MOLECULAR TYPING AND EPIDEMIOLOGY

Genotypic and phenotypic similarity of multiresistant *Acinetobacter baumannii* isolates in the Czech Republic

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The diversity of 103 clinical isolates of the *Acinetobacter calcoaceticus*–*Acinetobacter baumannii* complex obtained between 1991 and 1997 from 17 Czech hospitals was studied by ribotyping, biotyping, plasmid profiling and antibiotic susceptibility testing. According to the *EcoRI* ribotypes, all but one of these isolates were identified to the DNA group level: 77 isolates were allocated to DNA group 2 (*A. baumannii*), 14 to DNA group 3, 10 to DNA group 13 *sensu* Tjernberg and Ursing and one to DNA group 1 (*A. calcoaceticus*). In total, 50 different *EcoRI* ribotypes and 10 biotypes were observed. Plasmids were found in 92% of the isolates and a high variability in plasmid profiles was found in isolates of the same DNA group. The combination of typing profiles allowed two predominant groups (termed A and B) to be distinguished among the *A. baumannii* isolates (37 and eight isolates, respectively) that shared a specific ribotype and were highly similar in other properties. These two groups comprised both sporadic and outbreak isolates and were found in most localities. Group A and B isolates were markedly more resistant to antibiotics than most of the remaining isolates, thus representing 85% of all multiresistant isolates. The features of groups A and B corresponded to those of two epidemic clones identified recently among hospital strains in north-western Europe.

Introduction

During the last decades, bacteria of the genus *Acinetobacter* have emerged as important nosocomial pathogens. These micro-organisms can cause a wide range of clinical disorders, including pneumonia [1, 2], bacteraemia [2, 3], burn infections [4] and urinary tract infections [5], particularly in severely ill, hospitalised patients. Numerous outbreaks of nosocomial infections with *Acinetobacter* spp. have been reported and these are often associated with the spread of multiresistant strains [1, 3, 5–8]. The genus is taxonomically heterogeneous and, by DNA–DNA hybridisation, 21 DNA groups have been described, seven of which have species rank [9–12]. DNA group 1 (*A. calcoaceticus*), DNA group 2 (*A. baumannii*) and the unnamed DNA groups 3 and 13 *sensu* Tjernberg and Ursing are closely related genetically and very similar phenotypi-

cally [13]. Therefore, it has been proposed to classify these groups together in the '*A. calcoaceticus*–*A. baumannii* (*Acb*) complex' [13]. *A. baumannii* is the species isolated most frequently from clinical specimens, but strains of the other members of the *Acb* complex are common in patient specimens and are also isolated during hospital outbreaks [2, 12, 14, 15]. Phenotypic identification of the DNA groups included in the *Acb* complex has been shown to be of doubtful value [13] and therefore species identity is not certain in studies that use only phenotypic tests. However, several genotypic methods have now been described that enable accurate differentiation between genomic groups of the *Acb* complex [16–18].

Many recent studies on nosocomial *Acinetobacter* isolates have focused on either an evaluation of various typing methods or on an epidemiological investigation of hospital outbreaks. Most of these studies dealt with geographically limited collections of strains, i.e., isolated in one hospital or several hospitals located close to each other. Results obtained in different laboratories are difficult to compare

because of variations in the methods used. However, a different strategy was followed in one study in that the various characteristics of *A. baumannii* strains from geographically distant localities of north-western Europe were compared [19]. With a combination of typing methods it was shown that most outbreak strains belonged to two distinct groups (clones), whereas sporadic strains were more heterogeneous.

The aim of the present study was to analyse the diversity of clinical isolates belonging to the *Acb* complex collected over a period of several years from different Czech hospitals. A combination of genotypic and phenotypic methods was applied to identify the organisms to DNA group and strain level. These methods have been used repeatedly for the characterisation of acinetobacters [12, 15, 19] and allowed the results to be compared with those of other studies.

