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Identification of Epidemic Strains of *Acinetobacter baumannii* by Integrase Gene PCR

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Forty-eight clinical *Acinetobacter* isolates with different epidemic behavior were investigated for the presence of integrons and plasmids and for antibiotic susceptibility. Integrons were demonstrated in 50% of the strains by an integrase gene PCR. Epidemic strains of *Acinetobacter baumannii* were found to contain significantly more integrons than nonepidemic strains. Also, the presence of integrons was significantly correlated with simultaneous resistance to several antibiotics. Plasmids were detected in 42% of the strains. However, there was no significant correlation between the numbers of plasmids and integrons in *Acinetobacter* species strains, no significant difference in the number of plasmids between epidemic and nonepidemic *A. baumannii* strains, and no significant correlation between the presence of plasmids and antibiotic resistance. Hence, it is likely that integrons play an important role in antibiotic resistance and thereby in the epidemic behavior of *A. baumannii*. Because the integrase gene PCR identified almost three-quarters of the epidemic *A. baumannii* isolates (17 of 23), this seems to be a rapid and simple technique for the routine screening and identification of clinical *A. baumannii* isolates with epidemic potential.

Acinetobacter baumannii is an important opportunistic pathogen responsible for severe nosocomial infections, especially in intensive-care-unit (ICU) patients (3). The majority of infections are of epidemic origin, and treatment has become difficult because many strains are resistant to a wide range of antibiotics, including broad-spectrum β-lactams, aminoglycosides, and fluoroquinolones (13, 20, 24, 27). Studies of antibiotic resistance mechanisms in *A. baumannii* have demonstrated the presence of specific genes located on transferable plasmids and transposons (1, 22, 26). Natural transformation has been described in *Acinetobacter calcoaceticus*, but its role in the genetic spread of antibiotic resistance within clinical *A. baumannii* isolates has yet to be defined (15).

In recent years, a novel mechanism of resistance gene dissemination among bacteria has been described (25). This mechanism is based on the location of these genes on integrons. Integrons are conserved, transposon-like DNA elements which have the ability to capture and mobilize gene cassettes. Insertion and excision of these cassettes occur via a site-specific recombinase that belongs to the integrase family. A distinguishing feature of an integron is the presence of three components within the conserved 5′ region: (i) an integrase gene (*intI*) encoding the IntI integrase, (ii) a gene (*attI*) encoding the cassette integration site, and (iii) one or more promoters responsible for the expression of gene cassettes if present. Based on the sequence of their *intI* genes, four classes of integrons have been described, three of which (classes 1 to 3) contain antibiotic resistance gene cassettes. At present, approximately 60 different gene cassettes have been identified, most of which encode resistance to antibiotics (6, 8, 17, 25). Class 1 integrons are predominantly associated with a *sulI* gene as part of a 3′-conserved segment (25). Integrons of class 2 include transposon Tn*7* and relatives (9, 16). In class 3 only one integron has been described (2, 21). The majority of integrons belong to class 1 and have been found predominantly in clinical isolates of gram-negative bacteria, including *Acinetobacter* species (14, 18, 19, 23; M. E. Jones, E. Peters, A. M. Weersink, A. Fluit, and J. Verhoef, Letter, Lancet **349:**1742–1743, 1997). *A. baumannii* strains may vary considerably in their epidemiological potential, and those strains that have been known to spread widely and rapidly among hospitalized patients have been designated epidemic *A. baumannii* strains. Antibiotic resistance has been shown to be one of the factors which can influence the nosocomial dissemination of *A. baumannii* (5). In this study the presence of integrons and plasmids was investigated in a collection of unrelated epidemic and sporadic *Acinetobacter* isolates from different parts of the world. In addition, the association of integrons and plasmids with antibiotic resistance and epidemic behavior was determined.

MATERIALS AND METHODS

Strains. The *Acinetobacter* strains used in the present study comprise two sets of isolates. The first set consisted of 25 isolates recovered from patients from 25 independent hospital outbreaks in 11 countries (Table 1). *Acinetobacter* strains from The Netherlands were obtained from recognized nosocomial outbreaks, and strains from other countries were obtained from reported outbreaks. These epidemic strains were each isolated from at least three different patients. The second set consisted of 25 *Acinetobacter* species strains that were isolated only once from patients in each outbreak hospital; these strains were defined as nonepidemic, or sporadic.

