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Complexity of *Pseudomonas aeruginosa* infection in cystic fibrosis: combined results from esterase electrophoresis and rDNA restriction fragment length polymorphism analysis

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SUMMARY

Esterase electrophoretic typing and restriction fragment length polymorphism of ribosomal DNA regions (ribotyping) were used to differentiate 102 *Pseudomonas aeruginosa* clinical isolates obtained from chronic lung infection in 23 patients with cystic fibrosis (CF) and two reference strains (including the type strain ATCC 10145). Twenty-five zymotypes were obtained with the former method and 16 ribotypes with the latter. Combination of the two typing systems led to the finding of 30 different types. Our data highlights the physiopathological complexity of *P. aeruginosa* infection in CF as, in six individual cases, several types were found among isolates from a given patient. On the other hand, two unique types were found in two and three patients respectively, raising the possibility of cross-infections.

INTRODUCTION

Pseudomonas aeruginosa chronic lung infection is a major cause of morbidity and mortality in cystic fibrosis (CF) patients. Precise epidemiological data are necessary for the understanding of the disease physiopathology and for an optimal patients' management. Typing of *P. aeruginosa* strains isolated from patients with CF is often imprecise by the currently available phenotyping systems (O-serotyping, biotyping, phage-typing, pyocin-typing or determination of anti-microbial susceptibility profiles) [1]. Recently, genome fingerprinting by field inversion gel electrophoresis (FIGE) [2] and restriction fragment length polymorphism (RFLP) in the exotoxin A [3–7] or pilin gene regions [4, 5, 8] have been used as molecular approaches to *P. aeruginosa* epidemiology in CF. Esterase electrophoretic typing [9–11] and RFLP of rDNA regions [10] have been shown to provide discriminative data for epidemiological screening in non-CF patients' *P. aeruginosa* strains. The present study makes use of these two methods to

differentiate isolates within and between 23 *P. aeruginosa* chronic lung infections in CF patients.

MATERIALS AND METHODS

Strains and clinical features

We studied 102 clinical *P. aeruginosa* strains collected from 23 unrelated CF patients regularly followed at the Gastroenterology Unit of the Hôpital Robert Debré in Paris, France, as well as two reference strains. All patients had chronic lung *P. aeruginosa* infection. Chronic infection was defined as repeated positive cultures from sputum (direct examination: presence of polymorphonuclear leukocytes and Gram negative bacilli, culture: *P. aeruginosa* $> 10^6$ c.f.u. per ml of sputum) over a period of at least 6 months and/or as the presence of more than two precipitins against *P. aeruginosa* [12]. Clinical strains were isolated from sputum between January 1989 and April 1990. One to four strains were analysed for each sample depending upon the existence or not of colony macroscopic appearance dissimilarity. Isolates were identified as *P. aeruginosa* based upon typical morphology, oxidase reaction, ability to produce pigments, growth at 42 °C and biochemical tests (API–20 NE system – API, Les Balmes, France). O-serotyping was performed with O-antisera (Diagnostics Pasteur, Marnes La Coquette, France). Reference strains were the type strain of the species, ATCC 10145 and strain ATCC 27853.

Esterase electrophoresis

Bacteria were grown for 18 h at 37 °C in L broth [13] with constant shaking and harvested by centrifugation. Bacterial pellets were washed with a 60 mM Tris-glycine buffer pH 8·7, resuspended in the same buffer and disrupted by sonication for 18 min at 4 °C. Crude extract supernatants containing 40–60 mg of proteins per ml, were stored at −20 °C until used [14].

Horizontal slab-gel electrophoresis was performed according to the method of Uriel [15] in a composite polyacrylamide-agarose gel (7 and 1·4%, respectively) in a discontinuous Tris-glycine buffer pH 8·7 at constant voltage (7 V/cm) until the bromophenol blue marker had run 13 cm. The relative mobility (M_F value) is the moving distance of the esterase band as a percentage of the moving distance of the dye front. M_F values were compared by running bacterial extracts in contiguous tracks on the same gel; in some experiments, the order of the extracts on the gel was changed [16].