Materials and methods

Strains

From 1991 to 1997, *c.* 400 clinical isolates belonging to the *Acb* complex were received by the National Institute of Public Health from different diagnostic laboratories in the Czech Republic. In total, 103 isolates were selected from these 400 isolates. The selected isolates were from hospitalised patients only and differed as widely as possible in place and time of isolation. They originated from 40 different services of 17 hospitals in 14 Czech cities (Fig. 1; Table 1 [20]). Seven isolates representative of different groups of epidemiologically and genetically related isolates, i.e., those collected during incidents of epidemic spread and indistinguishable by typing (*Hind*III restriction analysis of whole-cell DNA and biotyping), were included in this collection. The isolates were from various clinical

specimens, including sputum and tracheal aspirate (33 isolates), wound swabs (23), urine (22), drainage fluid (7), blood (7), intravenous catheters (6), nasopharyngeal swabs (4) and bile (1). The following strains were used as reference strains for different DNA groups of the *Acb* complex: ATCC strain 17902 (*A. calcoaceticus*), ATCC strain 19606 (*A. baumannii*), ATCC strain 19004 (DNA group 3) and ATCC strain 17903 (DNA group 13 *sensu* Tjernberg and Ursing (TU)); in addition, strains 5804 ('close to TU13') and 10169 ('between DNA groups 1 and 3') were kindly provided by P. Gerner-Smidt, Statens Serum Institut, Copenhagen, Denmark. Strains RUH 875 and RUH 134 represented two epidemic clones of *A. baumannii* recognised recently [19].

Phenotypic identification

All isolates had the basic properties of the genus *Acinetobacter* [21], i.e., gram-negative, strictly aerobic, oxidase-negative, non-motile coccobacilli. The isolates were placed in the *Acb* complex on the basis of the biochemical tests suggested by Bouvet and Grimont [20], including growth in brain heart infusion broth at 37°, 41° and 44°C, aerobic production of acid from glucose in O/F medium, gelatine hydrolysis and utilisation of 14 different carbon sources. Carbon source utilisation was tested on agar plates containing the defined medium of Cruze *et al.* [22] supplemented with substrate 0.1% w/v. Each plate was inoculated with a drop of cell suspension in saline prepared from an overnight culture and incubated at 30°C for 6 days. Utilisation of substrates was evaluated by comparing growth on supplemented plates with that on the control plate containing the mineral base only, with growth of even isolated colonies in the inoculum zone being considered positive.

Fig. 1. Geographical location of hospitals in the Czech Republic from which the isolates belonging to the *A. calcoaceticus*–*A. baumannii* complex originated.

Biotyping

Biotyping was based on utilisation of levulinate, citraconate, L-phenylalanine, phenylacetate, 4-hydroxybenzoate and L-tartrate [20].

Antibiotic susceptibility testing

Antimicrobial susceptibilities were determined by a disk diffusion method on Mueller-Hinton Agar (Oxoid). The antimicrobial agents tested ($\mu\text{g}/\text{disk}$) were: ampicillin + sulbactam (10 + 10); ticarcillin (75); ceftazidime (30); imipenem (10); gentamicin (10); tobramycin (10); amikacin (30); ofloxacin (5); and co-trimoxazole (sulphamethoxazole + trimethoprim: 23.75 + 1.25) (Oxoid). The isolates were classified as susceptible, intermediate or resistant according to the recommendations of the National Committee for Clinical Laboratory Standards [23].

