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Comparison of Ribotyping and Pulsed-Field Gel Electrophoresis for Molecular Typing of *Acinetobacter* Isolates

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Seventy-three isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex, including 26 isolates from 10 hospital outbreaks, were typed by ribotyping with *Eco*RI and *Cla*I and by pulsed-field gel electrophoresis (PFGE) of genomic DNA after digestion with *Apa*I. Ribotyping with *Eco*RI distinguished 31 ribopatterns. Digestion with *Cla*I generated another eight ribotypes. PFGE, in contrast, identified 49 distinct patterns with seven variants. Both methods detected all outbreak-related isolates. By ribotyping, nine epidemiologically unrelated strains could not be differentiated from outbreak strains, in contrast to only one isolate not identified by PFGE. Thus, PFGE was more discriminating than ribotyping. However, ribotyping is known to generate banding patterns specific to each DNA group in the *A. calcoaceticus-A. baumannii* complex that may be used for taxonomic identification of the strains. PFGE was shown to lack this property. Both methods are therefore useful for strain differentiation in epidemiological studies of *Acinetobacter* isolates.

In recent years, strains of the genus *Acinetobacter* have been increasingly recognized as important nosocomial pathogens (2, 3, 9, 17, 26). Resistance to multiple antibiotics is a frequent finding with these organisms (23). Clinical manifestations may range from colonization of the skin and mucous membranes to serious disease with substantial morbidity and mortality. Currently, 19 DNA groups have been identified by DNA-DNA hybridization methods (8, 12, 29). Six closely related DNA groups have been assembled in the so-called *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex because they are phenotypically very similar and often impossible to differentiate from each other (12, 13). Three of these are common in clinical culture material, namely, *A. baumannii* (DNA group 2), the unnamed DNA group 3, and Tjernberg and Ursing's DNA group 13 (29). *A. baumannii* seems to be the most prevalent *Acinetobacter* species isolated from clinical specimens in hospitals (6, 22), and most hospital outbreaks have been attributed to this species (15, 16, 25, 27). However, in these reports the strains have been identified phenotypically only and therefore the identification may not be fully reliable. Various typing methods have been employed in the epidemiological investigation of outbreaks due to *Acinetobacter* species. The most widely applied methods have focused on variations in such phenotypic properties as antimicrobial susceptibility, biotypes, serotypes, phage types or cell envelope protein profiles (7, 18, 31). Many of these traditional typing procedures, however, lack sufficient reproducibility, typeability, and discriminatory power. More recently, molecular techniques, such as plasmid typing, ribotyping, and analysis of genomic DNA by pulsed-field gel electrophoresis (PFGE) and by arbitrarily primed PCR, that directly compare variations in the DNA of bacterial isolates have been applied to epidemiological investigations of *Acinetobacter* isolates (10, 11, 15, 16, 25). Currently, it is not known which of the available DNA typing methods is best suited to determine the relatedness of *Acinetobacter* isolates during the investigation of an outbreak or for ongoing surveillance. We therefore compared two of the most widely applied DNA-based typing methods, ribotyping and PFGE, for the epidemiological assessment of *Acinetobacter* strains.

The *Acinetobacter* isolates used in the present study comprise two sets of isolates. Set A contained 22 isolates from eight well-characterized outbreaks (G-I to G-VI, G-VIII, and G-X) and 18 epidemiologically unrelated isolates. All these isolates were collected within a period of 3 years in Cologne, Germany, and were identified as *A. baumannii* by the use of the simplified identification scheme of Bouvet and Grimont (6). From each outbreak, between two and four representative isolates were selected for this study. Outbreak G-I isolates represented an outbreak involving 10 patients with respiratory tract infections due to *A. baumannii* in a neurological intensive care unit. Outbreak G-II represents dissemination of a strain of *A. baumannii* in a rehabilitation unit and involved 12 patients with urinary tract infections. Outbreak G-III isolates were from an outbreak in a neonatal intensive care unit. Isolates representative of outbreaks G-IV, G-V, G-VI, G-VIII, and G-X were recovered during a complex epidemic situation involving five different epidemic clones and affecting more than 150 patients in multiple intensive care units in a tertiary care center. The outbreak isolates have been described previously (25) and have been characterized by plasmid profiles, antibiograms, biotyping, and PFGE patterns. Set B contained 27 previously described strains from Denmark (11, 12) and 5 more recent Danish isolates, 2 of which were recovered during an outbreak in a burn unit (D-I). In addition, the *A. baumannii* type strain, ATCC 19606^T, was investigated. All strains tested are listed in Table 1, along with their origins, biotypes as defined by Bouvet and Grimont (6), PFGE patterns, and ribotypes as determined and then arbitrarily named here.