Identification of strains. Presumptive identification of the isolates was performed by the analytical profile index procedure (API 20NE system; bioMérieux, Marcy l'Etoile, France). Species identification was confirmed by amplified fragment length polymorphism (AFLP), as described previously (11). All strains

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TABLE 1. Epidemiological data and results of integron and plasmid analysis of 50 clinical *Acinetobacter* species strains used in this study

Strain no.[a]	Location of hospital[b]	Yr of isolation	Body site or tract	Ward type	No. of patients involved	Presence of plasmids	Integron PCR result	Integrase gene PCR result[c]
1O	Amsterdam (NL)	1994	Respiratory	Surgical	36	−	+	1
2O	Groningen (NL)	1987	Urine	Surgical	10	−	+	1
3O	Amsterdam (NL)	1995	Respiratory	ICU	5	+	−	2
4O	Amsterdam (NL)	1997	Respiratory	ICU	10	+	−	−
5O	Rotterdam (NL)	1997	Skin/mucosa	Burns unit	6	+	−	−
6O	Rotterdam (NL)	1997	Digestive	ICU	6	−	+	1
7O	Dordrecht (NL)	1995	Skin/mucosa	ICU	5	+	+	1
8O	Utrecht (NL)	1997	Digestive	ICU	6	−	+	1
9O	Nottingham (U.K.)	1992	Respiratory	ICU	37	−	+	1
10O	Brussels (B)	1990	Blood	Surgical	9	−	−	1
11O	New York (U.S.)	1991	Digestive	ICU	59	−	−	−
12O	Trieste (I)	1996	Respiratory	ICU	>3	+	+	1
13O	Madrid (S)	1992	Respiratory	ICU	>100	−	+	1
14O	Ghent (B)	1991	Unknown	ICU	>8	−	+	1
15O	Leeds (U.K.)	1993	Respiratory	ICU	5	−	−	−
16O	Paris (F)	1991	Unknown	ICU	31	−	+	1
17O[d]	Berlin (G)	1990	Respiratory	ICU	Unknown	+	−	−
18O	Freiburg (G)	1994	Unknown	ICU	5	+	+	1
19O[d]	Berlin (G)	1992	Respiratory	ICU	13	+	−	−
20O	Hamburg (G)	1996	Respiratory	ICU	3	−	−	−
21O	St. Etienne (F)	1993	Respiratory	Surgical	15	−	+	1
22O	Vienna (A)	1996	Respiratory	ICU	13	−	+	1
23O	Barcelona (S)	1986	Respiratory	ICU	25	−	+	1
24O	Prague (CR)	1994	Skin/mucosa	Burns unit	Unknown	−	+	1
25O	Toronto (Can)	1993	Respiratory	ICU	121	+	−	−
1N[e]	Amsterdam (NL)	1994	Respiratory	ICU	1	−	−	−
2N	Groningen (NL)	Unknown	Unknown	Unknown	1	Not tested	+	1
3N[f]	Amsterdam (NL)	1997	Skin/mucosa	Outpatient	1	−	−	−
4N	Amsterdam (NL)	1997	Skin/mucosa	Surgical	1	−	+	1
5N	Rotterdam (NL)	1997	Respiratory	Pediatric	1	−	−	−
6N	Rotterdam (NL)	1997	Blood	ICU	1	−	−	−
7N	Dordrecht (NL)	1997	Blood	ICU	1	+	−	−
8N[g]	Utrecht (NL)	1997	Digestive	ICU	1	−	−	−
9N	Nottingham (U.K.)	1994	Respiratory	ICU	1	−	−	−
10N	Brussels (B)	1990	Respiratory	Urology	1	+	+	1
11N	New York (U.S.)	Unknown	Respiratory	Internal	1	+	−	−
12N	Trieste (I)	1993	Urine	Urology	1	−	+	1
13N	Madrid (S)	1992	Respiratory	ICU	1	+	+	1
14N[e]	Ghent (B)	1993	Unknown	ICU	1	−	−	−
15N	Leeds (U.K.)	1993	Respiratory	ICU	1	+	−	−
16N[f]	Paris (F)	Unknown	Unknown	Unknown	1	+	−	−
17N	Kiel (G)	Unknown	Respiratory	Unknown	1	−	−	−
18N[f]	Freiburg (G)	1994	Unknown	Pediatric	1	+	−	−
19N	Berlin (G)	Unknown	Urine	Unknown	1	−	+	1
20N	Hamburg (G)	1997	Respiratory	ICU	1	−	−	−
21N[f]	St. Etienne (F)	1992	Pus	Geriatric	1	+	−	−
22N	Vienna (A)	1996	Blood	Oncology	1	+	−	−
23N	Barcelona (S)	1996	Urine	Surgical	1	−	+	1
24N	Prague (CR)	1992	Skin/mucosa	Burns unit	1	+	−	−
25N[f]	Toronto (Can)	1993	Skin[h]	Unknown	1	+	−	−