Ribotyping

Whole-cell DNA was prepared by the method of Grimberg *et al.* [24], with minor modifications. Briefly, 1 ml of nutrient broth culture grown overnight at 30°C was harvested by centrifugation and the pellet was washed with 1 ml of TNE (10 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl, pH 8.0) and resuspended in 150 μl of the same buffer. Then 150 μl of TNE containing Triton X-100 2% v/v and lysozyme (final concentration 5 mg/ml; Serva, Heidelberg, Germany) was added and the suspension was incubated at 37°C for 20 min. Proteinase K (final concentration 1 mg/ml; Serva) was added to the lysed bacterial cell suspension and incubation was continued for 2 h at 65°C. Restriction endonuclease digestion of DNA samples was performed for 3 h at 37°C with 20 U of *EcoRI* (Fermentas, Vilnius, Lithuania) in 25 μl of incubation buffer supplied by the enzyme manufacturer. Electrophoretic separation of DNA fragments was done in agarose 0.7% w/v gels in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). Phage λ DNA (Fermentas) digested with *HindIII* and *StyI* (Fermentas) was used as molecular size markers. DNA fragments were transferred on to Zeta-Probe nylon membranes (BioRad) with a vacuum blotter (BioRad) and 10 $\times$ SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) as the transfer solution. A 16S and 23S *E. coli* rRNA (Boehringer) probe was labelled with digoxigenin-11-dUTP by M-MuLV reverse transcriptase (Boehringer) and random priming in accordance with the instructions given in the DIG DNA Labelling and Detection Non-Radioactive Applications Manual (Boehringer). The labelled probe used for detection of the molecular markers was obtained by random priming of phage λ DNA with digoxigenin-11-dUTP and a DIG DNA Labelling Kit (Boehringer). Pre-hybridisation, hybridisation (at 65°C) and washes were done as specified in the Zeta-Probe Blotting Membranes Instruction Manual (BioRad). Immunodetection of hybridised DNA was performed with a DIG Nucleic Acid Detection Kit

(Boehringer). Identification to the DNA group level was performed by visual comparison of the patterns with those of a set of reference patterns as described by Gerner-Smidt [16]. Similar ribotypes were compared in adjacent lanes on the same gel; they were considered different even if there was a difference in the position of only one band.

Plasmid analysis

To isolate plasmid DNA, the method of Kado and Liu [25] was modified as follows. Pelleted cells from 0.6 ml of an overnight culture were resuspended in 1 ml of saline and 26 μl of N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulphonate (Boehringer) 1% w/v in 100 mM citric acid were added. After incubation at 37°C for 10 min, cells were pelleted and resuspended in 0.15 ml of TE buffer (10 mM Tris.HCl, 1 mM EDTA, pH 8.0). To this was added 300 μl of lysing solution (SDS 3% w/v, 50 mM Tris, 32 mM NaOH) and the mixture was incubated for up to 15 min at 37°C. The lysed bacterial suspension was extracted once with 0.9 ml of water unsaturated phenol:chloroform (1:1), and the aqueous phase was incubated with RNase A (Sigma) 20 $\mu\text{g}/\text{ml}$ at 37°C for 30 min. The DNA was electrophoresed on agarose 0.7% w/v gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide and photographed under UV light. Supercoiled DNA ladder (D-5292; Sigma) was used to provide molecular markers. For restriction endonuclease digestion, aqueous DNA solution was purified of phenol by extraction with diethylether, precipitated with 800 μl of ethanol and dissolved in 30 μl of TE buffer. To verify the structural identity of 8.7-kb plasmids that were found frequently in *A. baumannii* isolates, a digoxigenin-labelled probe was prepared from one of these plasmids. The plasmid was extracted from isolate no. 632 which did not contain any other plasmids, purified in a caesium chloride gradient [26], and labelled by random priming with digoxigenin-11-dUTP and a DIG DNA labelling kit. Plasmid or whole-cell DNA digested with *HindIII* (Fermentas) was transferred on to a nylon membrane and hybridised with the probe. All steps for restriction endonuclease digestion, vacuum-transfer, hybridisation and detection were performed as described for ribotyping (see above).

Results

Identification

The properties and epidemiological data of the 103 isolates studied are given in Table 1. According to their *EcoRI* ribotypes, 77 isolates were allocated to DNA group 2, 14 isolates to DNA group 3, 10 isolates to DNA group 13TU and one isolate to DNA group 1. Isolate no. 417 could not be allocated unambiguously to any of these DNA groups. The pattern of isolate no. 417 had bands in common with both DNA group 2 and

Table 1. Taxonomic and epidemiological data for 103 isolates of the *A. calcoaceticus*-*A. baumannii* complex*

