



Comparative Assessment of Different PCR-Based Typing Methods of *Pseudomonas aeruginosa* Isolates

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Introduction: *Pseudomonas aeruginosa* is one of the important causes of nosocomial infections. Analyzing the diversity of these isolates is important to control the diseases caused by them. Studies of molecular epidemiology depend on the application of typing methods.

Purpose: This study aims to assess the performance of PCR- based typing techniques (RAPD, ribotyping, tDNA, and ERIC) in determining the genetic diversity of 44 *P. aeruginosa* urinary isolates.

Methods: Performance parameters were analyzed for each of the tested methods. The banding pattern was assessed by calculating polymorphism, genotypic gene diversity and the effective multiplex ratio. Moreover, strain diversity, typeability, and discriminatory power were used to measure the efficiency of typing methods. The congruence among typing methods was calculated by Rand's and Wallace coefficients.

Results: P-640 among RAPD primers and Ribo-2 among ribotyping primers were more informative as they gave high strain diversity, the highest number of clusters, and highest discriminatory power (ISD=70.45%, 29 clusters at 70% cutoff, DI=0.97 and ISD=75%, 25 clusters at 70% cutoff DI=0.969, respectively). Comparison of typing methods showed that RAPD-PCR gave the highest mean percent polymorphism per assay (76.85%) followed by ERIC-PCR. ERIC-PCR outperformed in most marker parameters; highest mean number of alleles, number of monomorphic bands per assay unit, mean genotypic gene diversity, effective multiplex ratio, and assay efficiency index. Calculated congruence revealed that individual methods demonstrate moderate to poor predictive power. Interestingly, this power increased by combining data obtained from another method.

Conclusion: RAPD primer (P-640) had more discrimination power followed by ribo-2 and ERIC. The performance and predictive power of typing methods can be improved by combining data obtained from different methods as ERIC+OPA-02 and ERIC+P-640 combinations gave complete typeability and discrimination of isolates. ERIC, ERIC+OPA-02, and ERIC+P-640 combinations can provide finer discrimination and classification of *P. aeruginosa* strains than the other tested methods.

Keywords: *P. aeruginosa*, PCR-based molecular typing, discrimination power, typeability, congruence

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Introduction

P. aeruginosa is a Gram-negative bacillus. It is one of the most important Gram-negative bacteria causing nosocomial infections. In most cases, it is resistant to antibiotics and associated with a high mortality rate. It represents around 10% of

urinary tract infections and accounts for about 35% of mortality due to bacteremia.^{1,2}

Bacterial strain typing is significant for diagnosis, treatment, and epidemiological surveillance of bacterial infections.³ Bacterial typing can be divided into conventional epidemiological typing and DNA-based typing methods. Conventional epidemiological typing methods include antibiogram, phage typing, and serotyping.⁴ While DNA-based typing methods include many techniques as restriction endonucleases of genomic and plasmid DNA, southern hybridization, pulsed gel electrophoresis (PFGE), PCR-based methods for plasmid and chromosomal profiling, microarray, and the last revolution, whole-genome sequencing.^{5,6}

PCR-based typing relies on the amplification of a tiny amount of specific DNA sequence. It includes different methods: PCR-RFLP, PCR-ribotyping, randomly amplified polymorphic DNA (RAPD), repetitive extragenic palindromic (Rep)-PCR, enterobacterial repetitive intergenic consensus (ERIC)-PCR, and PCR sequencing.⁷

RAPD is known as arbitrary primed PCR (AP-PCR) as it amplifies ambiguous regions of the genome. The primers used are short in length (about 10 bp). The discrimination power of RAPD depends on the number and sequence of the primers used and the amplification conditions.⁸

ERIC-PCR fingerprinting targets highly conserved central inverted repeats of 126 bp located in extragenic regions of bacterial genomes. The position of these regions differs between species and strains, so they are considered genetic markers.^{5,9} ERIC-PCR is an advantageous and fast method; thus it is paralleled with other PCR techniques.⁶

