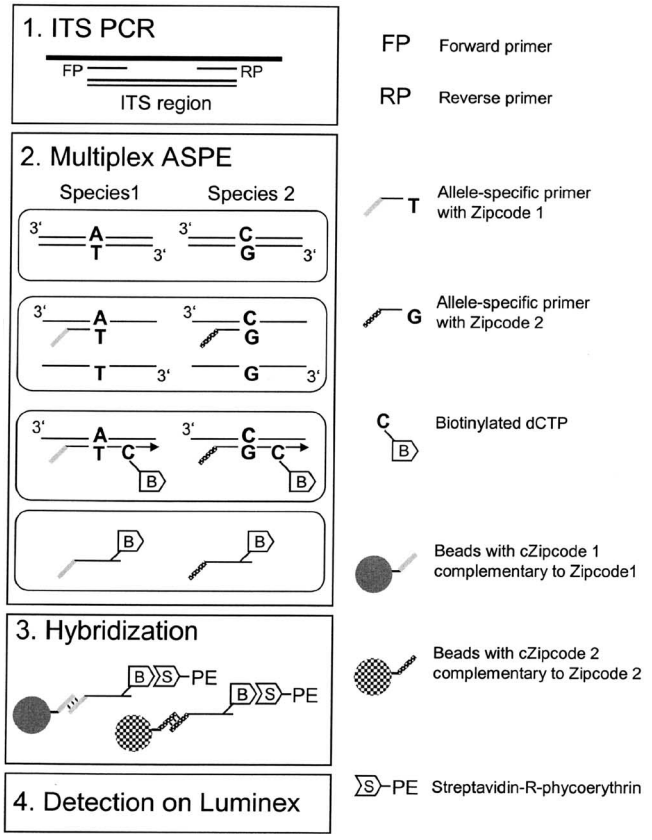

FIG. 1. Overview of species identification by microsphere-based array. (Step 1) ITS PCR. The ITS region of 13 distinct *Acinetobacter* species was amplified, and the PCR product was treated with shrimp alkaline phosphatase and exonuclease I. (Step 2) Multiplex ASPE. ASPE primers with specific ZipCode sequences overlapped the SNP site in the ITS region, and only the correctly hybridized primer was extended. ASPE primers were extended by *Tsp* DNA polymerase, and biotinylated dCTP was incorporated into the extended primers. (Step 3) Hybridization. The extended primers with specific ZipCodes were hybridized with specific beads with cZipCodes. Streptavidin–R-phycoerythrin bound to the biotin in the ASPE primers. (Step 4) Detection on the flow cytometer. Specific beads were sorted, and the fluorescent signals of phycoerythrin were measured.

TABLE 2. ASPE species-specific primers and measurement values

Primer	Identified species	ZipCode[a]	Primer sequence	Signal range (MFI)		Minimum ratio[d]
				Negative[b]	Positive[c]	
P-1	*A. calcoaceticus*	20	CCTACAAGGAGTAATAAGACATGA	0–26	2,792–3,523	107.4
P-2	*A. baumannii*	17	GATCTTGGTTTATTAACTTCTGTGATTTCAT	0–445	9,831–11,011	22.1
P-3	Genospecies 3	27	ACCCCAAACAGTCGTCAAC	7–282	6,069–6,256	21.5
P-4	*A. haemolyticus*	3	GAATACAGTCTAAGTTGACTGGTTTG	0–31	4,916–5,963	158.6
P-5	*A. junii*	14	GATGAATAATCACAAGCTGCTAG	0–31	14,445–15,698	466.0
P-6	Genospecies 6	22	AACAAGTTGTTCTTCTTGAAGATA	0–45	5,895–7,200	131.0
P-7	*A. johnsonii*	24	TGCTGAATACAGAAAAACAGAG	0–33	11,210–12,082	339.7
P-8	*A. lwoffii*	37	CTCTCCTAGTCTCCACCATC	57–336	3,472–3,979	10.3
P-10	Genospecies 10	40	TCCTAGTCTCCACCACTACTAC	8–1,686[e]	7,457–7,631	4.4
P-11	Genospecies 11	41	GTCAACGGTTCGACTCCGT	0–48	11,084–11,632	230.9
P-12	*A. radioresistens*	44	AGCAGTAATGCAGAAAAACAGATATG	0–30	5,480–9,747	182.7
P-13	Genospecies 13TU	50	CACCATGACTTTGACTGGTTA	0–361	12,153–13,457	33.7
P-16	Genospecies 16	49	GTTGCAAACTTAATTGCTACTATTC	0–91	4,962–6,162	54.5
UniA	All species	62	AACTAAGCTAAGTCCCCAGC	NA[f]	9,127–14,703	NA