Ribotyping was performed with restriction enzymes *Eco*RI and *Cla*I as described previously (12). In brief, cells were lysed by a modified EDTA-sodium dodecyl sulfate method. DNA was extracted with phenol-chloroform and was digested with *Eco*RI or *Cla*I. After agarose gel electrophoresis, the separated fragments were transferred to a nylon membrane and hybridized with a digoxigenin-11-dUTP-labelled cDNA probe derived from a commercially available *Escherichia coli* 16S and 23S rRNA preparation by random priming with reverse transcriptase. Arabic numerals were used to indicate the

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TABLE 1. Typing results for *Acinetobacter* isolates used in the present study

Strain	Outbreak[a]	Typing result					Source[e]	Sample site
		DNA group[b]	Biotype[c]	*Eco*RI[d]	*Cla*I[d]	PFGE type		
4419	No	13	9	13A	13d	I	DH-5	Urine
9894	No[f]	13	9	13A	13c	II	DH-2	Sputum
9836	No[f]	13	9	13A	13c	II	DH-2	Sputum
353	D-II	13	9	13A	13a	III	DH-3	Sputum
387	D-II	13	9	13A	13a	III	DH-3	Sputum
St-11681	G-VIII	13	9	13A	13c	IV	GH-1	Blood
St-7961	G-VIII	13	9	13A	13c	IV	GH-1	Blood
St-8195	G-VIII	13	9	13A	13c	IVa	GH-1	Catheter
St-2312	G-VIII	13	9	13A	13c	IVa	GH-1	Blood
10716	No[f]	13	9	13C	13e	V	DH-6	Sputum
10717	No[f]	13	9	13C	13e	V	DH-6	Sputum
12112	No	13	9	13C	13e	Va	DH-1	Blood
53937bb	No	13	9	13E	13b	VI	DH-6	No information
50853-82	No	2	2	2A	2a	VII	DH-3	Cerebrospinal fluid
189	No	2	2	2A	2a	VII	DH-3	Skin
St-15598	G-V	2	2	2A	2b	VIII	GH-1	Catheter
St-14970	G-V	2	2	2A	2b	VIII	GH-1	Catheter
St-1650	G-X	2	3	2A	2c	IX	GH-1	Blood
St-1954	G-X	2	3	2A	2c	IX	GH-1	Blood
St-20421	No	2	9	2A	2c	X	GH-1	Blood
9771	No	2	6	2C	2c	XI	DH-8	Urine
10074	No	2	19	2C	2c	XII	DGP-15	Urine
ATCC 19606	No	2	6	2C	2c	XIII		
14544	D-I	2	5	2C	2j	XIV	DH-9	Burn
14552	D-I	2	5	2C	2j	XIV	DH-10	Burn
10086	No	2	6	2I	2h	NT[g]	DH-18	Urine
W-8832	No	2	6	2I	2h	XV	GH-2	Wound swab
St-18748	No	2	6	2I	2h	XVI	GH-6	Catheter
W-8334	No	2	9	2I	2h	XVII	GH-2	Wound swab
10508	No[f]	2	6	2L	2g	XVIII	DH-6	Skin
10073	No[f]	2	6	2L	2g	XVIII	DH-6	Blood
M-13546	No	2	6	2L	2e	XIX	GH-2	Tracheal aspirate
St-13641	No	2	6	2L	2g	XX	GH-2	Blood
U-10247	G-II	2	6	2L	2g	XXI	GH-3	Urine
U-11177	G-II	2	6	2L	2g	XXIa	GH-3	Urine
U-11432	G-II	2	6	2L	2g	XXIb	GH-3	Urine
V-4316	No	2	6	2L	2g	XXII	GH-5	Wound swab
V-12334	No	2	9	2L	2g	XXIII	GH-2	Wound swab
14554	No	2	6	2L	2g	XXIV	DH-10	Burn
W-5420	G-I	2	1	2M	2a	XXV	GH-2	Tracheal aspirate
U-1901	G-I	2	1	2M	2a	XXV	GH-2	Tracheal aspirate
M-3317	No	2	6	2N	2d	XXVI	GH-2	Tracheal aspirate
St-284	G-III	2	6	2O	2f	XXVII	GH-4	Blood
St-14733	G-III	2	6	2O	2f	XXVII	GH-4	Blood
St-20820	G-IV	2	6	2P	2d	XXVIII	GH-1	Blood
St-21359	G-IV	2	6	2P	2d	XXVIII	GH-1	Catheter
St-16706	G-IV	2	6	2P	2d	XXVIIIa	GH-1	Blood
V-12561	No	2	6	2Q	2i	XXIX	GH-7	Abscess
M-7360	No	2	9	2Q	2k	XXX	GH-7	Tracheal aspirate
M-3789	No	2	9	2R	2j	XXXI	GH-8	Tracheal aspirate
St-12084	No	2	9	2S	2l	XXXII	GH-1	Blood
St-17093	G-VI	2	9	2T	2m	XXXIII	GH-1	Blood
V-7459	G-VI	2	9	2T	2m	XXXIII	GH-1	Tracheal aspirate
St-17108I	G-VI	2	9	2T	2m	XXXIIIa	GH-1	Blood
St-17108II	G-VI	2	9	2T	2m	XXXIIIa	GH-1	Blood
St-17306	No	2	9	2U	2g	XXXIV	GH-6	Blood
U-9560	No	2	9	2U	2n	XXXV	GH-8	Urine
V-12277	No	2	9	2V	2d	XXXVI	GGP-1	Wound swab
W-4329	No	2	9	2W	2o	XXXVII	GH-2	Gastric juice
10790	No	3	8	3F	3c	XXXVIII	DH-6	Dialysate
10089	No	3	18	3F	3c	XXIX	DH-20	Urine
9907	No	3	N[h]	3L	3c	XXXX	DH-4	Urine
10078	No	3	5	3M	3c	XXXXI	DGP-16	Vagina
12174a	No	3	8	3N	3c	XXXXII	DH-5	Urine
10088	No	3	8	3O	3c	XXXXIII	DGP-19	Ulcer
12398	No	3	9	3P	3d	XXXXIV	DH-5	Sputum