Isolate no. *	DNA group	EcoRI ribotype	Biotype†	Plasmid profile‡	Plasmid pANI	Antibiotic resistance pattern		Date	Origin		Specialty§
						ST	Of Ge		City§		
360	2	I	6	1	+	ST	Of Ge	8/94	Plzeň		ICU
421	2	I	6	1	+	ST	Of Ge	4/96	Plzeň		Sur
15	2	I	6	1	+	ST	Ge	6/91	Praha		BU
56	2	I	6	1	+	ST		8/92	Praha		BU
127	2	I	6	1	+	ST	Ge	5/93	Praha		BU
186	2	I	6	1	+	ST	Ge	10/93	Praha		BU
409	2	I	6	2	+	ST	Ge	4/96	Brno 1		Sur
437	2	I	6	2	+	ST	Ge	5/96	Brno 1		Sur
469	2	I	6	2	+	ST	Ge	1/97	Č. Budějovice		Sur
470	2	I	6	2	+	ST	Ge	1/97	Č. Budějovice		ICU
654	2	I	6	2	+	ST	Ge	3/96	Praha		Sur
472	2	I	6	u	+	ST	Of Ge To Am	1/97	Č. Budějovice		ICU
632	2	I	11	3	+	ST	Of Ge	11/94	Praha		BU
385	2	I	11	3	+	ST	Of Ge	3/96	Příbram		Sur
387	2	I	11	3	+	ST	Of Ge	3/96	Sedčany		ICU
110	2	I	11	4	+	ST	Of Ge	3/93	Praha		Ort
626	2	I	11	4	+	ST	Of Ge	7/94	Praha		Ort
303	2	I	11	u	+	ST	Of Ge	6/94	Kladno		ICU
392	2	I	11	5	+	ST	Of Ge	4/96	Sedčany		ICU
321	2	I	11	5	+	ST	Of Ge	8/94	Tábor		NH
322	2	I	11	5	+	ST	Of Ge	8/94	Tábor		Sur
331	2	I	11	5	+	ST	Of Ge	8/94	Tábor		ICU
207	2	I	11	6	+	ST	Of Ge	11/92	Liberec		ICU
7	2	I	11	6	+	ST	Of Ge	2/91	Praha		BU
642	2	I	11	6	+	ST	Of Ge	3/95	Praha		Sur
340	2	I	11	7	+	ST	Of Ge To Am	1/94	Ostrava 1		BU
352	2	I	11	7	+	ST	Of Ge To Am	8/94	Ostrava 2		ICU
353	2	I	11	7	+	ST	Of Ge To Am	8/94	Ostrava 1		Sur
408	2	I	11	7	+	ST	Of Ge To Am	4/96	Plzeň		ICU
188	2	I	11	7	+	ST	Of Ge To Am	10/93	Praha		Med 1
630	2	I	11	7	+	ST	Of Ge To Am	11/94	Praha		BU
639	2	I	11	7	+	ST	Of Ge To Am	2/95	Praha		RU
281	2	I	11	7	+	ST	Of Ge To Am	7/94	Příbram		ICU
309	2	I	11	8	+	ST	Of Ge To Am	8/94	Kladno		ICU
290	2	I	11	8	+	ST	Of Ge To Am	10/94	Příbram		ICU
356	2	I	11	8	+	ST	Of Ge To Am	7/94	Slaný		ICU
357	2	I	11	u	+	ST	Of Ge To Am	7/94	Slaný		ICU
471	2	II	2	NF	-	ST	Ge	1/97	Č. Budějovice		ICU
220	2	II	2	NF	-	ST	Ge To Am	1/93	Kladno		Neo
24	2	II	2	NF	-	ST	Ge	9/91	Praha		BU
48	2	II	2	NF	-	ST	Ge	12/91	Praha		BU
78	2	II	2	NF	-	ST	Of Ge	12/92	Praha		BU
141	2	II	2	NF	-	ST	Of Ge	6/93	Praha		Neu
330	2	II	2	NF	-	ST	Ge	7/94	Tábor		Ort
455	2	II	2	u	-	ST	Of Ge	9/96	Jihlava		ICU

Table 1. (Continued)