[a] Strains with the same number originated from the same location, except for isolates 17O and 17N. O, outbreak strains; N, nonoutbreak strains. Strains were identified as *A. baumannii* except where otherwise specified.

[b] Country names are abbreviated in parentheses as follows: A, Austria; B, Belgium; Can, Canada; CR, Czech Republic; F, France; G, Germany; I, Italy; NL, The Netherlands; S, Spain; U.K., United Kingdom; U.S., United States.

[c] 1, *intI1* detected; 2, *intI2* detected; −, no integrase gene detected.

[d] *Acinetobacter* DNA group 13.

[e] Nontypeable.

[f] *Acinetobacter* DNA group 3.

[g] *A. calcoaceticus* (DNA group 1).

[h] Strain isolated from a hand wash of one of the nursing staff.

belonged to the *A. calcoaceticus-A. baumannii* complex (*A. calcoaceticus* [$n = 1$], *A. baumannii* [$n = 40$], *Acinetobacter* genospecies 3 [$n = 5$], *Acinetobacter* genospecies 13 [$n = 3$]), except for two (1N and 14N) which could not be identified at the species level and were therefore excluded from the final results (Table 1). Of all 25 epidemic strains, 23 were identified as *A. baumannii*.

Genomic DNA isolation. DNA was prepared from fresh overnight cultures grown on Luria-Bertani (LB) agar plates (Difco Laboratories, Detroit, Mich.) as described previously (4). Extracted DNA was resolved in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) supplemented with 10 μg of RNase (Sigma, St. Louis, Mo.). Purified DNA was aliquoted and stored at −20°C.

TABLE 2. Oligonucleotides for PCR analysis of integrons

Primer	Nucleotide sequence (5′ to 3′)
5′-CS[a]	GGC ATC CAA GCA GCA AG
3′-CS[a]	AAG CAG ACT TGA CCT GA
Int1F	CAG TGG ACA TAA GCC TGT TC
Int1R	CCC GAG GCA TAG ACT GTA
Int2F	TTG CGA GTA TCC ATA ACC TG
Int2R	TTA CCT GCA CTG GAT TAA GC

[a] Described by Lévesque et al. (12).

PCR amplification. PCR amplifications were carried out in 20-μl volumes containing 5 μl of template DNA, 0.2 mM (each) deoxynucleoside triphosphate (dNTP), 2 μl of 10× PCR buffer, 1 U of *Taq* polymerase (Perkin-Elmer [PE] Applied Biosystems, Foster City, Calif.), 1.5 mM $MgCl_2$, and 1.25 μM each primer. PCR amplification was performed with the GeneAmp PCR System 9700 thermal cycler (PE Applied Biosystems). Amplification products were resolved by electrophoresis at 120 V for 2 h on 2% agarose gels with 0.5× Tris-borate-EDTA buffer containing ethidium bromide and were visualized under UV light. All PCR amplifications were performed in duplicate.