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TABLE 1—*Continued*

Strain	Outbreak[a]	Typing result					Source[e]	Sample site
		DNA group[b]	Biotype[c]	*Eco*RI[d]	*Cla*I[d]	PFGE type		
10084	No	3	18	3Q	3c	XXXXV	DH-20	Umbilicus
14526	No	3	8	3R	3a	XXXXVI	DH-6	Sputum
14577	No	3	8	3S	3b	XXXXVII	DH-1	Blood
5804	No	n1[i]	9	n1-V	n1-v	NT	DH-23	Blood
10090	No	n1	8	n1-Y	n1-y	NT	DGP-21	Ulcer
10169	No	n2	8	n2-X	NT	XXXXVIII	DGP-9	Sputum
10095	No	n2	2	n2-Z	n2-z	XXXXIX	DH-18	Abscess

[a] No, epidemiologically unrelated isolate; I to X, outbreak number in Germany (G) or Denmark (D).
[b] DNA group as determined by ribotyping.
[c] Biotype as determined by the method of Bouvet and Grimont (6).
[d] Ribotyping result obtained with *Eco*RI or *Cla*I.
[e] GH, German hospital; DH, Danish hospital; GGP, German general practice; DGP, Danish general practice.
[f] Isolate pairs 9894 and 9836, 10716 and 10717, and 10508 and 10073 were recovered from different departments of the same hospitals; an epidemiological link can therefore not be excluded.
[g] NT, not typeable.
[h] N, biotype not described before, assimilating only tartrate.
[i] n, not belonging to any previously described DNA group in the *A. calcoaceticus-A. baumannii* complex.

DNA group, capital letters were used to define the *Eco*RI ribotype, and minuscules were used to define the *Cla*I ribotype. Isolates showing one band difference were assigned different types.