Esterases were stained on the gel [17, 18] using the following specific substrates: α-naphthyl acetate, β-naphthyl acetate, α-naphthyl propionate, β-naphthyl propionate, α-naphthyl butyrate, β-naphthyl butyrate and indoxyl acetate (Sigma, St Louis, Missouri, U.S.A.). Sensitivity of esterases to diisopropyl-fluorophosphate (DFP) (10^{-3} M) was also tested.

RFLP of rDNA regions

Bacterial DNA was prepared as published elsewhere [19]. Five μg of DNA were digested with *Hind* III, *Eco*R I, and *Bcl* I restriction enzymes (Boehringer, Mannheim, F.R.G.) according to the manufacturer's specifications and analysed by electrophoresis on 0·8% submarine agarose gels containing ethidium bromide.

DNA-fragment size-marker Raoul I (Appligene, Strasbourg, France) was used. Size-separated DNA restriction fragments were transferred to a nylon membrane (Gene Screen Plus, New England Nuclear Products, Boston, Massachusetts, U.S.A.) by the method of Southern [20]. Ribosomal 16+23 S RNA from *Escherichia coli* (Boehringer) was labelled by random oligopriming using a mixture of hexanucleotides (Pharmacia, Uppsala, Sweden) and cloned M-MLV reverse transcriptase (BRL, Gaithersburg, Maryland, USA) in the presence of [^{32}P]dCTP (2′ deoxycytidine-5′-triphosphate, 800 Ci/mmol, Amersham, Amersham, U.K.) [19]. Hybridization, washing and autoradiographic procedures were as described previously [19].

RESULTS

O-serotyping

Seventeen strains among the clinical isolates were serotypable (16.7%). They belonged to serogroup 1 (5 strains), serogroup 4 (3 strains), serogroup 6 (6 strains), serogroup 9 (1 strain), serogroup 10 (1 strain) and serogroup 11 (1 strain). The remaining clinical strains were polyagglutinable (2 strains, 1·9%), auto-agglutinable (12 strains, 11·8%) or non-typable (71 strains, 69·6%). Two strains of distinct serogroups (serogroups 10 and 6) were isolated from a single patient (AU). All other patients were infected by strains of unique serogroups.

Esterase electrophoretic typing

Fig. 1 shows typical esterase patterns, here obtained on 5 different *P. aeruginosa* strains, after β-naphthyl propionate staining of the electrophoretic gel. Four main esterase categories can be defined based upon their activity towards the tested substrates and their sensitivity or resistance to DFP [11]. All hydrolysed β-naphthyl propionate. They were numbered P_1–P_4 in order of decreasing mobility towards the anode. Seven additional bands that could not be classified in one of these main categories were detected in some strains. On the whole, the 104 strains of *P. aeruginosa* could be divided into 25 zymotypes (1–25) according to their esterase band patterns (Table 1).

Ribotyping

RFLP in the rDNA regions was analysed on 52 *P. aeruginosa* strains. As an example, Fig. 2 shows some of the patterns obtained with *Hind* III, *Bcl* I and *Eco*R I, respectively. Each of the three restriction endonucleases tested produced six different RFLP patterns of the rDNA regions. Each distinct combination of patterns was used to define a ribotype. Altogether, 16 ribotypes were identified (A–P) (Table 1).

Combination of esterase and ribotyping

Combination of the two sets of results allows the delineation of 30 types among the 104 strains of *P. aeruginosa* (Table 1); each type being designated as the association of a given zymotype to a specific ribotype (i.e. ATCC 10145 corresponds to type 9/A). When several isolates were available for a given patient, they belonged to the same type in 8 cases but to 2 types in 4 cases and to 3 types in 2 cases. Based upon this typing strategy, identical strains among different patients