Isolate no.*	DNA group	EcoRI ribotype	Biotype†	Plasmid profile‡	Plasmid pAN1	Antibiotic resistance pattern	Origin		
							Date	City§	Specialty§
C 10	2	III	6	10	+	ST Of Ge Am AS Ti	7/91	Praha	BU
125	2	III	6	10	+	ST Of Ge Am AS Ti	5/93	Praha	BU
D 304	2	IV	6	11	-	ST Of Ge Ti	6/94	Kladno	ICU
301	2	IV	6	11	-	ST Of Ge Ti	6/94	Slaný	ICU
47	2	u	6	u	-	ST Of Ge To	12/91	Praha	BU
191	2	u	9	u	-	ST Of Ge To Am Ti Cz	11/93	Praha	BU
657	2	u	2	9	-	ST Of Ge Am Ti Cz	4/96	Praha	Neu
335	2	u	9	u	-	ST Of Ge To	7/94	Tábor	NH
60	2	V	9	u	-		11/92	Praha	Med 2
E 103	2	V	9	12	-		3/93	Praha	BU
169	2	V	9	13	-		9/93	Praha	Med 1
609	2	V	9	13	-		1/94	Praha	BU
4	2	u	5	u	-		2/91	Praha	BU
33	2	u	9	u	-	ST	10/91	Praha	BU
45	2	u	6	u	-		11/91	Praha	BU
67	2	u	1	u	-		11/92	Praha	BU
70	2	u	9	u	-		11/92	Praha	BU
80	2	u	9	u	-	ST			Sur
81	2	u	6	NF	-		1/93	Praha	PS
88	2	u	9	12	-		2/93	Praha	BU
143	2	u	11	u	-		6/93	Praha	Paed
146	2	u	16	NF	-		7/93	Praha	Ort
190	2	u	8	u	-		11/93	Praha	BU
201	2	u	6	u	-		7/92	Liberec	ICU
203	2	u	8	u	-		7/92	Jablonec	Med
254	2	u	11	u	-		6/93	Č. Budějovice	Paed
324	2	u	8	u	-		8/94	Tábor	Med
329	2	u	2	u	-		7/94	Tábor	ICU
407	2	u	9	u	-		3/96	Tábor	ICU
410	2	u	11	u	-		4/96	Brno 2	Paed
601	2	u	13	u	-		11/93	Praha	Med 2
615	2	u	15	ND	-		1/94	Praha	BU
F 12	13TU	VI	9	u	-		7/91	Praha	BU
41	13TU	VI	9	u	-		10/91	Praha	BU
84	13TU	VII	9	u	-		1/93	Praha	BU
G 386	13TU	VII	9	14	-		3/96	Příbram	ICU
430	13TU	VII	9	14	-		5/96	Příbram	ICU
H 63	13TU	VIII	9	15	-		11/92	Praha	Sur
105	13TU	VIII	9	15	-		3/93	Praha	Paed
106	13TU	u	11	u	-		3/93	Praha	BU
258	13TU	u	9	u	-		6/93	Č. Budějovice	Neo
395	13TU	u	9	u	-		4/96	Příbram	ICU
I 76	3	IX	11	16	-		12/92	Praha	Ort
104	3	IX	11	16	-		3/93	Praha	Ort

Table 1. (Continued)

Isolate no.*	DNA group	EcoRI ribotype	Biotype†	Plasmid profile‡	Plasmid pANI	Antibiotic resistance pattern	Origin		
							Date	City§	Specialty§
14	3	u	8	u	—		6/91	Praha	BU
36	3	u	atyp.¶	u	—		10/91	Praha	BU
82	3	u	8	u	—		1/93	Praha	Ort
95	3	u	11	u	—		2/93	Praha	Uro
119	3	u	8	u	—		4/93	Praha	BU
145	3	u	8	u	—		7/93	Praha	NH
202	3	u	8	u	—		7/92	Jablonec	Med
241	3	u	8	u	—		6/93	Znojmo	RU
243	3	u	11	u	—		5/93	Znojmo	Uro
328	3	u	11	u	—		8/94	Tábor	Ort
336	3	u	1	u	—		8/94	Tábor	Uro
627	3	u	8	u	—		7/94	Praha	Ort
13	1	u	2	ND	—		7/91	Praha	BU
417	unident.	u	15	ND	—		4/96	Brno 3	Med