[a] Selected from the *Mycobacterium tuberculosis* genome (4).
[b] Range of MFI for all strains showing negative MFI after subtracting the MFI of the PCR negative-control value for the given primer.
[c] Range of MFI for all strains showing positive MFI after subtracting the MFI of the PCR negative-control value for the given primer.
[d] The lowest recorded positive MFI divided by the highest recorded negative MFI.
[e] The genospecies 5 strain cross-reacted with P-10 at about 23% of the signal of the genospecies 10 strain.
[f] NA, not applicable.

initial denaturation temperature of 95°C for 5 min, followed by 30 min of incubation at 40°C. After being incubated, the mixture was centrifuged at 8,000 rpm and the supernatant carefully discarded. Seventy microliters of 1× TMAC solution containing 10 ng/μl streptavidin–R-phycoerythrin (Molecular Probes, Eugene, OR) then was added to each sample and incubated for 10 min in the dark at 40°C.

(iv) Detection on flow cytometer and calculation. Samples were measured on the basis of fluorescence intensity in a Bio-Plex 200 suspension array system (Bio-Rad Laboratories, Inc., Hercules, CA). The median fluorescence intensities (MFI) were calculated from 100 replicate measurements with a digital signal processor and Bio-Plex Manager 4.1.1 software. The minimal ratio is the lowest recorded positive MFI divided by the highest recorded negative MFI. Values twice the minimal ratio were used as a threshold for defining positive events.

Sensitivity and multiple-species detection. The sensitivity of the microsphere-based array was tested by spiking pooled blood from three healthy individuals with serial dilutions of *A. baumannii* cells ranging from 1 to 10^4 CFU per ml and serial dilutions of genomic DNA ranging from 10 to 10^{-4} ng. The total DNA of each sample was extracted using a QIAamp DNA blood kit.

To test the multiple-species detection capability, 10 pg of genomic DNA from genospecies 3 and 13TU was used, while the amount of *A. baumannii* was increased gradually from 100- to 10,000-fold to simulate the inconsistent ratios of mixed infections.

Antimicrobial susceptibility testing. MICs of the antimicrobial agents ciprofloxacin, imipenem, meropenem, ampicillin-sulbactam, aztreonam, gentamicin, cefazidime, and cefepime for the clinical isolates were determined by an agar dilution method (32) according to guidelines of the Clinical and Laboratory Standards Institute (5).

RESULTS

Confirmation of ITS PCR products. ITS products were electrophoresed on 2% agarose gels to confirm the success of PCR amplification. ITS fragments of 593 to 706 bp were successfully amplified from 13 reference strains belonging to different species as well as 92 clinical *Acinetobacter* species isolates (data not shown).

ASPE assay and specificity. Table 2 shows the negative and positive ranges for all species-specific ASPE primers and minimum ratios. The positive MFI of 13 species-specific primers ranged from 2,792 to 15,698, with minimum ratios from 4.4 to 466. The *Acinetobacter* genus-specific primer (UniA) was positive for all 13 reference strains, and the MFI ranged from 9,127 to 14,703. The species-specific primer P-10 generated a low minimum ratio of 4.4, because it partially cross-reacted with genospecies 5. In addition, we also tested the specificity of species-specific ASPE primers for the other 14 non-*Acinetobacter* bacterial species that frequently cause nosocomial infections (see Fig. S1 in the supplemental material). No cross-reactivity with the 58 non-*Acinetobacter* isolates was found. This suggested that all species-specific ASPE primers have a high specificity for the identification of *Acinetobacter* spp.