PFGE was performed as described previously (25). Briefly, genomic DNA in agarose plugs was digested with restriction endonuclease *Apa*I or *Sma*I. Slices were loaded into the wells of a 1.2% agarose gel, and genomic DNA was separated by PFGE at 175 V for 18 h using Pharmacia-LKB equipment. The pulse time was increased from 5 to 20 s. All isolates were evaluated by at least two independent electrophoretic runs. Isolates with identical restriction profiles were assigned the same type. Isolates that differed by one or two band shifts consistent with a single genetic event were assigned a subtype

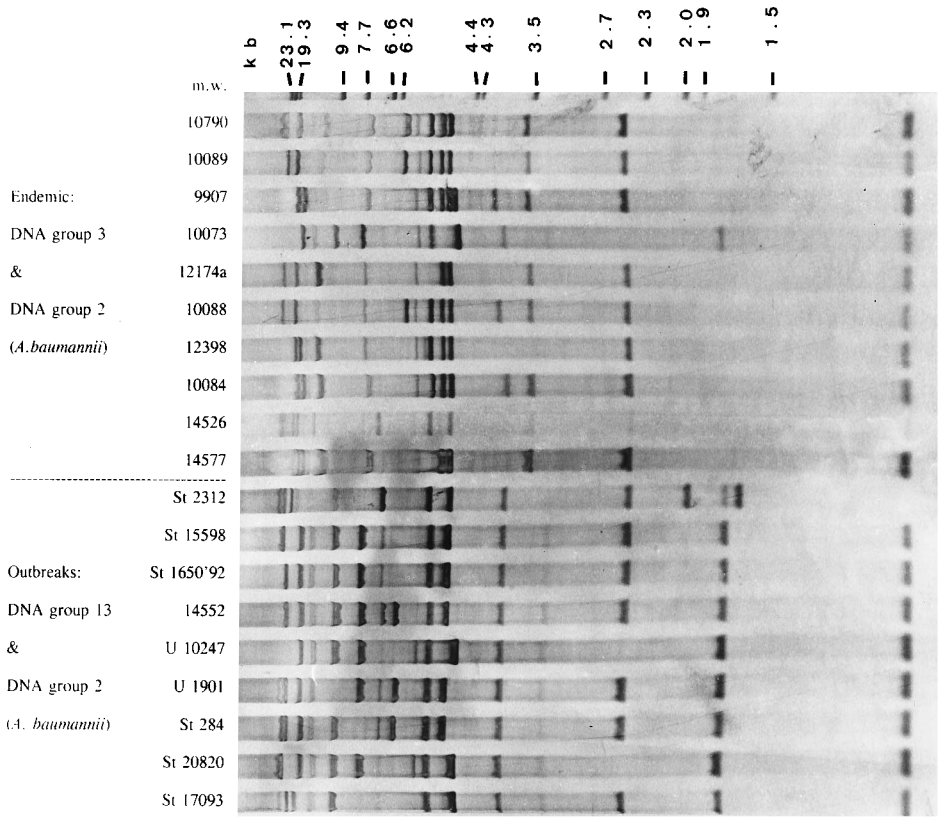

FIG. 1. *Eco*RI ribotypes of sporadic and epidemic strains of *Acinetobacter*. The 10 strains above the dashed line are sporadic strains of DNA group 3, except strain 10073, which belongs to DNA group 2 (*A. baumannii*). The nine strains below the line represent different outbreaks. Eight strains belong to DNA group 2 (*A. baumannii*), and one (St 2312) belongs to unnamed DNA group 13. The molecular size marker (m.w.) is a mixture of phage λ digested with *Hin*dIII and *Sty*I.

