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ORIGINAL PAPER

A Comparative Evaluation of PCR Ribotyping and ERIC PCR for Determining the Diversity of Clinical *Pseudomonas Aeruginosa* Isolates

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Abstract

Two typing methods were evaluated, utilizing 62 clinical strains of *Pseudomonas aeruginosa*, to assess their usefulness as tools to study the bacterial diversity within this complex group. Genetic diversity was determined by PCR ribotyping and enterobacterial repetitive intergenic consensus (ERIC) PCR. By these methods, 9 and 36 genotypes were found, respectively. The result showed that ERIC PCR analysis is a more discriminatory method than PCR ribotyping analysis and traditional serotyping scheme. We suggest that maximum discrimination can be achievied by a combination of these methods.

K e y w o r d s: *Pseudomonas aeruginosa*, serotyping, ribotyping, ERIC PCR

Introduction

Pseudomonas aeruginosa is a versatile Gram-negative bacillus that grows plant and animal tissues. People with respiratory, gastrointestinal and urinary tracts infection, burn victims, individuals with cancer, and patients hospitalized in intensive care units are particularly affected by *P. aeruginosa* mostly due to nosocomial spreads and cross contaminations (Ahmed *et al*., 2002; Ruimy *et al*., 2001).

Molecular typing methods discriminate between of the same species by use of their chromosomal differences. The discriminatory power is much higher than that obtained by classical phenotypic methods such as serotyping, phage typing, pyocin typing and biotyping (Speert 2002; Bennekov *et al*., 1996). DNA typing methods have been frequently used for investigating the diversity of collections of *P. aeruginosa*. These methods include pulsed-field gel electrophoresis (PFGE) (Kersulyte *et al*., 1995; Renders *et al*., 1996), ribotyping (Dawson *et al*., 2002; Fiett *et al*., 1998; Liu *et al*., 1996), and PCR-based fingerprinting methods (Czekajło-Kołodziej *et al*., 2006; Dawson et al., 2002; Dąbrowski *et al*., 2003; Fiett *et al*., 1998; Hernandez *et al*., 1997; Liu *et al*., 1996).

The present investigation compares the results of PCR ribotyping (date which concentrate on the analysis of the segments of the ribosomal genes) and ERIC-PCR (assays which utilize primers targeting highly conserved repetitive sequence elements in the whole bacterial genome) for clinical *P. aeruginosa* isolates.

Experimental

Materials and Methods

Isolates source, identification and O serotyping. A total of 62 strains of *P. aeruginosa*, were originally isolated from a variety of clinical specimens: faeces (26), urine (11), blood (1), bronchial washings (9), sputum (1), wound swab (9), throat swab (2), ulceration swab (1), swab from skin round tracheotomy (1) and from ear (1). The bacteria were obtained from 62 patients from different wards of the municipal hospital, main hospital and outpatients' department in Siedlce (Poland), between December 2005 and March 2006. The strains were identified as *P. aeruginosa* according to biochemical patterns in the Api 20NE system (bio Mérieux). We also identified *P. aeruginosa*

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by PCR amplification of 16S ribosomal RNA (Kingsford and Raadsma, 1995). All isolates resulted in a positive reaction. The control strain NCTC 6749 was also examined.

Stock cultures were stored in TSB (tripticase soy broth, Difco) containing 20% glycerol at –80ºC.

Serotyping was performed by slide agglutination using commercially available O antisera (Sanofi Diagnostics Pasteur) as recommended by the manufacturer.

DNA extraction. Isolates were grown in trypticase-soya broth at 37°C for 24 h and DNA was extracted by using the Genomic DNA Pre Plus (A&A Biotechnology, Gdańsk, Poland).