PCR-ribotyping is a technique used to detect polymorphism in intergenic regions of rRNA or tRNA. Intergenic spacer regions (ISRs) are exposed to inferior evolutionary pressure and thus demonstrate broader genetic variations that hang on the number and type of tRNA genes between 23S and 16S sequences. Two PCR-based methods are used for identifying these ISRs. The first method; PCR-ribotyping which is used for identification and discrimination between bacterial strains and species utilizing primers that amplify the sequences between 16S and 23S genes. The second technique is tDNA-PCR which amplifies intergenic spacers between tRNA genes. These genes are conserved and present in several copies along the bacterial genome. Besides, they are grouped and disconnected by spacers whose length and sequences are subjected to great variation.¹⁰

Assessment of the efficiency of the typing method depends on several factors such as typeability and discrimination. Typeability is simply the percentage of bacterial isolates that can be positively typed by the typing marker.¹¹ The discriminatory power of a typing method can be estimated by the number of types obtained by this method and their relative frequencies.¹² It can be expressed by discrimination index (DI) which can be defined as the probability that two random isolates from the test population are distinguished into different typing groups.^{5,11,12}

An important characteristic for an ideal marker is high polymorphism with consistent distribution within the genome. In addition, it should give an adequate resolution of genetic differences. The efficiency of molecular markers can be evaluated in assessing genetic diversity by measuring various parameters. The number of total alleles, allele range, % polymorphism, effective multiplex ratio, assay efficiency index (AEI), and index of strain diversity are performance parameters that can estimate the discriminatory power of the typing methods.¹³ Welsh and McClelland show that comparing polymorphisms in genomic fingerprints can distinguish strains.¹⁴

The correspondence between typing methods can be assessed quantitatively by calculating two coefficients, Rand (R) and Wallace (W) coefficients. They are used to explore the concordance between typing methods. The value of the W coefficient indicates the probability that two strains are classified as the same type by either of the two methods. This indicates that results obtained by one method can be predicted by the other method.¹⁵

The present work aims to compare the efficiency of different PCR-based typing methods (RAPD, ribotyping, tDNA, and ERIC) to discriminate between strains of *P. aeruginosa* isolated from UTIs. Moreover, the concordance of different typing methods and their combinations was evaluated.

Materials and Methods

Bacterial Isolates

Forty-four *P. aeruginosa* isolates were cultured from urine samples obtained from patients at Urology and Nephrology Center, Mansoura University, Egypt.

Isolates were selected based on their colony morphology on Pseudomonas isolation agar (Oxoid). All isolates were confirmed as *P. aeruginosa* using the VITEK 2 system (bioMerieux, Marcy l'Etoile, France) using *P. aeruginosa* (Schroeter) Migula (ATCC 27853) as

a quality control strain. Confirmed isolates were stored at -80°C for further studies.

This research was approved by the ethics committee of the faculty of Pharmacy, Mansoura University (code 2020–127). Isolates were a part of the routine hospital laboratory procedure.

Isolation of Bacterial DNA

Genomic DNA was obtained by the modified boiling method of Englen and Kelley.¹⁶ Briefly, pure bacterial colonies were mixed with 100 μL DNase/RNase-free water and the suspension was held at 95°C for 10 min. The lysate was centrifuged and transferred to a new Eppendorf and used as a template DNA.

PCR-Based Typing

RAPD typing was carried out using six primers: D-10514,^{17,18} D-14306,¹⁷ OP-13,^{19,20} P-640,^{16,21} OPA-02,²² and AP3.^{23,24} PCR ribotyping was done using three sets of universal primers: Ribo-1,²⁵ Ribo-2,²⁶ and Ribo-3.¹⁰ ERIC-PCR-reaction was performed using the sequences ERIC1 and ERIC2.²⁷ The tDNA-PCR amplified ISRs between the tRNA genes using the conserved primers T5A and T3B.¹⁰ The primers' sequences were illustrated in Table 1. All primers were obtained from Invitrogen (Thermo Scientific). The amplifications were performed in the thermocycler machine (Model TC96K, AccuLab[®], USA). The temperature profile and the PCR

conditions were conducted as described previously for each primer. The PCR products of all typing methods were electrophoresed in 1.5% agarose gel with the DNA molecular weight marker (GeneRuler 100 bp Plus DNA Ladder, Thermo Scientific) and visualized in a gel documentation system (Model Gel Doc 1.4, 1189; AccuLab[®]). DNA fingerprints were recorded and compared visually.