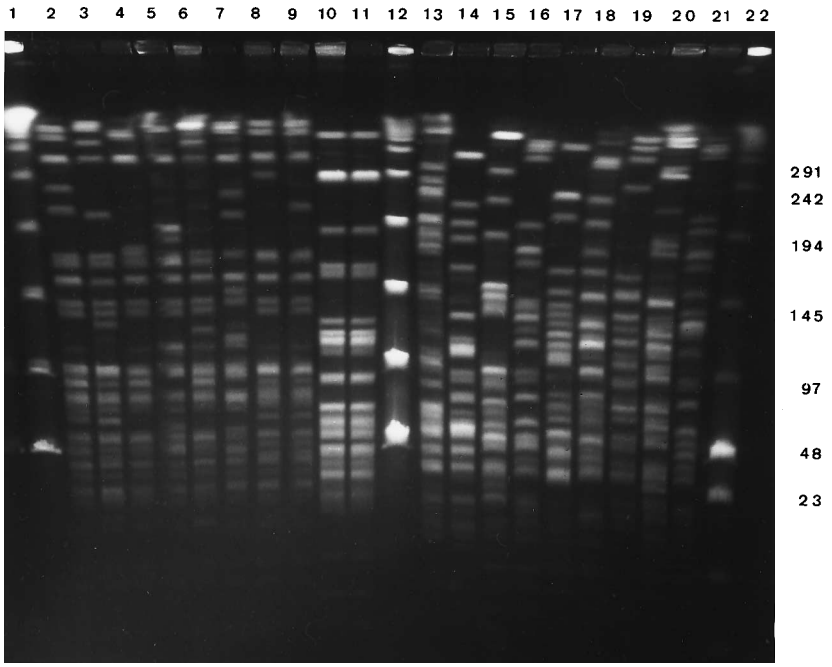

FIG. 2. Representative PFGE patterns of 19 strains in the *A. calcoaceticus-A. baumannii* complex after digestion with *Apa*I. Lanes: 1, 12, and 22, lambda DNA concatemers (band sizes are expressed on the right in kilobases); 2 to 7, epidemiologically unrelated *A. baumannii* (DNA group 2) strains (2, 10508; 3, 14554; 4, M-13546; 5, St-13641; 6, V-4316; 7, V-12334); 8 and 9, *A. baumannii* (DNA group 2) strains representing outbreak G-II (8, U-10247; 9, U-11177); 10 and 11, *Acinetobacter* DNA group 13 strains representing outbreak G-VIII (10, St-11681; 11, St-7961); 13 to 21, epidemiologically unrelated DNA group 3 strains showing highly variable patterns (13, 10790; 14, 10089; 15, 9907; 16, 12174a; 17, 10088; 18, 12398; 19, 10084; 20, 14526; 21, 14577).

or variant (20). Roman numerals were used to indicate major restriction types, and minuscules were used to indicate subtypes.

In total, 73 *Acinetobacter* isolates were studied; 46 were *A. baumannii* (DNA group 2), 10 were of *Acinetobacter* DNA group 3, 13 were of *Acinetobacter* DNA group 13, and 4 strains belonged to two newly described DNA groups within the *A. calcoaceticus-A. baumannii* complex, as was shown by characteristic *Eco*RI banding patterns (11, 12). Using *Eco*RI, we found 31 different ribotypes among these isolates. All of the isolates were typeable, and ribotyping yielded between 10 and 15 bands per strain (Fig. 1). Cleavage of DNA by *Cla*I yielded another eight ribotypes among strains not differentiated by *Eco*RI. By combining the results obtained with both enzymes, 21 ribotypes were distinguished among *A. baumannii* (DNA group 2) strains, *Acinetobacter* DNA group 3 strains were assigned to 9 ribotypes, and *Acinetobacter* DNA group 13 strains were split into 5 ribotypes. The remaining four strains showed unique ribotypes. All outbreak-related isolates were correctly allocated to the same ribotype. However, 16 epidemiologically unrelated strains were classified among the outbreak strains by ribotyping with *Eco*RI (13A, 2A, 2C, and 2L). In addition, nine epidemiologically unrelated strains were assigned to only four *Eco*RI ribotypes (2I, 2Q, 2U, and 3F). When both enzymes were used for ribotyping, nine unrelated strains were still indistinguishable from strains in the outbreak clusters, among them strains 10508, 14554, St-13641, V-4316, and V-12334; all these strains were of ribotype 2L-2g, identical to strains U-10247 and U-11177 representing outbreak G-II, but exhibited clearly distinct patterns by PFGE (Fig. 2).

When these isolates were studied by PFGE after digestion of genomic DNA with *Apa*I, they were shown to belong to 49 distinct patterns with seven variants. All but three strains were typeable (DNA of strain 10086 was only partially cut by *Apa*I and could not be evaluated), and PFGE generated 15 to 20 bands per strain. By using *Sma*I, the remaining three strains could be readily distinguished and showed unique patterns (data not shown). Among *A. baumannii* (DNA group 2) strains, 31 major restriction patterns could be distinguished. Ten different patterns were found for *Acinetobacter* DNA group 3 strains, whereas *Acinetobacter* DNA group 13 strains were split into six PFGE patterns. All outbreak-related strains were correctly identified, and only one unrelated strain was included in the outbreak-related clusters. Nine Danish strains that were considered unrelated a priori were assigned to four PFGE types (II, V, VII, and XVIII); these strains were also grouped together by ribotyping, as well as by biotyping and plasmid typing (data not shown). Three pairs of these were recovered from patients in the same hospital during the same period, and thus, an epidemiological link between them could not be ruled out. These results were further confirmed by using *Sma*I restriction endonuclease for PFGE (data not shown). All other unrelated strains were assigned to distinct PFGE types. No DNA group-specific PFGE patterns could be established.