PCR ribotyping. Polymorphisms were sought in the 16S-23S spacer region of the rRNA genes by the DNA amplification using the primers complimentary to the conserved regions of the 16S and 23S bacterial rRNA genes. Primer sequences used were

Table I
Compilation of PCR ribotyping and ERIC PCR ribotyping data for isolates of *Pseudomonas aeruginosa*

No of isolate	Sero-type	PCR ribotype	ERIC PCR genotype	Source of isolation	Hospital/ ward
1	P1	2	1	urine	main/1*
2	P6	3	2	urine	main/2
3	P1	1	3	wound	main/3
4	P6	1	4	faeces	municipal/4
5	P6	1	4	faeces	main/4
6	P6	1	5	urine	main/5
7	P10	4	6	urine	main/6
8	P6	1	4	faeces	main/4
9	P1	1	7	sputum	main/7
10	P6	1	8	faeces	main/8
11	P6	1	8	faeces	municipal/4
12	P6	6	8	faeces	main/8
13	P6	1	8	faeces	municipal/4
14	P6	1	9	faeces	municipal/4
15	P6	1	10	the reference strain NCTC6749	
16	PMF	1	11	wound	municipal/9
17	P6	1	12	wound	main/3
18	P6	1	12	wound	main/2
19	P15	1	13	bronchial washings	main/7
20	P6	1	8	faeces	municipal/4
21	P6	1	8	faeces	municipal/4
22	P6	1	9	faeces	municipal/4
23	P6	1	14	throat swab	main/7
24	P6	1	15	skin	main/7
25	P6	5	15	faeces	main/8
26	P6	6	16	faeces	main/8
27	PMF	1	17	wound	main/2
28	P6	1	18	bronchial washings	main/7
29	P6	1	15	wound	main/9
30	P6	1	19	urine	outpatients' department
31	P6	1	15	faeces	municipal/4
32	P6	1	20	urine	outpatients' department
33	P1	1	21	urine	main/6
34	PMA	9	22	bronchial washings	main/5
35	P6	1	23	bronchial washings	main/7
36	P6	1	26	faeces	main/4
37	P6	1	26	faeces	main/8
38	P6	1	15	faeces	municipal/4
39	P6	1	24	bronchial washings	main/10
40	P1	1	21	wound	main/3
41	P6	5	26	faeces	municipal/4
42	PMC	7	25	throat swab	main/7
43	P6	1	8	faeces	main/8
44	P6	1	27	urine	outpatients' department
45	P6	5	26	faeces	municipal/4
46	P6	6	27	wound	main/9
47	P6	8	26	faeces	main/4
48	P3	8	22	bronchial washings	main/10
49	P9	9	28	urine	main/11
50	P6	8	26	faeces	municipal/4
51	PMA	8	22	bronchial washings	main/10
52	P6	8	8	faeces	municipal/4
53	P6	1	29	faeces	main/8
54	PMA	8	30	ulceration	municipal/9
55	P6	8	12	wound	main/2
56	P6	8	10	bronchial washings	main/7
57	P6	8	31	urine	main/8
58	P6	8	32	faeces	main/8
59	P6	1	33	faeces	main/8
60	P16	8	34	blood	main/12
61	PMA	8	36	bronchial washings	main/7
62	P6	8	35	urine	outpatients' department
63	PMA	8	36	ear	outpatients' department

*1. Obstetric-gynaecological ward; 2. Orthopaedic-traumatical ward; 3. Orthopaedic ward; 4. Infectious ward; 5. Urologic ward; 6. Rehabilitation ward; 7. Intensive care units ward; 8. Infantile ward; 9. Surgical ward; 10. Neurological ward; 11. Pathology of pregnancy ward; 12. Oncology ward