Data Analysis

For data analysis, each band with a different size was assigned and marked by a binary system (1 for presence and 0 for the absence of band of a specific molecular weight). The banding patterns produced by the different tested markers were analyzed by constructing a dendrogram using the unweighted pair group method with arithmetic mean (UPGMA) to determine the relatedness between isolates using the Dice coefficient, with the program DendoUPGMA [Univertat Rovirai Virgili (URV), Tarragona, Spain].

The polymorphism percentage (PM%), monomorphism percentage (MM%) were calculated for each typing system.²⁸ Genotypic gene diversity (H_g) was calculated as described earlier^{28,29} by the following equation:

$$H_g = 1 - (\pi^2 - q_i^2)$$

where π ($\pi = 1 - q_i$) refers to the frequency of the dominant allele and q_i represents the frequency of the null allele (ie the number of isolates without the band).

Table 1 Sequence of the Primers Used in This Study

Primer Name	Nucleotide Sequence (5' to 3')	References	
RAPD	D-10514 (5'-TGGTGGCCTCGAGCAAGAGAACAAAG-3')	[17,18]	
	D-14306 (5'-GGTTGGGTGAGAATTGC-3')	[17]	
	OPA13 (5'-CAGCACCCAC-3')	[19,20]	
	P-640 (5'-CGTGGGGCCT-3')	[16,21]	
	OPA-02 (5'-TGCCGAGCTG-3')	[22]	
	AP3 (5'-TCA CGA TGC A-3')	[23,24]	
Ribotyping	Ribo-1 Fw (5'-TTGTACACACCGCCCGTCA-3') Rv (5'-GGTACCTTAGATGTTTCAGTTC-3')	[25]	
	Ribo-2 Fw (5'-GAAGTCGTAACAAGG-3') Rv (5'-CAAGGCATCCACCGT-3')	[26]	
	Ribo-3 Fw (5'-TTGTACACACCGCCCGTCA-3') Rv (5'-GGTACCTTAGATTGTTTCAGTTC-3')	[10]	
	ERIC	Fw (5'-ATGTAAGCTCCTGGGGATTAC-3') Rv (5'-AAGTAAGTGACTGGGGTGAGCG-3')	[27]
		tDNA Fw (5'-AGTCCGGTGCTCTAACCAACTGAC-3') Rv (5'-AGGTCGCGGGTTCGAATCC-3')	[10]

Abbreviations: Fw, forward; Rv, reverse.

The effective Multiplex Ratio (EMR) is the product of the fraction of polymorphic bands and the number of polymorphic bands for an individual assay where n_p is the number of polymorphic bands and n is the total number of bands. It was calculated according to the equation:²⁸

$$EMR(E) = n_p (n_p/n)$$

In addition, the assay efficiency index (AEI = Polymorphic bands/Total number of primers)²⁸ of each typing method was calculated.

To determine the index of strain diversity (ISD), that measured as the frequency of diverse genotypes, the following equation was used.³⁰

$$ISD(\%) = 100\%(\text{number of genotypes}/\text{total number of isolates})$$

The typeability of each typing method was calculated as the percentage of isolates that can be typed among the tested ones.^{13,31} For the discriminatory power of each typing method, it was assessed by calculating the discriminatory index (DI) according to the following Simpson's equation:³¹

$$DI = 1 - [1/N(N - 1)] \sum_{j=1}^S n_j(n_j - 1)$$

Where N: the total number of isolates, S: the total number of types, n_j : the number of isolates belonging to the j th type, and Non-typeable isolates were gathered into one group (untypeable group).

Calculation of the concordance of different typing methods and their combinations was done by the software described by Carrico et al,³² where Rand's (R) and adjusted Wallace's coefficients (W) were calculated.

Results

In the present study, the genetic diversity of 44 *P. aeruginosa* isolates was assessed using RAPD, ribotyping, tDNA, and ERIC typing techniques. The banding patterns were scored and were designed by a numeral for each primer.

RAPD Analysis

Among the 44 tested isolates, the typeability ranged from 70.5% to 97.7% (Table 2). RAPD-PCR typed 43 isolates by primer D-14306 and OP-13 (typeability 97.7%). Thirteen isolates were untypable by AP3 primer (typeability 70.5%). P-640 primer gave the highest strain diversity (ISD=70.45%), while D-14306 was the least marker in differentiating isolates (ISD=29.54%).