The aim of the present study was to compare two DNA-based typing methods for the epidemiological investigation of *Acinetobacter* isolates. Both ribotyping and PFGE are increasingly used in clinical and research microbiology laboratories and are applied to an ever-increasing number of organisms (11, 14, 19, 21, 28, 30). Both methods are highly reproducible, are applicable for typing virtually all strains, and allow objective evaluation of results. Ribotyping has the advantage that it can be used for taxonomic purposes and can identify acinetobacters to the DNA group level (11). In a previous investigation (12), the DNA group affiliations of 28 isolates were established by ribotyping and confirmed by DNA-DNA hybridization. In our study, each outbreak was characterized by a unique ribotype with both *Eco*RI and *Cla*I. However, ribotyp-

ing with *Eco*RI alone was less discriminatory than PFGE. When both *Eco*RI and *Cla*I were used for ribotyping, nine unrelated strains with different PFGE patterns showed a ribopattern indistinguishable from an outbreak pattern. In no instance could strains showing identical PFGE patterns be further differentiated by ribotyping. In other studies with *Serratia marcescens* and *Pseudomonas aeruginosa*, ribotyping has been used successfully for epidemiological purposes (4, 5), although some investigators have found this method less discriminatory than PFGE for the typing of *Enterococcus faecalis* (14) and methicillin-resistant *Staphylococcus aureus* (21).

PFGE has been used by several groups, including our own, to study epidemiological issues associated with acinetobacters (1, 15, 25, 27). This technique was shown to be a suitable method for differentiating strains from hospital outbreaks. PFGE and another DNA-based typing method, PCR fingerprinting, have recently been comparatively evaluated for epidemiological studies of *A. baumannii* and were found to be equally effective for differentiation of a limited number of outbreak strains (16). However, to assess the discriminatory power of a method, epidemiologically unrelated strains have to be included in the investigation. In the present study, PFGE using *Apa*I was highly sensitive for detecting outbreak-related strains and generated distinct digestion patterns with 35 of 47 unrelated isolates; three strains were typeable only after digestion of genomic DNA with another restriction endonuclease. However, the remaining nine Danish strains that were allocated to four PFGE patterns could not be distinguished by ribotyping or by other typing methods such as biotyping and plasmid profiling. As noted before, an epidemiological link for six of these (three pairs from three hospitals) could not be ruled out. The link between the remaining three strains may be clonal in a broad sense.

One of the German outbreak strains (G-VIII) was phenotypically identified as *A. baumannii* biotype 9. This strain showed the typical ribopattern for DNA group 13. Thus, it does undoubtedly belong to this DNA group and not to DNA group 2 (*A. baumannii*). This result is yet another example of the insufficiency of the phenotypical methods for identification of strains of the *A. calcoaceticus-A. baumannii* complex. None of the German outbreak strains were identified as belonging to DNA group 3 by ribotyping, and none of the Danish strains described in the present paper originated from outbreaks. In a previous study from Cologne (24), no strain phenotypically identified as belonging to DNA group 3 was related to an outbreak. The only outbreaks caused by DNA group 3 strains that have been described occurred in The Netherlands (10). Strains from this DNA group show considerable heterogeneity, as judged by ribotyping, PFGE (Fig. 1 and 2), and plasmid profiling (described in this study and in reference 24). Thus, it seems that strains from this DNA group are not as easily maintained in a hospital setting as strains from DNA groups 2 and 13.

In summary, both ribotyping and PFGE analysis of genomic DNA are useful to delineate outbreaks of nosocomial *Acinetobacter* infections. However, PFGE appears to be more discriminatory than ribotyping. Ribotyping, on the other hand, may be used for identification of strains to the DNA group level, whereas PFGE is not useful in this context.

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