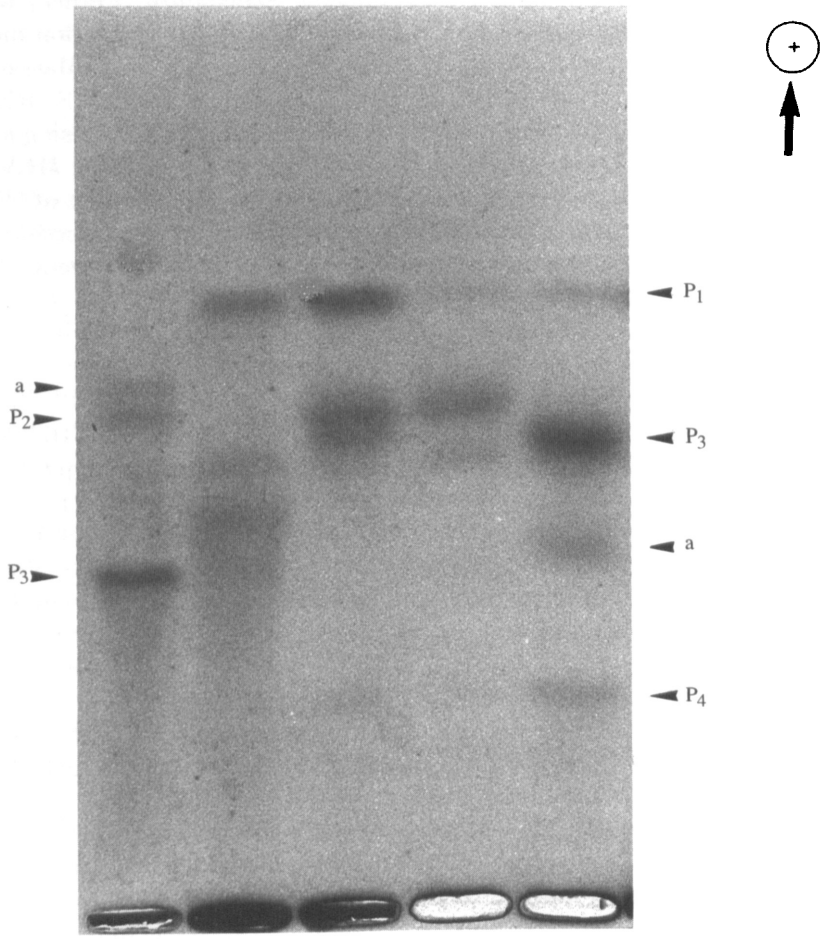

Fig. 1. Representative esterase electrophoretic patterns of five *P. aeruginosa* strains obtained after staining by β-naphthyl propionate. P_1, esterase P_1; P_2, esterase P_2; P_3, esterase P_3; P_4, esterase P_4; a, additional esterase.

were observed only with type 9/B (strains DU 3, LA 14, 18 and LE 3) and type 9/H (strains CH 1 and MI 1).

DISCUSSION

As noted by others [1–3, 8], O-serotyping is of limited value for epidemiological studies of *P. aeruginosa* infection in CF patients. In the present study, 83·3% of the isolates were either polyagglutinable, autoagglutinable or non-typable. On the other hand, when serotype is compared to genotype, it appears that genetically indistinguishable strains may present with different serotypes [3, 4, 8]. For instance, *P. aeruginosa* isolates of patient AU were indistinguishable both by esterase electrophoretic typing and by ribotyping but exhibited two distinct serotypes (see results and Table 1). It has been formerly postulated that these examples of serotype changes represent a subtle genetic change within a strain such as phage conversion, gene duplication or phase variation [3].