Sensitivity and multiple-species detection. To determine the sensitivity of the ASPE assay, a distinct number of *A. baumannii* cells (from 1 to 10^4 CFU per assay) were spiked into pooled human blood and detected by the ASPE assay (Fig. 2A). By relying on the 2× minimal ratio, the sensitivity of the ASPE assay was as few as 100 CFU per ml of blood, and the smallest amount of genomic DNA required for detection for all four species of the ACB complex was 1 pg (data not shown). To evaluate whether the ASPE assay could accurately detect individual species in a mixture of multiple species, genomic DNA from three major *Acinetobacter* clinical strains, *A. baumannii*, genospecies 3, and genospecies 13TU, were mixed in various ratios, and PCR amplification and an ASPE assay were conducted (Fig. 2B). Despite the fluorescence signals of genospecies 3 and 13TU being partially influenced by the presence of DNA from other species, the results of the ASPE assay clearly discriminated the three *Acinetobacter* species from the multiple DNA sources at inconsistent ratios, all exceeding the 2× minimal ratio.

Species identification of clinical isolates. To further explore the feasibility of the ASPE assay for the identification of clinical *Acinetobacter* species isolates, 92 clinical isolates previously assigned to *A. baumannii* by the API 20NE system were analyzed by ITS sequencing and the ASPE assay (Table 3). The species identities of clinical isolates were assigned according to the maximum similarities between the ITS sequences and the ITS database of the NCBI. A total of 56 isolates were identified as *A. baumannii* by the ASPE assay as well as by ITS sequenc-

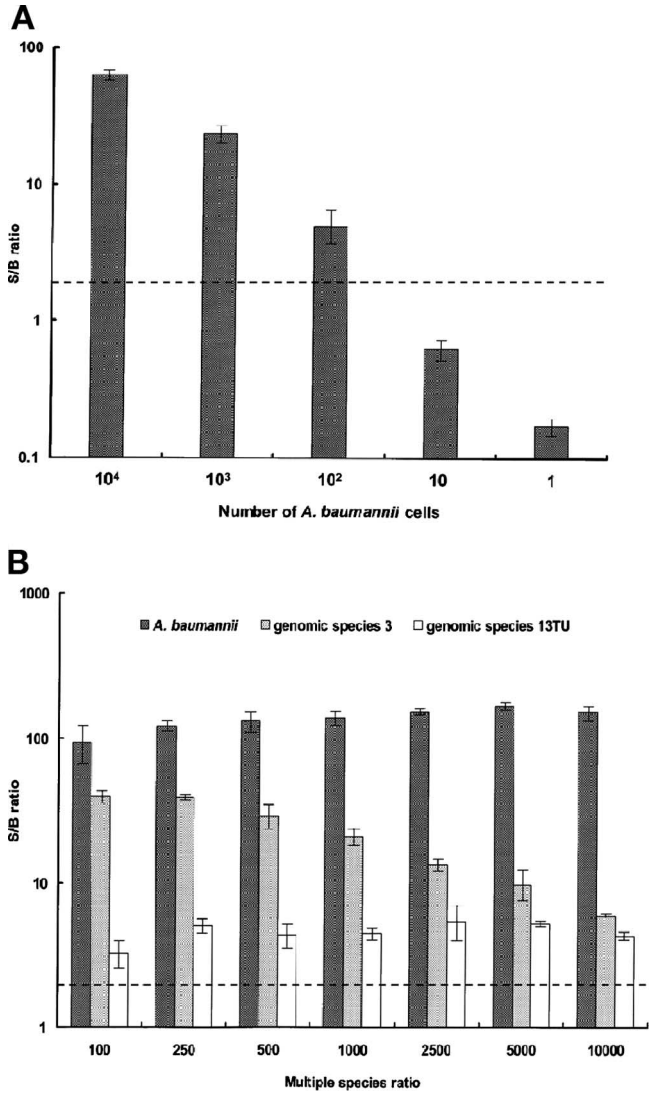

FIG. 2. Sensitivity and detection of distinct DNA ratios of multiple species by the ASPE assay. (A) A distinct number of *A. baumannii* cells (from 10^4 to 1 cell per assay) were spiked into pooled human blood and detected by the ASPE assay. The dotted line indicates the 2× minimal ratio. (B) Genomic DNAs of *A. baumannii*, genospecies 3, and genospecies 13TU were mixed in distinct ratios. The amounts of DNA of genospecies 3 and genospecies 13TU were invariable (10 pg), while the amount of DNA of *A. baumannii* was increased progressively from 100- to 10,000-fold. PCR amplification and the ASPE assay then were conducted to detect the distinct DNA ratios of multiple species. Each column represents the mean (error bars indicate standard deviations). The S/B ratio is the MFI after subtracting the PCR negative-control value and dividing by the PCR negative-control value.

ing. Among the 36 non-*A. baumannii* isolates identified by the ASPE assay, 10 were found to exhibit results discrepant from those of ITS sequencing.