PCR amplification for the detection of class 1 integron cassettes (integron PCR) was performed with primers 5′CS and 3′CS, as described previously (12). For PCR detection of the IntI1 and IntI2 integrase genes (integrase gene PCR), oligonucleotide primers based on the *intI1* and *intI2* genes were designed (Table 2). Primers IntIF and IntIR were used to amplify a 160-bp fragment of the *intI1* gene. The combination of primers Int2F and Int2R amplified a fragment of 288 bp, specific for the *intI2* gene. The positions of the primers relative to the integron are indicated in Fig. 1. PCR amplification of integrase gene type 1 and type 2 was performed simultaneously for 35 cycles: 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 30 s of extension at 72°C.

DNA sequencing of PCR products. Template PCR products were purified with the Qiaquick PCR Purification kit (Qiagen, Chatsworth, Calif.). Purified PCR products were sequenced with dye terminators on an ABI 377 automatic sequencer (Applied Biosystems). DNA sequences were compared to the National Center for Biotechnology Information (NCBI) database.

Isolation of plasmids. From each strain, plasmid DNA was prepared in duplicate, as described by Hartstein et al. (10), with minor modifications. Briefly, isolates were grown on LB agar plates at 37°C for 24 h. Cells from half of the plate were suspended in 1.5 ml of a solution containing 2.5 M NaCl–10 mM EDTA (pH 8.0), 250 μl of 0.5% alkyltrimethylammonium bromide (ATAB), 250 μl of 1% Triton X-100, and 200 μl of lysozyme (10 mg/ml). After incubation in a water bath at 56°C for 15 min, protein was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and plasmid DNA was precipitated with ice-cold isopropanol. The precipitate was collected by centrifugation and dissolved in 80 μl of TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.2]). After addition of 1 μl of RNase (500 μg/ml), the solutions were incubated at 37°C for 30 min. Ten microliters of the samples and 5 μl of running dye were loaded onto the gel and run for 18 h at 25 V. Gels were stained with ethidium bromide and photographed under UV illumination.

Antibiotic susceptibility. MICs of selected antimicrobial agents were determined with the Vitek System (bioMérieux, Vitek, Inc., Hazelwood, Mo.). Thirteen antibiotics were tested: ampicillin, ampicillin-clavulanate, piperacillin-tazobactam, cefuroxime, cefotaxime, ceftazidime, imipenem, meropenem, gentamicin, tobramycin, amikacin, ciprofloxacin, and trimethoprim-sulfamethoxazole. MIC data were interpreted according to the guidelines of the NCCLS.

RESULTS

Epidemiological data for the different strains and hospital outbreaks are shown in Table 1. Almost three-quarters of the outbreaks (18 of 25) occurred in ICUs and affected 3 to more than 100 patients.

Detection of class 1 integrons by integron PCR. For the detection of complete class 1 integrons, PCR amplification was performed with primers for the 5′- and 3′-conserved segments. This PCR also permitted the determination of the size of any inserted gene cassette. Integrons with various insert sizes were found in 44% (22 of 48) of the *Acinetobacter* species strains. The range of inserted gene cassette sizes detected varied from 800 to 3,000 bp. Sixty-five percent (15 of 23) of epidemic *A. baumannii* isolates were integron positive. Strikingly, in non-epidemic *Acinetobacter* species isolates, the frequency of integron carriage was only 30% (7 of 23).

Detection of class 1 and class 2 integrons by integrase gene PCR. PCR detection of the *intI1* and *intI2* genes demonstrated the presence of integrons in two more strains, compared to the integron PCR. Overall, the integrase gene PCR resulted in a frequency of integron-positive isolates of 50% (24 of 48). All the integrons were found in isolates of *A. baumannii* (24 of 40). Class 1 integrons were detected in 58% (23 of 40) of the *A. baumannii* isolates, whereas only one *A. baumannii* strain (3O) contained a class 2 integron (Fig. 2). The correlation between the presence of integrons, as determined by integrase gene PCR, and the epidemic character of the *Acinetobacter* strains was statistically significant ($P < 0.05$).

Sequence analysis. To confirm that primers Int1 and Int2 correctly identified the *intI1* and *intI2* genes, amplification products of strain 1O and 3O were sequenced. The sequences of these products were 100% identical to previously published sequences of the *intI1* and *intI2* genes.