It was reported earlier that using at least two typing systems improves

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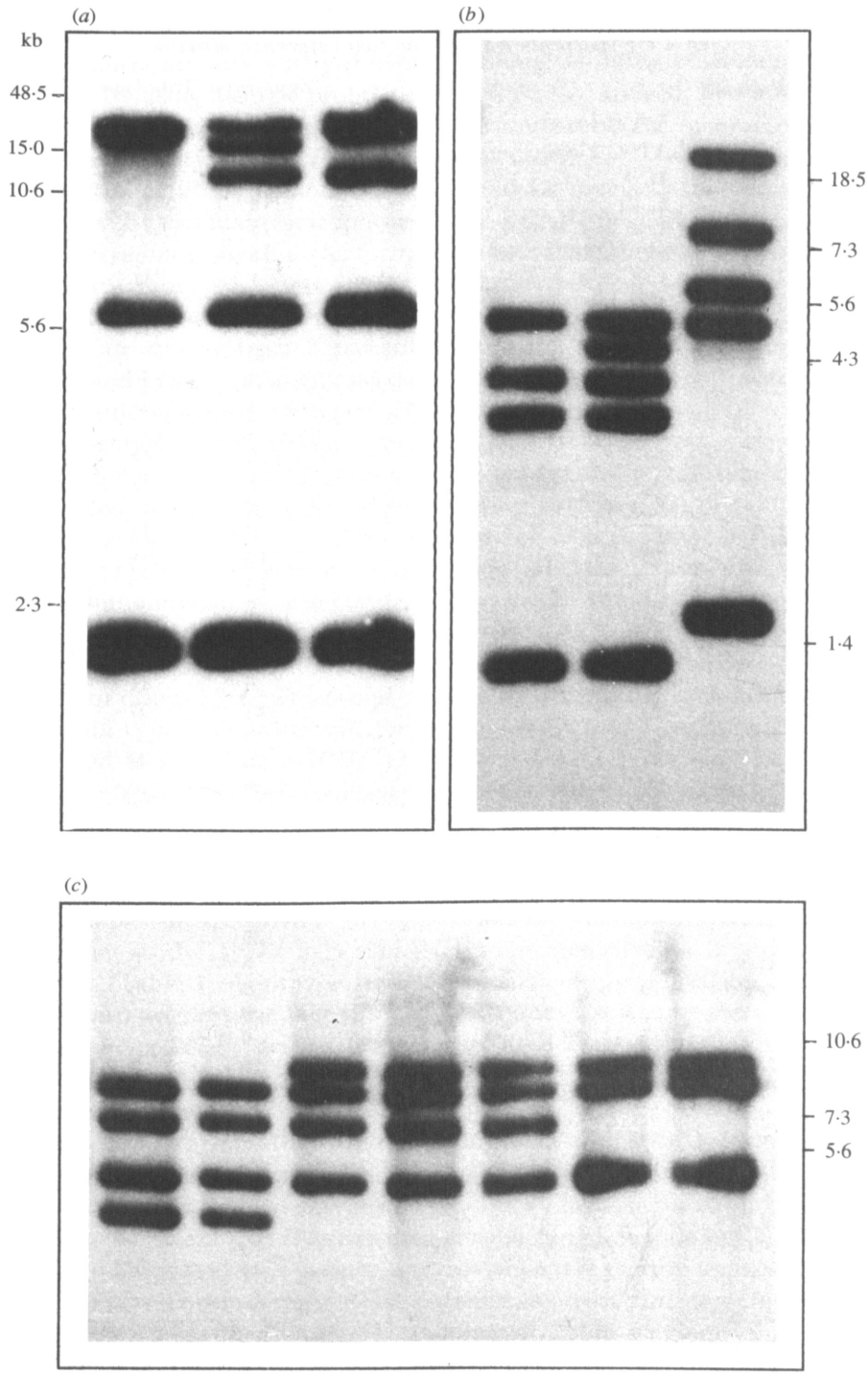

Fig. 2. Representative RFLP patterns of *P. aeruginosa* rDNA regions obtained by digestion with three restriction enzymes. (*a*) *Hin*d III; (*b*) *Bcl* I; (*c*) *Eco*R I.

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Table 1. *Zymotyping and ribotyping of the 102* P. aeruginosa *isolates obtained from CF patients and of the two reference strains*