Amplification using primer D-10514 yielded 27 genotypes through amplifying 15 bands ranging from 150 bp to 2000 bp. It clustered the isolates into 22 clusters at 67% cutoff. At cutoff 72%, two clusters could be further subdivided into two groups. One of these clusters was subdivided into three groups at 77% cutoff and into four groups at 80% cutoff. Six clusters were found to contain isolates with 100% similarity (Figure 1A).

Primer D-14306 generated 13 genotypes by amplifying 10 bands ranging approximately from 500 bp to 2000 bp. It classified the isolates into 7 clusters at 70% cutoff where they grouped them into 10 clusters at 83% cutoff, 12 clusters at 90% cutoff, and 13 clusters at 97% cutoff. Cluster No. one comprised the highest number of isolates (27) with 100% similarity. Three isolates were different at all levels of similarity (Figure 1B).

Table 2 Typeability, Index of Strain Diversity, and Cluster Analysis of 44 *P. aeruginosa* Strains Using Different PCR-Based Typing Methods

Typing Method		Typeability (%)	ISD(%)	No. of Clusters	Cutoff	No. of Clusters at 70% Cutoff
RAPD-PCR genotyping	D-10514	90.9	61.36	22	67%	23
	D-14306	97.7	29.54	7	70%	7
	OP-13	97.7	56.81	19	62%	23
	OPA-02	81.8	40.90	10	65%	13
	P-640	86.4	70.45	27	65%	29
	AP3	70.5	59.09	25	70%	25
Ribotyping	Ribo-1	97.7	18.18	8	97%	8
	Ribo-2	97.7	75.00	19	60%	25
	Ribo-3	100	29.54	10	75%	10
tDNA-PCR		97.7	47.72	15	65%	18
ERIC-PCR		81.8	72.72	28	75%	28

Abbreviation: ISD, index of strain diversity.