Detection of plasmids. Plasmids were found in 42% (20 of 48) of the *Acinetobacter* strains. The distribution of plasmids in epidemic isolates was 36% (9 of 25) versus 48% (11 of 23) for

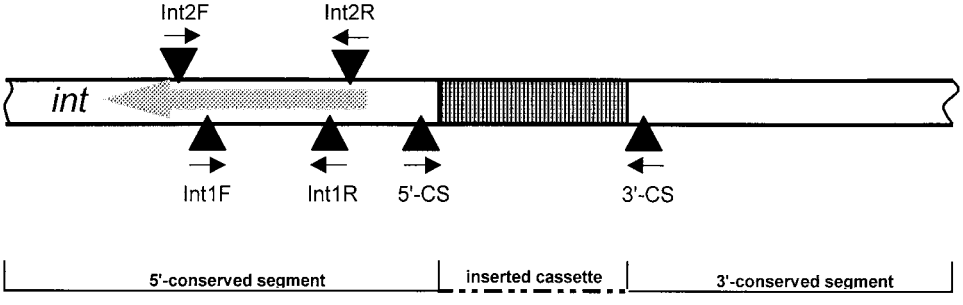

FIG. 1. Scheme for PCR detection of class 1 and class 2 integron structures. The grey arrow shows the direction of transcription. Primers 5′CS and 3′CS are specific to the 5′- and 3′-conserved segments of class 1 integrons, respectively, and were used to amplify the variable regions of class 1 integrons (integron PCR). Primers Int1F and Int1R were used to detect IntI1 integrase (integrase gene 1 PCR). Primers Int2F and Int2R were used to detect IntI2 integrase (integrase gene 2 PCR).

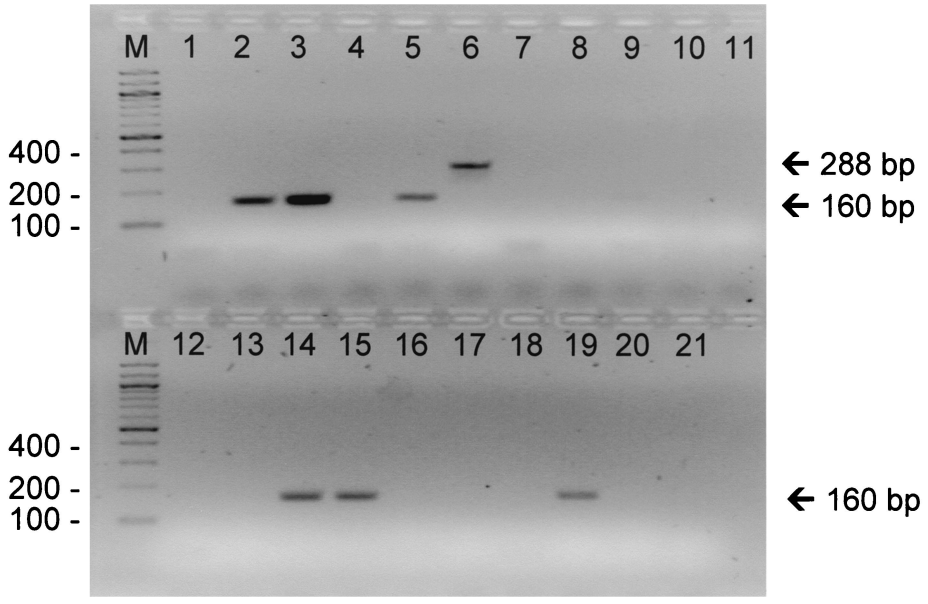

FIG. 2. Gel image of representative PCR amplification products from different clinical strains of *Acinetobacter* species after amplification with *intI1*- and *intI2*-specific primers. Lane M, molecular weight marker; lane 1, PCR-negative buffer control; lanes 2 to 11, strains 1O, 2O, 3N, 4O, 3O, 5O, 5N, 6N, 7N, and 8N, respectively; lanes 12 to 21, strains 9N, 11O, 6O, 7O, 14N, 15O, 15N, 16O, 2O, and 20N, respectively. Strain designations are as presented in Table 1.

nonepidemic isolates; this difference was not significant. Interestingly, only six isolates of *A. baumannii* contained both an integron and a plasmid.