u, unique profile; NE, no plasmids found; ND, not determined; ST, co-trimoxazole; Of, ofloxacin; Ge, gentamicin; To, tobramycin; Am, amikacin; AS, ampicillin + sulbactam; Ti, ticarcillin; Cz, ceftazidime; underlined symbols indicate intermediate susceptibility; BU, burns unit; ICU, intensive care unit; Med, medical; Neo, neonatology; Neu, neurology; NH, nursing home; Ort, orthopaedic; Paed, paediatrics; PS, plastic surgery; RU, rehabilitation unit; Sur, surgery; Uro, urology.

*The isolates were classified successively according to DNA group, ribotype and plasmid profile; each box indicates a group (labelled A–I) of isolates identical in ribotype and similar in other properties; each of the underlined isolates is representative of a group of genetically related isolates that were collected during incidents of epidemic spread.

†Biotype as determined by Bouvet and Grimont [20].

‡Plasmid profiles were compared for identity only in the isolates belonging to the same DNA group.

§Different hospitals in the same city, or different specialties of the same type in a single hospital, are numbered.

¶Atypical biotype: growth on phenylacetate, absence of growth on L-phenylalanine.

DNA group 13TU, but some other bands of these DNA groups were missing in this isolate (Fig. 2). When compared with the reference strains of two additional DNA groups, several common bands with strain no. 5804 ('close to TU13') were found (data not shown). All isolates identified as *A. baumannii* or DNA group 13TU showed growth at 44°C, whereas all isolates identified as DNA group 3 and isolate no. 417 grew at 41°C, but not at 44°C. The single isolate identified as *A. calcoaceticus* grew at 37°C only.

Ribotypes

Twenty-nine different *Eco*RI ribotypes were observed among the isolates of *A. baumannii* (Table 1). Ribotypes I and II were found in 37 and eight isolates, respectively, representing together as many as 58% of all *A. baumannii* isolates. Ribotype V was revealed in four isolates, ribotypes III and IV were each shared by two isolates, and 24 ribotypes were found only once. Isolates of DNA group 13TU showed six different ribotypes, with ribotypes VI, VII and VIII represented by two, three and two isolates, respectively, with three ribotypes represented by only one isolate. DNA group 3 isolates showed the highest heterogeneity, as only one of 13 different ribotypes observed was found in two isolates, while the other profiles were recorded only once.

Biotypes

Biotyping according to Bouvet and Grimont allowed 10 biotypes to be distinguished. *A. baumannii* isolates belonged to biotypes 1, 2, 5, 6, 8, 9, 11, 13, 15 and 16. Biotype 11 was the most frequent (28 isolates), followed by biotypes 6, 9 and 2 (20, 11 and 10 isolates respectively). Biotypes 1, 8 and 11 were encountered among the isolates of DNA group 3, with biotype 8 being predominant (seven isolates). Isolate no. 36 appeared atypical as it grew on phenylacetate but not on L-phenylalanine. Isolates of DNA group 13TU belonged to biotype 9, except for isolate no. 106 which was of biotype 11. The single *A. calcoaceticus* isolate belonged to biotype 2, while isolate no. 417 was of biotype 15.

Antibiograms

All isolates were susceptible to imipenem and, according to their susceptibility to the other antibiotics, could be divided into two main groups. The 'susceptible' group included 48 isolates susceptible to all antibiotics, i.e., 22 *A. baumannii* isolates and all non-*A. baumannii* isolates. The 'resistant' group included 52 *A. baumannii* isolates resistant to two or more antibiotics, including at least one of the aminoglycosides. The remaining three *A. baumannii* isolates were resistant only to co-trimoxazole.