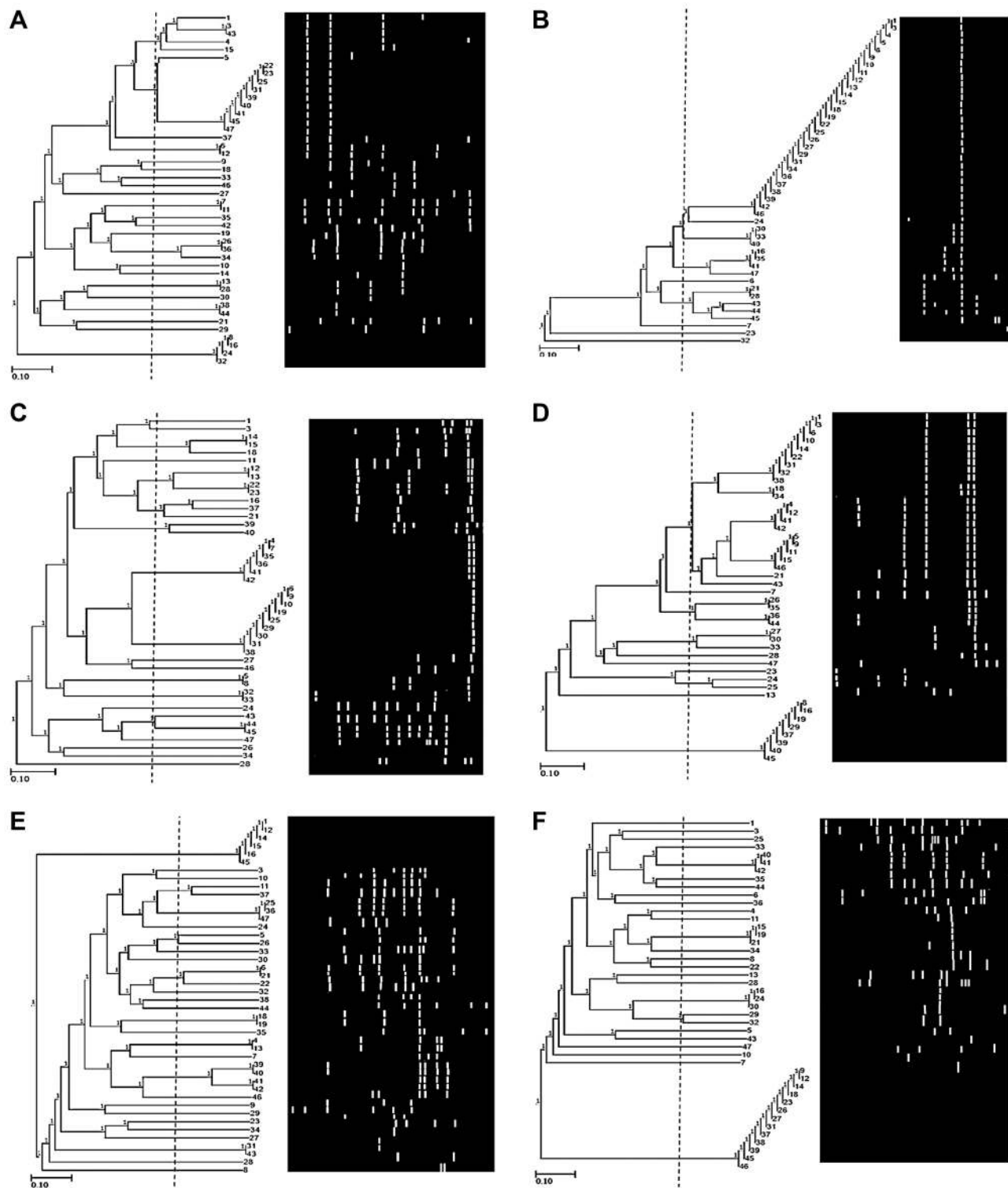


Figure 1 Dendrogram of 44 *P. aeruginosa* isolates using UPGMA algorithm and Dice coefficient for D-10514 primer (A), D-14603 primer (B), OP-13 primer (C), OPA-02 primer (D), P-640 primer (E), and AP3 (F). Patterns are schematically represented.

OP-13 and OPA-02 primers generated 25 genotypes (22 different bands ranging from 300 bp to 2500 bp) and 18 genotypes (12 bands ranging from 300 bp to 2500 bp), respectively (Figure 1C and D). The isolates were

classified by OP-13 into 19 clusters at 62% cutoff where they were grouped into 25 clusters at 97% cutoff. Eight Clusters showed 100% similarity between their isolates. On the other hand, OPA-02 classified the isolates into 10,

14, 17, and 18 clusters at 65%, 76%, 83% and 97% cutoff, respectively. Eight Clusters showed 100% similarity between their isolates.

Thirty-one different genotypes were obtained by P-640 primer by amplifying 24 bands ranging from 250 bp to >3000 bp. P-640 primer classified the isolates into 27 clusters at 65% cutoff where they grouped them into 31 clusters at 97% cutoff where four clusters were further subdivided each into two groups. Eight clusters showed 100% similarity between their isolates with cluster No. 1 comprised six isolates (Figure 1E).

AP3 primer gave 26 genotypes with band sizes ranging between 250bp and 3000bp (25 bands). The isolates could be clustered into 25 clusters with two isolates showing similarity at 70% cutoff. Isolates (40, 41 and 42), (15, 19 and 21) and (16, 24 and 30) showed 100% similarity. Cluster No. 25 comprises the highest number of isolates (Figure 1F).

The number of the obtained amplicons in all tested isolates was specific for each of the tested RAPD primers. All tested primers showed PM% ranged between 70% to 86.7%. A number of 82 out of 108 total alleles was polymorphic. AP3 generated a maximum of 25 alleles followed by P-640 primer with 24 alleles. D-14306 generated a minimum of 10 alleles (Table 3).

The calculated EMR ranged between 4.9 and 14.44 (mean EMR=10.44). Gene diversity (H_g) values ranged between 0.3 and 1.36 with an average of 0.97. A total of

770 amplicons were observed and AEI was 13.67. D-14306 primer revealed minimum values of H_g and EMR (Table 3).

PCR Ribotyping

The ribotyping assay was performed using three different markers. Both Ribo-1 and Ribo-2 primers typed 43 out of 44 tested isolates (typeability 97.7%). Ribo-3 primer typed all isolates resulting in 100% typeability (Table 2).