Antibiotic susceptibility and integron carriage. Susceptibility to 13 different antibiotics was related to the presence or absence of an integron within the *Acinetobacter* strains. Table 3 shows the antibiotic susceptibilities of integron-positive and integron-negative isolates to each of the antibiotics tested, expressed in terms of the MIC at which 50% of the isolates tested were inhibited (MIC_{50}). Integron carriage was significantly associated with an increase in antibiotic resistance. In addition, integron-positive *A. baumannii* strains showed resistance to a significantly higher number of different antibiotics compared to integron-negative isolates (Fig. 3). Eighty percent (20 of 25) of the epidemic strains were resistant to five or more of the antibiotics tested. Strikingly, all integron-positive strains except one (23 of 24) showed resistance to five or more of the antibiotics tested. Integron detection identified 74% (17 of 23) of the epidemic *A. baumannii* isolates.

DISCUSSION

The aim of our study was to investigate the possible role of integrons and plasmids in the epidemic behavior of clinical

TABLE 3. Antibiotic susceptibilities of integron-positive *A. baumannii* and integron-negative *Acinetobacter* species isolates

Antibacterial agent	Susceptibility of isolates				P^b
	Integron positive (n = 24)		Integron negative (n = 24)		
	MIC_{50} (mg/liter)	% S[a]	MIC_{50} (mg/liter)	% S	
Amikacin	32	38	1	77	<0.01
Gentamicin	32	4	0.25	69	<0.001
Tobramycin	32	25	0.25	77	<0.001
Ciprofloxacin	8	17	0.25	88	<0.001
Trimethoprim-sulfamethoxazole	160	21	5	73	<0.001
Ampicillin	64	0	16	27	
Amoxicillin-clavulanic acid	64	8	4	69	<0.001
Piperacillin-tazobactam	64	42	4	73	<0.05
Cefuroxime	64	4	64	4	NS
Cefotaxime	32	8	16	38	<0.05
Ceftazidime	16	38	4	81	<0.01
Imipenem	2	83	2	92	NS
Meropenem	1	96	1	96	NS

[a] Percentage of strains that are susceptible according to NCCLS breakpoints.
[b] Statistical significance of the difference between the number of susceptible integron-positive isolates and the number of susceptible integron-negative isolates (by Fisher's exact test). NS, not significant.

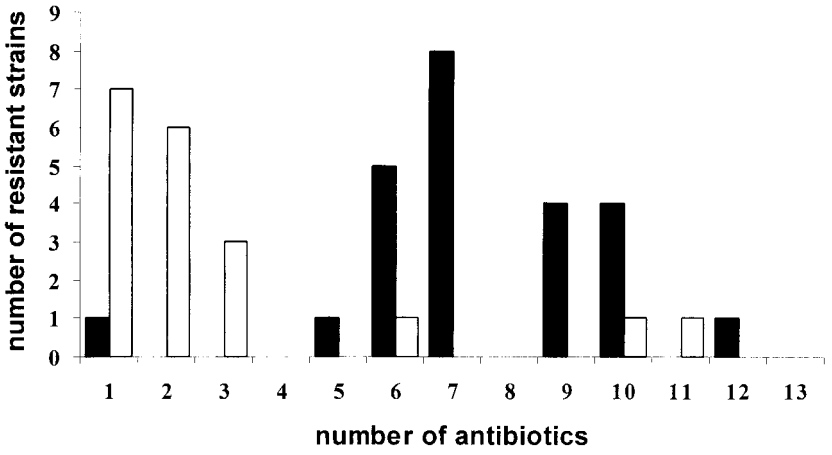

FIG. 3. Comparison of resistance among integron-positive *A. baumannii* isolates (solid bars) and integron-negative *Acinetobacter* species isolates (open bars) in terms of the numbers of antibiotics to which isolates were resistant. Five integron-negative strains (two epidemic and three nonepidemic strains) were susceptible to all antibiotics tested.

isolates of *A. baumannii*. The epidemic *A. baumannii* isolates as well as the sporadic *Acinetobacter* species strains included in this study were obtained from hospitals in 11 different countries. Almost half of these strains (48%) carried integrons. Integrons, however, were found significantly more often in epidemic *A. baumannii* strains than in sporadic isolates. Possibly, these genetic structures play an important role in the epidemic behavior of *A. baumannii*.