Fig. 2. Examples of *Eco*RI ribotypes showing those for 15 clinical isolates and four reference strains of the *A. calcoaceticus*-*A. baumannii* complex. Isolate nos 7 and 24 represent groups A and B (Table 1), respectively; isolate no. 417, ungrouped; HG, hybridisation (DNA) group; MW, mol. wt markers (phage λ DNA digested with *Hind*III and *Sty*I).

Plasmid analysis

Plasmids were detected in all isolates of DNA groups 3 and 13TU, and in 68 (89%) of 76 *A. baumannii* isolates, ranging in size between 2 kb and >100 kb. Plasmid profiles contained one to six bands and were compared for similarity only in the isolates belonging to the same DNA group. In total, 37, eight and 13 different profiles were found among the isolates of DNA groups 2, 13TU and 3, respectively. Some different profiles shared one or more plasmids of similar size. These profiles were observed mostly in isolates with an identical ribotype. Each of the ribotype I isolates carried a plasmid of *c.* 8.7 kb (Fig. 3). Plasmids of this size were not found in any of the other isolates, except for two isolates with ribotype III. As isolates with ribotypes I and III mostly carried one to four additional plasmids, the structural similarity of the

Fig. 3. Plasmid profiles of 13 *A. baumannii* isolates characterised by the presence of an 8.7-kb plasmid (pAN1). Except for isolate no. 10, all belonged to the most prevalent *EcoRI* ribotype. MW, mol. wt marker (supercoiled DNA ladder, Sigma D-5292); Chr, chromosomal DNA.

8.7-kb plasmids was investigated by hybridisation of *HindIII* restriction profiles of plasmid DNA with a probe made from the 8.7-kb plasmid (designated pAN1) from isolate no. 632. In each case, three fragments were detected, 4.8, 2.6 and 1.3 kb in size, corresponding to the *HindIII* restriction profile of purified plasmid pAN1 DNA. The presence of sequences homologous with pAN1 in the remaining isolates of the *Acb* complex was investigated by hybridisation of the pAN1 probe with *HindIII* restriction profiles of whole-cell DNA. No positive signal was recorded for 59 isolates, while five isolates yielded one band each of varying sizes (data not shown). The localisation of these sequences (i.e., on plasmid or chromosome) was not studied.

Correlation between the ribotypes and other characteristics

The combination of typing profiles allowed nine groups of isolates to be defined (labelled A–I) that shared a specific ribotype and were highly similar in other properties (Table 1). Group A included 37 *A. baumannii* isolates with ribotype I that belonged to biotypes 11 and 6 (25 and 12 isolates respectively). Moreover, all isolates of this group contained a pAN1 plasmid. The group A isolates originated from 22 hospital specialties in 11 cities. Isolates impossible to differentiate by any of the methods used came from the same locality and also from geographically distant hospitals. Group B included eight *A. baumannii* isolates with ribotype II that belonged to biotype 2. Plasmid DNA was not detected in six isolates of this group, while a plasmid of different size was present in each of two other isolates. The group B isolates originated from six hospital specialties in five cities. Except for isolate no. 56 (group A), which was resistant only to co-trimoxazole, all isolates in groups A and B were resistant to two or more antibiotics. Therefore, 44 (85%) of 52 'resistant' isolates belonged to these two groups. Seven other groups each included two to four isolates with the same ribotype, biotype and similar plasmid profiles (at least one plasmid of similar size). Apart from isolates of group G (DNA group 13TU), isolates belonging to the same group originated either from the same hospital or from two nearby hospitals (group D).

Characteristics of strains RUH 875 and RUH 134

Strain RUH 875 (representative of epidemic clone I) had ribotype I and harboured a pAN1 plasmid; strain RUH 134 (representative of epidemic clone II) had ribotype II and did not contain plasmid DNA.

Discussion

In this study, *A. baumannii* was the most frequent group among isolates of the *Acb* complex. All multi-resistant and outbreak isolates belonged to this species.