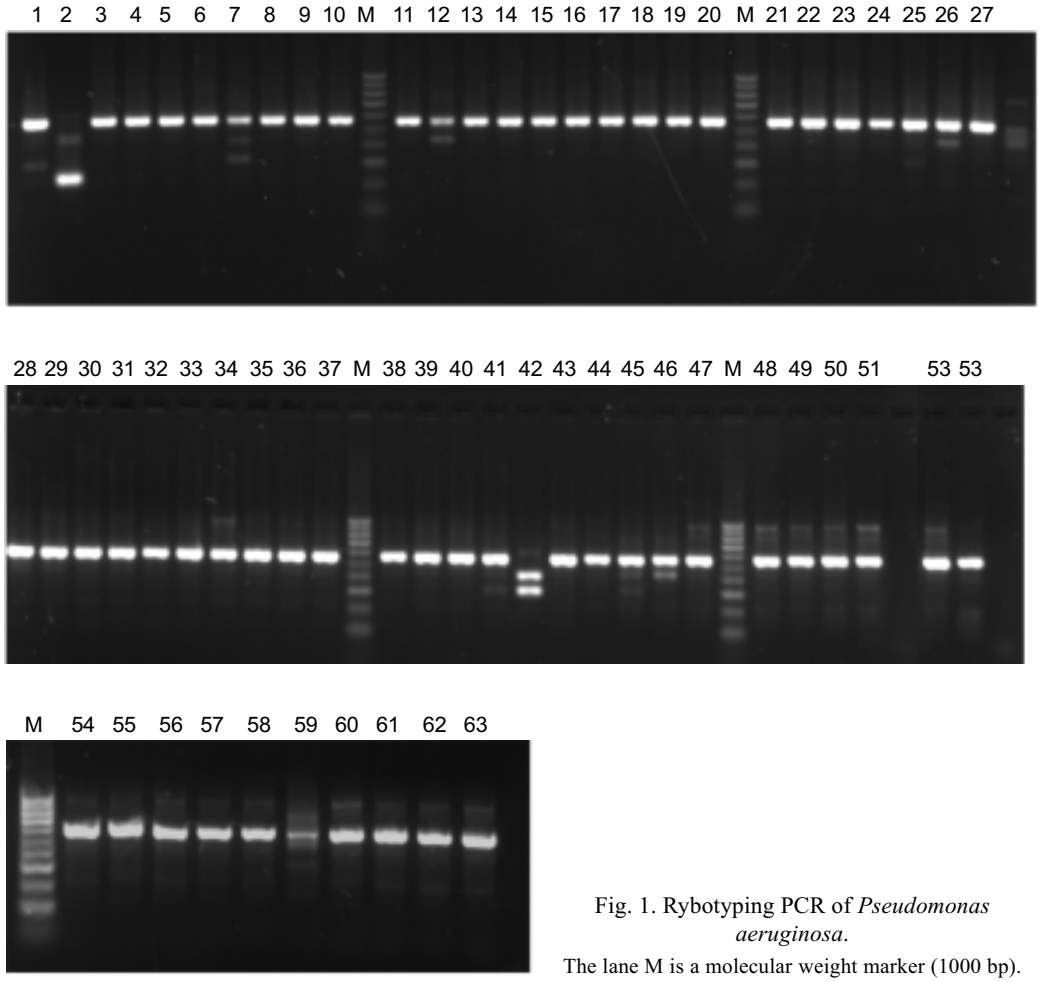

Fig. 1. Rybotyping PCR of *Pseudomonas aeruginosa*.
The lane M is a molecular weight marker (1000 bp).

5'-GAAGTCGTAACAAGG-3' and 5'-CAAGGCATCCACCGT-3'. Amplification was carried out with a 10x PCR buffer (100 mM Tris-HCl, 1 mM DTT, 0.1 mM EDTA, 100 mM KCl, 0.5% Nonidet P40, 0.5% Tween 20) in a total reaction of 50 µl containing 2.5 mM dNTP, 50 mM $MgCl_2$, 10 µM of each primer, 2 µl of genomic template DNA, and 1 unit of Taq DNA polymerase. PCR ribotyping was carried out according to Dawson *et al*. (2002) using a PTC-100 Programmable Thermo Controller (MJ Research) according to the following procedure. Initial denaturation at 94ºC for 3 min followed by 35 cycles of PCR consisting of denaturation at 94°C for 1 min, annealing at 55°C for 7 min, and extension at 72°C for 2 min; in the last cycle, the extension time was 5 min. The PCR product (10 µl) was analysed on a 2% (w/v) agarose gel in the TBE buffer (5.4 g/l Tris-Base, 2.75 g/l Boric acid, 0.37 g/l EDTA (pH 8.0) and photographed under the UV light.

Enterobacterial Repetitive Intergenic Consensus (ERIC). Enterobacterial Repetitive Intergenic Consensus (ERIC) primer sequences were used in PCR to detect differences in the number and distribution of these bacterial repetitive sequences in the bacterial genome. ERIC PCR was carried out using the primer sequences ERIC-1R, 5'-CACTTAGGGGTCCTCGAATGTA-3' and ERIC-2, 5'-AAGTAAGTGACTGGGGTGAGCG-3' to amplify the regions in the bacterial genome placed between the ERIC sequences (Versalovic *et al*., 1991). Amplification and electrophoresis were the same for the PCR ribotyping except for the following difference: annealing was at 52°C for 1 min.