Ribo-1 primer distinguished the typable isolates within eight genotypes (ISD=18.18%) through amplifying eight bands (500bp to 2000 bp) (Figure 2A). Ribo-2 primer amplified 20 visible bands (200bp to 2000 bp) resulting in 33 genotypes with high ISD=75.00% (Figure 2B). The third primer (Ribo-3) classified the tested isolates into 13 distinct genotypes with ISD= 29.54% by amplifying 10 different DNA fragments ranging approximately from 100 bp to 1500 bp (Figure 2C).

Ribo-1 primer showed the highest similarity between isolates where it classified them into 7 clusters at 97% cutoff. Cluster No. 2 contained 23 (52.27%) isolates. Ribo-2 primer classified the isolates into 19 clusters at 60% cutoff while it grouped them into 30 clusters at 80% cutoff where 3 clusters were further subdivided into two groups and four clusters were subdivided into 3 groups. At 97% cutoff, 32 clusters were found, five of them contained isolates with 100% similarity. Ribo-3 primer classified the isolates into 10 clusters at 70% cutoff and 13 clusters at 97% cutoff where 3

Table 3 Polymorphism Detected by the Used PCR-Based Typing Primers

Typing Method	Total Alleles	MB	PB	MM%	PM%	Total Amplicons	Allele Range	H_g	EMR	AEI
RAPD-PCR genotyping										
D-10514	15	2	13	13.3	86.7	135	150–2000	1.36	11.27	13.67
D-14306	10	3	7	30	70	68	500–2000	0.30	4.9	
OP-13	22	6	16	27.3	72.7	148	300–2500	1.14	11.64	
OPA-02	12	2	10	16.7	83.3	127	300–2500	0.58	8.33	
P-640	24	7	17	29.2	70.8	173	250->3000	1.12	12.04	
AP3	25	6	19	24	76	119	250–3000	1.30	14.44	
Ribotyping										
Ribo-1	8	2	6	25	75	83	500–2000	0.30	4.5	9.3
Ribo-2	20	6	14	30	70	171	200–2000	0.53	9.8	
Ribo-3	10	2	8	20	80	105	100–1500	0.30	6.40	
tDNA-PCR	16	5	11	31.3	68.7	120	100–3000	0.68	7.56	11
ERIC-PCR	21	6	15	28.6	71.4	149	200–3000	1.41	10.71	15

Abbreviations: MB, number of monomorphic bands; PB, number of polymorphic bands; MM (%), monomorphism percentage; PM (%), polymorphism percentage; H_g , genotypic gene diversity; EMR, effective multiplex ratio; AEI, assay efficiency index.