These findings are consistent with previous studies [14, 17, 20] and corroborate findings of the major epidemiological and clinical importance of *A. baumannii* among the other nomenclatures and DNA groups of *Acinetobacter*. DNA group 3, also isolated frequently from hospital patients and found to be associated with hospital outbreaks [2, 15], was the second most numerous group. The fact that 10% of the isolates belonged to DNA group 13TU, not included in the Bouvet and Grimont scheme [9, 20], is indicative of the importance of this group.

Ribotyping allows both species identification and the detection of genetic relatedness between strains at the subspecies level [27]. Previous studies have shown a wide diversity of *EcoRI* ribotypes in the *Acb* complex and this finding proved useful for typing hospital isolates [16, 28, 29]. The diversity in *EcoRI* ribotypes among DNA group 3 isolates, was confirmed, with most of these isolates having a unique ribotype. *A. baumannii* was different in that >50% of the isolates belonged only to two ribotypes. The isolates with identical ribotypes were also similar in other characteristics such as plasmid profile, biotype and susceptibility to antimicrobial agents. Such a correspondence of genotypic and phenotypic characteristics can be explained by a common clonal origin [30]. Therefore, the two most numerous groups of isolates, A and B, seem to represent two evolutionary lineages of *A. baumannii* dispersed widely in hospitals throughout the Czech Republic.

A separate recent study [19] compared the genotypic and phenotypic properties of outbreak and sporadic *A. baumannii* strains from different north-western European cities and found that most outbreak strains could be placed in two similarity groups (designated as clones I and II), whereas sporadic strains were more heterogeneous. Strain RUH 875 (representative of clone I) was identical in ribotype with group A in the present study, while strain RUH 134 (representative of clone II) was identical in ribotype with group B. Strains of clone I belonged to biotypes 6, 11 and 19, while isolates of group A belonged to biotypes 6 and 11. Similarly, strains of clone II belonged to biotypes 1, 2 and 9, while isolates of group B belonged to biotype 2. Both clones I and II, and groups A and B were characterised by a higher degree of resistance to antibiotics compared with most of the other strains. In addition, the cryptic plasmid pAN1, characteristic of the group A isolates, was also detected in strain RUH 875, while no plasmid was found in either strain RUH 134 or most group B isolates. Thus, the north-western European clones corresponded with the two Czech groups in their genotypic and phenotypic characteristics.

Previous studies have pointed out the predominant frequency of certain biotypes amongst *A. baumannii* isolates, namely biotypes 1, 2, 6 and 9 [14, 20]. The most frequent biotype in the present study was biotype

11, found rather rarely in other studies. When comparing the properties of the *A. baumannii* isolates belonging to the same biotype, the frequent biotypes 6 and 9 were found to comprise genotypically heterogeneous isolates. In contrast, except for a few cases, isolates of biotype 11 had indistinguishable ribotypes, i.e., belonged to group A. Therefore, the frequent occurrence of biotype 11 in the Czech Republic is the result of its predominance among the strains of a highly prevalent clonal group.

A high frequency of genetically related strains among the outbreak and multiresistant strains may affect the efficacy of some typing methods used in epidemiological investigations of *acinetobacter* infections, i.e., in the case of the concurrent spread of different strains of the same clone. Some electrophoretic methods, such as ribotyping with *EcoRI* or cell envelope protein analysis, are apparently not useful for subtyping these strains [19], and the efficacy of other methods remains to be evaluated. Of these methods, macro-restriction analysis has been shown to be more discriminatory than ribotyping in *A. baumannii* [31] and may prove useful in this context. Moreover, the variations that were found in plasmid profiles and biotypes may also be of use in the further differentiation of isolates of the most prevalent Czech group.

In summary, almost half the Czech hospital isolates of the *Acb* complex fell into two groups of *A. baumannii*, highly similar to two clones which have been reported to predominate among the outbreak strains of north-western Europe. These groups comprised both outbreak and sporadic strains isolated within the last 7 years from all over the Czech Republic. Moreover, isolates of these groups were much more resistant to antibiotics than the remaining isolates. The finding that the multiresistance is linked to a limited range of genetically related strains is surprising and may be significant in the light of the progressive increase in resistance in *Acinetobacter* strains during the last decades.

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