All data analysis were performed using the computer software Unistat for Windows. Dice coefficient was calculated and compared to evaluate similarity among strains through the use of BIOGENE software.

Results

Serotyping. All clinical strains were agglutinable. 45 of 62 isolates reacted with serum P6 and 5 strains with serum P1. 8 strains were typed only by polyvalent sera: PMA (5), PMF (2), PMC (1). Individual isolates reacted with following sera: P9, P10, P15 and P16.

PCR ribotyping. *P. aeruginosa* belonged to 9 ribotypes (Table I, Fig. 1), which were showed in one to three bands. Bands varied between 220 and 900 bp. Nearly all strains (with the exception of 2 and 42 isolates) shared a common band of 560 bp. The cluster 1

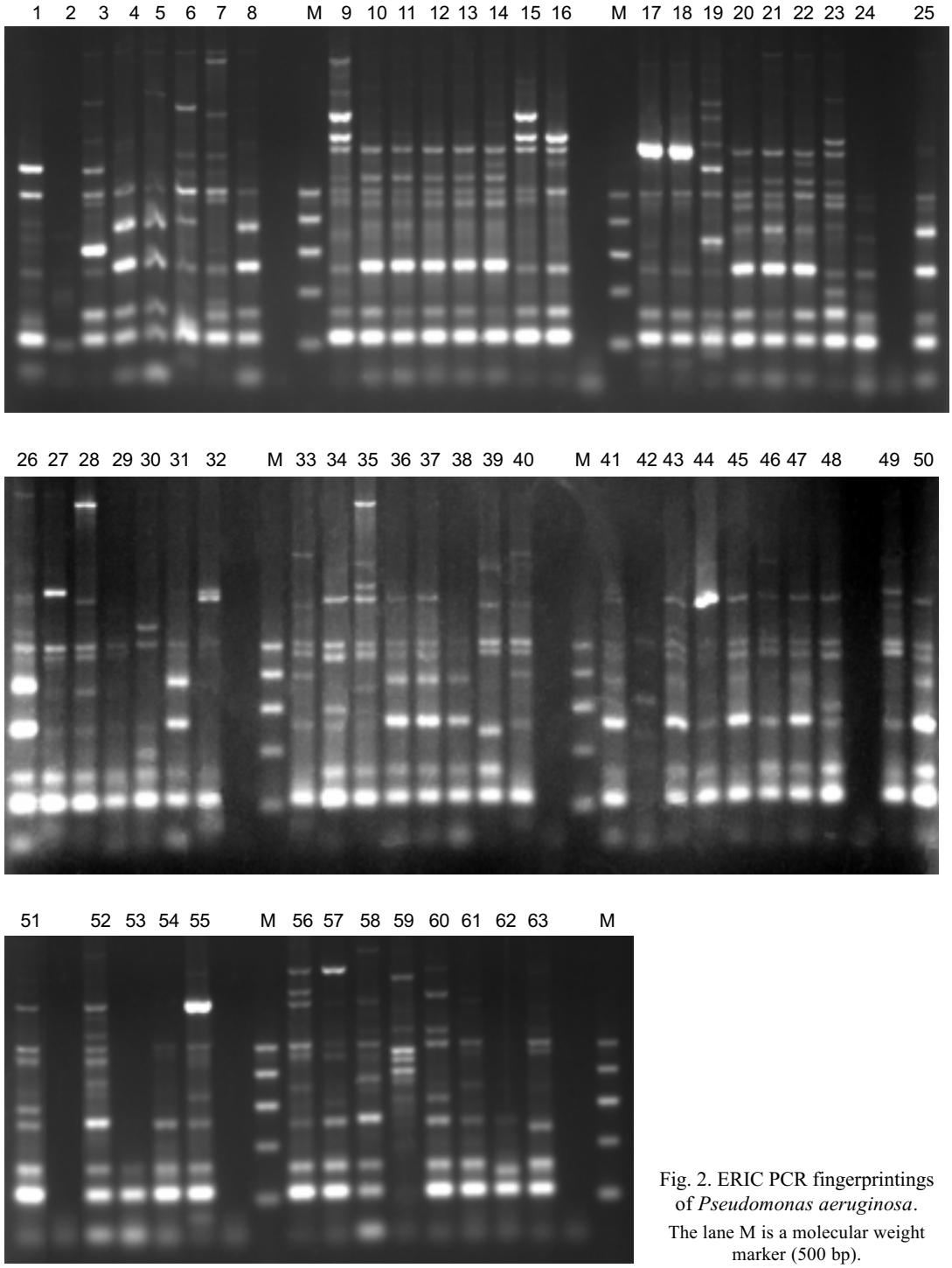

Fig. 2. ERIC PCR fingerprintings of *Pseudomonas aeruginosa*. The lane M is a molecular weight marker (500 bp).

predominated and contained 36 (58.1%) of *P. aeruginosa* isolates, and the reference strain of *P. aeruginosa* NCTC 6749. This ribotype showed only one band in size of 560 bp. The second major cluster was the 8 one, it contained 14 (22.6%) of all isolates. This type showed two bands in size of 560 and 900 bp. The isolates in clusters 1 and 8 were obtained from different clinical specimens of patients being treated at the main and municipal hospitals, and the out-patients' department. Clusters 5 and 6 consisted of three isolates. Cluster 5 contained isolates from faeces (serotype P6) of patients of the municipal (2 isolates) and main hospitals (1 isolate). Cluster 6 included isolates (serotype P6) from the faeces (2 isolates) and wounds (1 isolate) of patients of the main hospital. Cluster 9 had two isolates from urine (P9) and bronchial washings (PMA) of patients of the main hospital. The remaining *P. aeruginosa* isolates were grouped individually in clusters 2, 3, 4 (isolates from urine of patients belonging to the main hospital) and 7 (the isolate from throat swabings of a patient of the main hospital).

The predominant PCR ribotypes 1 and 8 were divided into 22 and 11 ERIC PCR groups, respectively.

ERIC PCR. ERIC PCR fingerprinting revealed 36 genetic patterns (Table I, Fig. 2). The clusters were shown in 2 to 12 bands between 110–1535 bp. Over half of isolates had 7 to 9 bands per pattern. The most characteristic products of PCR for *P. aeruginosa* were the following: 110, 150 and 510 bp; only one isolate (59) had not a band of 110 bp; five isolates (1, 2, 6, 42, 59) had not a band of 150 bp; and five (2, 42, 53, 59, 62) had not a band of 510 bp size. The other most frequently observed products of PCR were 260, 300, 350, 380, 470, 580, 720, 750, 950 bp.