Class 1 integrons were the most common integrons found in this collection of *A. baumannii* isolates. Only one clinical isolate, strain 3O, was found to contain a class 2 integron structure. Other investigators have also found predominantly class 1 integrons in *Acinetobacter* species (23). A survey by Gonzalez et al., however, demonstrated predominantly class 2 integrons in *A. baumannii* isolates from Chilean hospitals (7). Possibly the strains from the latter study were more genetically related.

In the present study, two different PCR assays were used to detect either class 1 integrons by amplification of any inserted gene cassette or class 1 and class 2 integrons by detection of the specific *intI1* and *intI2* genes. The integron PCR could lead to false-negative results because (i) the number of inserted genes in the cassette could exceed the PCR extension capacity, which is optimized for DNA products of less than 2.5 kb; (ii) a strain can possess an integron without a gene cassette; and (iii) class 2 integrons do not contain the *sulI* gene at the 5′-conserved segment (18). The integrase gene PCR, however, detects both class 1 and class 2 integrons by amplification of two products of specific small sizes irrespective of the heterogeneity of the inserted gene cassettes. The integrase gene PCR was indeed more sensitive than the integron PCR and detected integrons in two outbreak strains which were negative in the integron PCR.

Integron-positive strains were significantly associated with resistance to multiple antibiotics. This is not surprising, since many antibiotic resistance gene cassettes encoding resistance to a wide range of antibiotics have been reported (6). However, this could not explain resistance to extended-spectrum β-lactams because such integron-encoded resistance genes have never been described. Resistance to extended-spectrum β-lactams could be due to a combination of integrons and resistance genes located on other genetic structures such as plasmids (14). The differences in ciprofloxacin resistance, however, cannot be explained this way, since transferable quinolone resistance encoded by integrons or plasmids has never been described. Most likely, changes in outer membranes of integron-positive *Acinetobacter* strains, induced by transferable β-lactam resistance, are responsible for these differences. Although plasmids were detected in a substantial number of strains, no significant correlations between plasmid carriage and either antibiotic resistance (data not shown) or epidemic behavior was found. These results indicate that antibiotic resistance in clinical isolates of *A. baumannii* is associated particularly with the presence of integrons. In the strains analyzed in this study, the integrons are probably not located on plasmids, since only six of the strains investigated harbored both integrons and plasmids.

The analysis of *A. baumannii* strains with known epidemic behavior demonstrates that early identification of epidemic strains may be possible by detection of integrons or multiple antibiotic resistance. The integrase gene PCR identified almost 75% of the epidemic *A. baumannii* strains. Multiple antibiotic resistance, defined as resistance to five or more antibiotics, showed good correlation with the presence of integrons and epidemic behavior of the strains. Susceptibility testing, however, has several disadvantages, including its laboriousness, the need for a pure bacterial culture, and subsequent overnight incubation. Also, the chosen cutoff level of five antibiotics is arbitrary and depends on the choice and the number of the antibiotics tested. PCR mapping of integrons, on the other hand, is a rapid and easy technique which can be performed on a single colony.

Integrons were also found in seven nonepidemic strains, which may be an indication of the epidemic potential of these strains. Another explanation for this finding could be the definition of the epidemic and nonepidemic phenotypes in terms of the capacity to spread to one or more patients. It is well known that many different circumstances, such as infection control measures, antibiotic policy, and susceptibilities of in-

dividual patients, can facilitate or prevent the dissemination of bacteria, including *A. baumannii*.

In conclusion, integrase gene PCR is a rapid, valuable procedure, which can be easily used in routine clinical microbiology laboratories for the detection of integrons in clinical *A. baumannii* isolates. It seems to be a rapid and simple tool for the routine screening of *A. baumannii* isolates in order to identify strains with epidemic potentials. This is important for the immediate introduction of specific infection control measures in the hospital setting in order to limit the nosocomial spread of these strains.

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