Patient	Strain	Zymotype	Ribotype
	ATCC 10145	9	A
	ATCC 27853	22	B
AP	1	10	C
AU	8, 20	18	A
	1, 2, 3, 4, 5, 6, 7, 10, 11, 12,	18	
	14, 15, 16, 17, 18, 19	18	
BA	1, 2, 3, 4, 5, 6, 7, 8, 9	1	D
BD	1	2	A
BH	1, 3	25	E
	2	25	
BO	5, 11	3	F
	9	4	F
	10	4	
CA	1	9	G
CE	3, 5	8	B
	2, 4, 6, 7, 8	8	
CH	1	9	H
	11	13	B
	3, 8	13	
	9, 10	7	B
CL	2	15	B
	1	15	
CO	1	6	H
	2	21	B
DU	1	9	I
	3	9	B
	2, 4, 5, 6	9	
EL	2, 6, 10	19	J
	4, 8, 9, 11, 12	19	
	5	24	J
GE	1, 2	12	K
GR	1, 2	20	B
	3, 4	20	
JO	1	5	L
LA	14, 18	9	B
	3, 5, 6, 7, 8, 9, 10, 11, 12,	9	
	13. 15. 16, 17, 19	9	
LE	2, 7	16	N
	1	16	
	5	17	N
	3	9	B
LY	1	9	M
ME	1	14	O
MI	1	9	H
SO	1	23	P
VE	1	11	H

epidemiologic analysis of *P. aeruginosa* [10, 21–23]. In this study, we have combined esterase electrophoretic typing and ribotyping. These two methods have already been shown individually to be powerful and readily applicable epidemiological tools [9, 11, 19, 24–30]. RFLPs in the exotoxin A [3–7] or pilin [4, 5, 8] gene regions have also been used successfully as specific genetic markers to differentiate *P. aeruginosa* strains. However, one advantage we see in our

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approach is that, as with genomic fingerprinting by FIGE [2], it is comprehensively applicable to other bacterial species. Esterases are ubiquitous in bacteria and rDNA sequences are sufficiently conserved among the eubacterial kingdom [31] to be explored by using a single probe, i.e. *E. coli* rRNA. Indeed, Pitt and colleagues [10] have used these two methods concomitantly for characterization of multiresistant serotype O 12 *P. aeruginosa* strains.

All the studied *P. aeruginosa* isolates could be precisely and unambiguously characterized by esterase electrophoresis and RFLP of rDNA regions. Among the studied *P. aeruginosa* strains, the former method discriminated 25 zymotypes and the latter 16 ribotypes. Some zymotypes and ribotypes were more frequently encountered. For instance, zymotype 9 was found in strains from 7 patients and ribotype B in strains from 8 patients (Table 1). Overall, the combination of ribotyping and zymotyping enhanced the discriminative power of each individual method in differentiating 30 types of strains.

A strict correlation between zymotype and ribotype was observed among isolates from 8 patients. For 7 of them, a unique zymotype/ribotype combination was recovered in all strains. For the remaining patient (CO), 2 zymotypes were found together with 2 ribotypes. Divergence between the 2 typing methods was found for 5 patients. For one of them (patient DU) the strains were of the same zymotype but differed by 2 separate ribotypes. In the 4 other cases, a unique ribotype corresponded to 2 distinct zymotypes.

The strain heterogeneity detected in six of our cases differentiate our findings from those of others [3, 6] who found a single strain only, in each patient. Indeed, several strain types were concomitantly demonstrated in 4 patients (BO, CO, DU and EL) and in one patient (CH), the various strain types were not recovered sequentially. This strain heterogeneity is independent of the range of time period from first to last isolate. These data, in agreement with those of Speert and colleagues [8], demonstrate the physiopathological complexity of *P. aeruginosa* infection in CF.

Patient to patient spread of *P. aeruginosa* has been demonstrated recently in CF [2, 6]. In our study, types 9/B and 9/H were identified in 3 and 2 patients, respectively (Table 1). However, an epidemiological interpretation of this result must take into account the fact that we find zymotype 9 to be the most frequent zymotype among 127 *P. aeruginosa* strains isolated from various human infections [11]. Still, it is worth mentioning that patients DU and LA both exhibiting type 9/B attended on the same day at the out-patient clinic.

In conclusion, we find that combination of two molecular typing methods, esterase electrophoresis and rDNA-RFLP determination constitutes a very valuable tool for the precise characterization of *P. aeruginosa* strains. Our results highlight the complexity of *P. aeruginosa* chronic lung infection in CF. Finally, the approach we have used is of general interest since it is readily applicable to other bacterial species encountered in CF patients such as *Haemophilus influenzae*, *Staphylococcus aureus* or *P. cepacia*.

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