ERIC PCR typing revealed 11 main genotypes, containing 2 to 8 isolates and 25 other unique patterns. Strains classified to the one genotypic type belonged to the same serotype and usually were isolated from the same source. It was observed that the incidence of the same genetic types of *P. aeruginosa* occurred in two different hospitals, so it was possible to postulate that the same clone was present in the environments of both hospitals. Five of genotypes consisted of 2 isolates. Genotype 9 included isolates obtained from faeces (serotype P6) of patients hospitalized in municipal hospital. Genotype 10 had the reference strain of *P. aeruginosa* NCTC 6749 (serotype P6) and the isolate obtained from the bronchial washings (serotype P6) of a patient from the main hospital. Genotype 21 consisted of isolates obtained from the urine (P1) and wounds (P1) of patients of the main hospital. Genotype 27 contained isolate from the urine (P6) of a patient from the outpatients' department, and from the wound (P6) of a patient of the main hospital. Genotype 36 contained isolates (PMA) obtained from bronchial washings of patients hospitalized in the main hospital, and from the ear of a patient from the outpatients' department. Three groups of genotypes consisted of three isolates. Genotype 4 included isolates obtained from faeces (P6) of two patients being treated at the main hospital, and one patient the municipal hospital. Genotype 12 had isolates obtained from the wounds (P6) of patients staying at the main hospital. Two isolates (17 and 18) showed identical patterns, indicating a high degree of similarity. Genotype 22 consisted of isolates obtained from the bronchial washings (serotype PMA) of patients hospitalized in the main hospital. Genotype 15 consisted of five isolates (P6), two of them were obtained from the faeces of patients of the municipal hospital, and the remaining three were isolated from the skin, faeces and wounds of patients of the main hospital. Genotype 26 contained six isolates from faeces (P6) of patients being treated at both main and municipal hospitals. Genotype 8 consisted of eight isolates from faeces (P6) of patients of the main and municipal hospitals.