Among the 15 isolates identified as genospecies 3 by ITS sequencing, 10 isolates were assigned to genospecies 3, 4 isolates were assigned to genospecies 13TU, and 1 isolate was identified as belonging to genospecies 3 and 13TU concurrently. Among the 17 isolates identified as genospecies 13TU by ITS sequencing, 13 isolates were assigned to genospecies 13TU, 3 isolates were assigned to genospecies 3, and 1 isolate was identified to be simultaneously genospecies 3 and genospecies 13TU by the ASPE assay. Three isolates were nonidentifiable by the ASPE assay. Using ITS sequencing, one was identified as *Acinetobacter* sp. strain ADP1, and the other two isolates showed an ITS similarity to the 13 reference species of 81 to 85%. In summary, 79 of the 88 clinical isolates could be assigned to species of the ACB complex using the ASPE assay, with an identification rate of 90%.

Antimicrobial susceptibilities. The antimicrobial susceptibility test showed that 56 clinical *A. baumannii* isolates were resistant to most antimicrobial agents, including ciprofloxacin, meropenem, ampicillin-sulbactam, aztreonam, gentamicin, cefazidime, and cefepime, with resistance rates of 54 to 95%, but only 32% were resistant to imipenem (Table 3). Fewer genospecies 3 and 13TU isolates than other isolates were susceptible to imipenem, meropenem, aztreonam, gentamicin, cefazidime, and cefepime, but more were susceptible to ciprofloxacin and ampicillin-sulbactam. Thus, the antimicrobial agent to which *A. baumannii* isolates were most susceptible was imipenem, but the agents to which genospecies 3 and 13TU isolates were most susceptible were ciprofloxacin and ampicillin-sulbactam.

DISCUSSION

Although other molecular methods for *Acinetobacter* species identification are used, they are not ideal for rapid or large-scale applications. The microsphere-based array platform can be finished within 8.5 h (20) and is very suitable for high-throughput applications. The ASPE assay is based on the design of a single-nucleotide polymorphism (SNP)-specific nucleotide at the 3′ end of each extension primer and can readily discriminate any SNP or mutant. The utilization of the ZipCode/cZipCode hybridization enabled universal hybridization with microspheres (29, 36). The cZipCode-coupled beads also can be used in other DNA-based tests, which will economize bead usage.

In this study, the results showed that the microsphere-based array platform can be applied to the multiplexed identification of 13 reference *Acinetobacter* spp. For the identification of 92 clinical isolates, 88 isolates belonging to the ACB complex have been discriminated, of which 56 *A. baumannii* isolates were accurately identified by the ASPE assay. The results show that the most common clinical *Acinetobacter* species isolates in Taiwan are *A. baumannii* isolates. However, discrepant identification results between the ASPE assay and ITS sequencing were found for nine isolates belonging to genospecies 3 and 13TU. It was presumed that the interspecies diversity between genospecies 3 and 13TU clinical strains was low.

To assess the specificity of the ASPE assay, 14 different non-*Acinetobacter* species nosocomial bacteria were subjected to the ASPE assay with all 13 species-specific ASPE primers and UniA. The data (see Fig. S1 in the supplemental material) clearly demonstrated that all ASPE primers were highly specific for their targeted genus and species. The high specificity of the ASPE assay may be advantageous in nosocomial pathogen detection. The sensitivity of the ASPE assay is fewer than 100 CFU per ml of blood, which suggests that the ASPE assay can detect target species in human blood. In addition, the result of

TABLE 3. Clinical isolates of *Acinetobacter* spp. identified by the ASPE assay, ITS sequencing, and antimicrobial susceptibility tests

Identified species	No. of clinical isolates identified by:		No. (%) of strains resistant to[a]:							
	ASPE assay	ITS sequencing	CIP	IPM	MEM	SAM	ATM	GEN	CAZ	FEP
A. baumannii	56	56	53 (95)	18 (32)	33 (59)	30 (54)	53 (95)	52 (93)	54 (96)	38 (69)
Genospecies 3	13[b]	15	0	6 (40)	5 (33)	1 (7)	7 (50)	5 (33)	6 (40)	4 (27)
Genospecies 13TU	19[c]	17	0	2 (12)	4 (24)	1 (6)	6 (35)	5 (29)	3 (18)	1 (6)
Genospecies 10	1	1	0	1 (100)	1 (100)	0	1 (100)	0	1 (100)	1 (100)
Acinetobacter sp. strain ADP1	NA[d]	1	0	0	0	0	0	1 (100)	0	0
Other species[e]	3	2	0	1 (50)	0	0	0	1 (50)	1 (50)	0