The other, 25 unique types were collected from the following clinical specimens: urine (9 isolates), wound (3 isolates), sputum (1), bronchial washings (4), faeces (5), ulceration (1), blood (1) and pharyngeal swab (2).

Nearly all strains (with the exception of 2, 42 and 59) showed over 40% of similarity according to Dice coefficient values. 16 genotypes containing 41 (66.1% of all) isolates revealed a high similarity (70–100%) (Fig. 3). Among them, the strains isolated from faeces formed the major group (92.3% of all isolates from faeces), and the isolates from urine belonged to the least group (18.2%). The same similarity is also characteristic for 7, 10, 11 genotypes, and 29 and 35 genotypes. The isolates belonging to these genotypes were probably epidemiologically related.

Discussion

The investigations of nosocomial infections caused by *Pseudomonas aeruginosa* are hampered by the inadequate discriminatory capacity of phenotypic markers, but with the advent of DNA-based techniques, stable and discriminatory epidemiological markers have become available (Speert, 2002).

In the present study, two PCR-based fingerprinting methods were used to investigate the genetic diversity of *P. aeruginosa* isolates from clinical specimens. The isolates were genetically diverse. Eleven major ERIC-PCR clonal groups, and 25 unique genotypes were obtained. The highly heterogeneous strains were isolated from urine. It was observed that among 11 isolates 6 different clusters appeared in the ribotyping and 9 clusters in the ERIC PCR genotyping. While the group of isolates from faeces showed the least genetic variations among all strains. In the group of 26 isolates, we detected 4 of PCR ribotypes and 9 of ERIC PCR genotypes. Dendrogram analysis enabled the division of strains into main groups (40–60% of similarity), then subgroups (63–78%) and genotypes (80–100%). Also others (Dawson *et al*., 2002; Liu *et al*., 1996; Syrmis *et al*., 2004) demonstrated sufficient discriminatory power of ERIC PCR for the investigation of clinical *P. aeruginosa* strains. The results of the study of Syrmis *et al*. (2004) showed that ERIC PCR identified six major clonal groups and 58 distinct clonal groups among 163 *P. aeruginsa* strains isolated from patients with *cystic fibrosis*. Liu *et al*. (1996) by using three methods-RAPD, ERIC PCR and 16S–23S spacer region based RAPD-indicated that the 47 isolates of *P. aeruginosa* that had caused bacteraemia in 19 cancer patients were indistinquishable. 17 other isolates that had caused bacteraemia in 10 cancer patients were discriminated into eight further groups. Twenty four environmental and non-cancer patient isolates were grouped into further distinct groups.

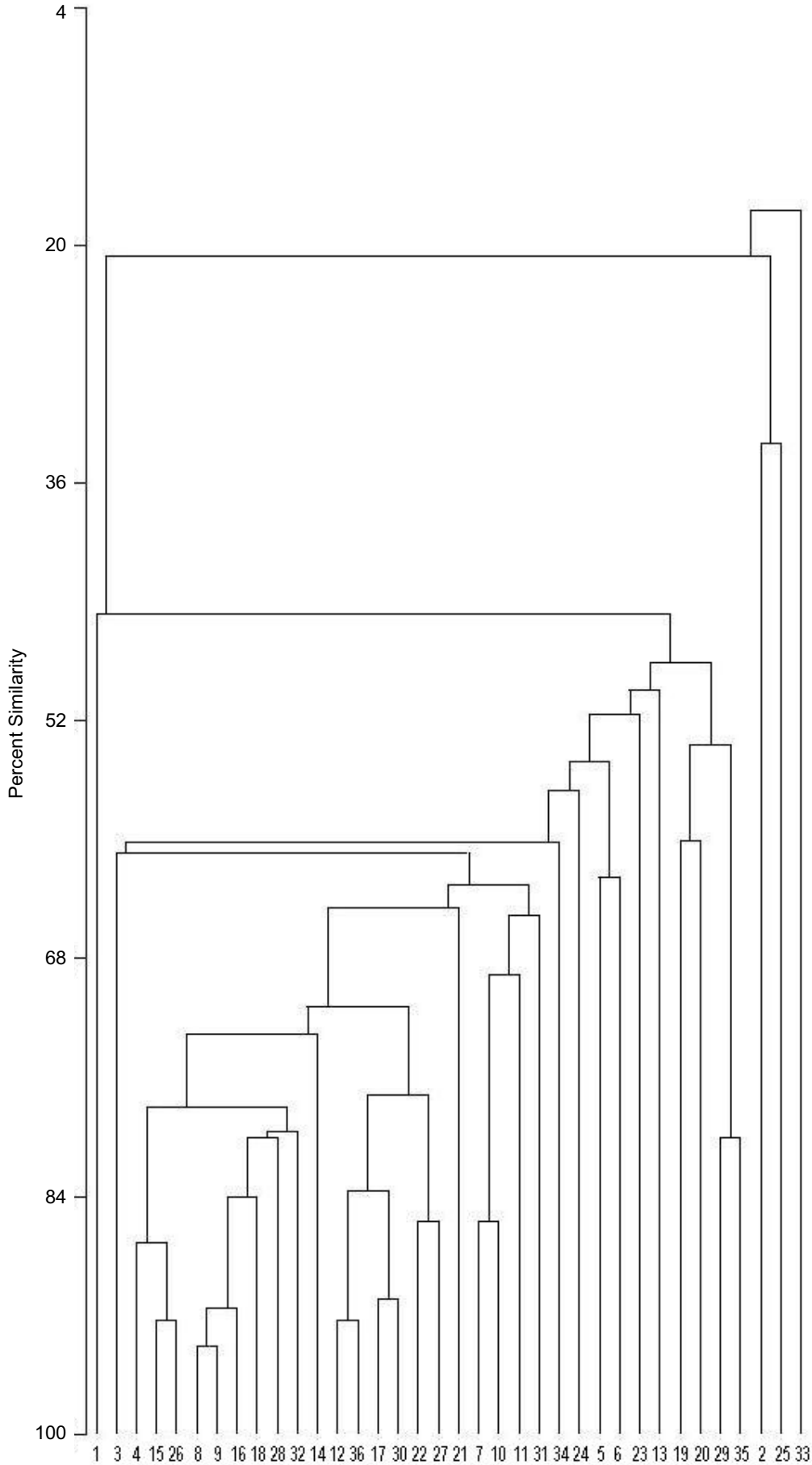

Fig. 3. Dendrogram demonstrating the genetic relationship among isolates *Pseudomonas aeruginosa.*

PCR ribotyping produced much minor diverse range of types and grouped isolates into 9 groups with a predominance of 2 main groups containing 80.3% of all strains. This was caused by the limited number of bands in each ribotype, which did not provide sufficient characteristic for confident differentiation. Also Martin *et al*. (1995) observed a low degree of heterogeneity of rRNA operons using ribotyping for molecular typing of strains of *P. aeruginosa* from patients with *cystic fibrosis*. Vinuesa *et al*. (1998) have found that PCR ribotyping can be improved by digestion of the PCR products with the restriction endonucleases. The study of Ferrus *et al*. (1998) successfully used EcoRI for epidemiological studies of *P. aeruginosa* isolated from infected patients at Valencia hospitals. The others demonstrated higher discrimination of ribotyping with PvuII enzyme than EcoRI; riboprinting by EcoRI had reduced banding patterns (Botes *et al*., 2003; Fiett *et al*., 1998).

Serotyping is not the method of high discriminatory power (Czekajło-Kołodziej *et al*., 2006; Hernandez *et al*., 1997). The observed strains were classified into nine different serotypes. However, most of the strains (72.6%) belonged to P6 serotype. This somatic antigen occured among all strains isolated from faeces of patients staying in two hospitals, strains obtained from urine (3 strains isolated from the main hospital and 4 from the outpatients' department), wounds (5 strains isolated from the main hospital), bronchial washings (4 strains isolated from main hospital), skin (1 strain from the main hospital) and throat swab (1 strain from the main hospital).

In conclusion, among all currently used methods ERIC PCR turned out to be a powerful tool for the study of clinical *P. aeruginosa* isolates diversity.

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