[a] The drug abbreviations and their MICs are the following: CIP, ciprofloxacin (≥4 mg/liter); IPM, imipenem (≥16 mg/liter); MEM, meropenem (≥16 mg/liter); SAM, ampicillin-sulbactam (≥16 mg/liter); ATM, aztreonam (≥32 mg/liter); GEN, gentamicin (≥32 mg/liter); CAZ, ceftazidime (≥32 mg/liter); and FEP, cefepime (≥32 mg/liter).

[b] Five isolates yielded results that were discrepant from those determined by sequencing, with four isolates that were assigned to genospecies 3 by sequencing being assigned to genospecies 13TU by ASPE and one isolate with genospecies 3 and genospecies 13TU signals concurrently.

[c] Four discrepant isolates, with three isolates that were assigned to genospecies 13TU by sequencing being assigned to genospecies 3 by ASPE and one with concurrent genospecies 3 and genospecies 13TU signals.

[d] NA, not applicable.

[e] Isolates identified as not belonging to the 13 reference *Acinetobacter* species.

the simulated multiple *Acinetobacter* species infections suggested that multiple species could be discriminated by the ASPE assay despite being present in the mixture at different ratios. Polymicrobial bacteremia was identified in 5 to 22% of bacteremia cases (25, 33), and blood cultures may be inefficient in detecting polymicrobial bacteremia (27). Therefore, these data suggested that the ASPE assay is useful for clinical application.

Further investigations are required to define the clinical significance of *Acinetobacter* spp. other than *A. baumannii* (2). In this study, a combination of the identified results and antimicrobial susceptibilities of the clinical isolates showed that most *A. baumannii* isolates were resistant to most antimicrobial agents other than imipenem, but the majority of genospecies 3 and 13TU isolates were susceptible to ciprofloxacin and ampicillin-sulbactam. There are a few reports describing the significant differences of antimicrobial susceptibility among members of the ACB complex. The antimicrobial patterns described in Korean reports by Lim et al. (21) and Lee et al. (19) are similar to those of our findings: almost all *A. baumannii* isolates were highly resistant to most antimicrobial agents except carbapenems, while *Acinetobacter* genospecies 3 and 13TU isolates mostly were susceptible to ciprofloxacin and ampicillin-sulbactam. In Hong Kong, Houang et al. also reported significant differences in the antimicrobial susceptibilities of isolates of *A. baumannii*, genospecies 3, and genospecies 13TU and suggested that the delineation of genospecies is important in surveillance studies of antimicrobial susceptibilities (10). During the study period, tigecycline and colistin, the two antimicrobial agents with encouraging activity against MDR *Acinetobacter* spp., were not available in Taiwan. In many countries, carbapenems (e.g., imipenem and meropenem) have been the drugs of choice against *Acinetobacter* infections and have retained better activity than other antimicrobial agents (2). However, carbapenem-resistant *Acinetobacter* spp. have been reported worldwide (1) and are rapidly increasing in prevalence, from 5.88% in 1993 to 21.5% in 2000 in Taiwan (12). Our results suggested that accurately differentiating genospecies 3 and 13TU from *A. baumannii* isolates is significant because of their differences in antimicrobial susceptibility. Ciprofloxacin or ampicillin-sulbactam might be a better choice than carbapenems for the treatment of the *Acinetobacter* genospecies 3 and 13TU infections. A recent clinical report in Taiwan suggested that combined carbapenem and ampicillin-sulbactam regimens were associated with a better outcome than the combination of carbapenem and amikacin or carbapenem alone (17). These conclusions might provide clinicians with information for the treatment of *Acinetobacter* infections.

In conclusion, the microsphere-based array is rapid and reliable and has a multiplex capability for the identification of *Acinetobacter* spp. This method may be of help in clinical applications. Furthermore, distinct resistance patterns among *Acinetobacter* spp. also were observed, thus highlighting the importance of accurate species identification.

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