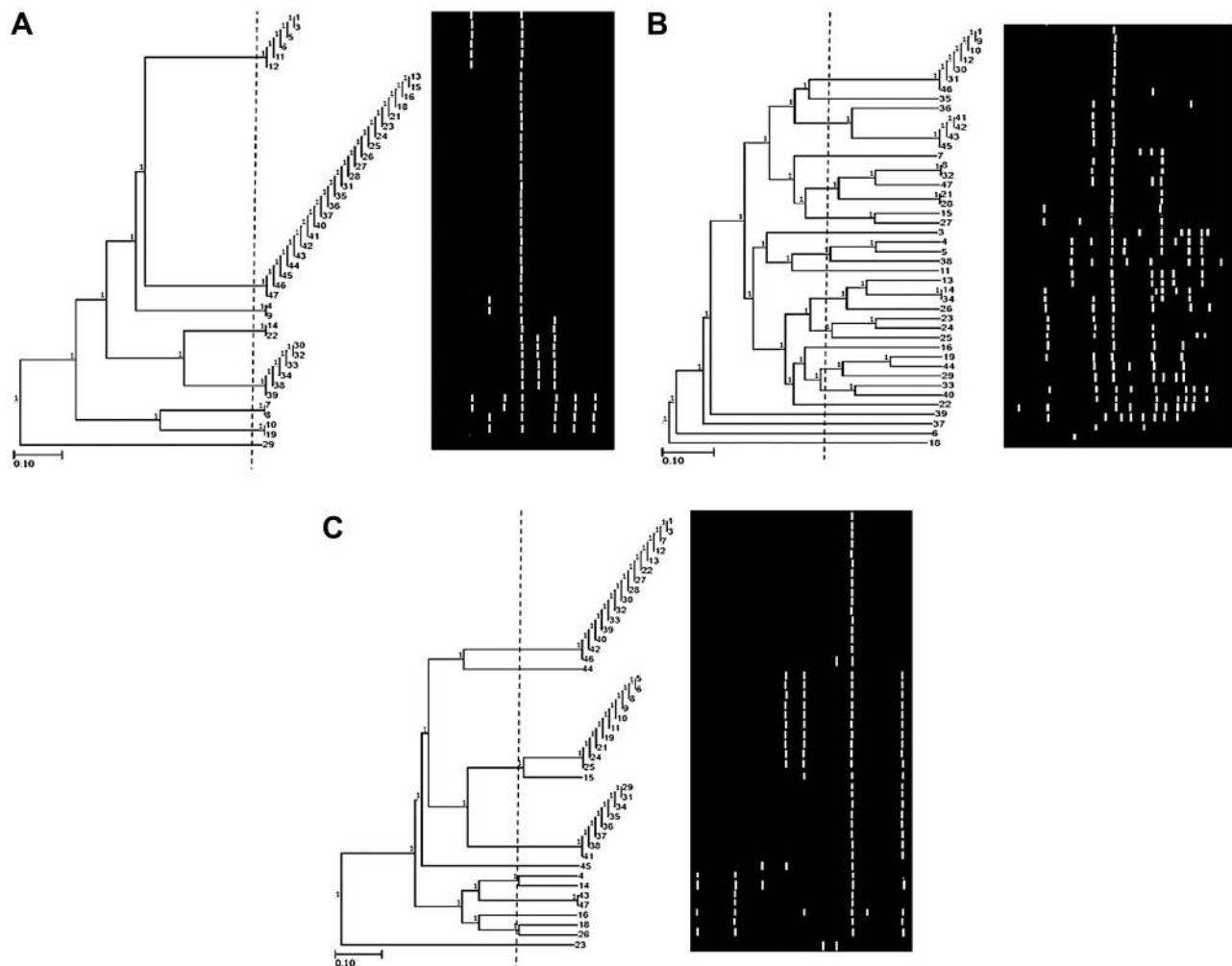


Figure 2 Dendrogram of 44 *P. aeruginosa* isolates using UPGMA algorithm and Dice coefficient for Ribo-1 primer (A), Ribo-2 primer (B), and Ribo-3 primer (C). Patterns are schematically represented.

clusters were further subdivided into two groups. Isolates of four clusters showed 100% similarity.

Ribotyping revealed a total of 38 alleles, with an average of 12.67 per primer. Twenty-eight alleles were polymorphic resulting in an average of 75% polymorphism. The maximum H_g and EMR were observed with the primer Ribo-2. A total of 359 amplicons were obtained by ribotyping primers and AEI was 9.3 as illustrated in Table 3.

tDNA-PCR

tDNA marker typed 97.7% of isolates. It demonstrated 21 different genotypes with moderate strain diversity (ISD= 47.72%) of isolates by amplifying 16 alleles that ranged from 100 bp to 3000 bp. Among the tested isolates, tDNA amplified 120 amplicons, AEI was 11. The H_g and EMR values were 0.68 and 7.56, respectively (Table 3).

tDNA primer classified the isolates into 15 clusters at 65% cutoff where they grouped them into 18 clusters at

75% cutoff and 20 clusters at 97% cutoff. Isolates of seven clusters showed 100% similarity (Figure 3A).

ERIC-PCR

The molecular typing using ERIC-PCR generated 21 different alleles (200 bp to 3000 bp) among the 36 typed isolates (Figure 3B), 81.8% typeability (Table 2). Six bands were monomorphic and 15 were polymorphic. The typable isolates were classified into 32 genotypes with ISD= 72.72%. Besides that, EMR was 15.43. A total of 149 amplicons were observed and AEI was 18 (Table 3).

ERIC primer clustered the isolates into 28 clusters at 75% cutoff. Two clusters (No. 3 and 7) could be further subdivided into two and three groups at 87% cutoff, respectively. There was 100% similarity between isolates (24 and 25), (9, 10 and 43), (15 and 18), and (11, 22 and 29). Cluster No.28 contains the highest number (8) of isolates with 97% similarity.

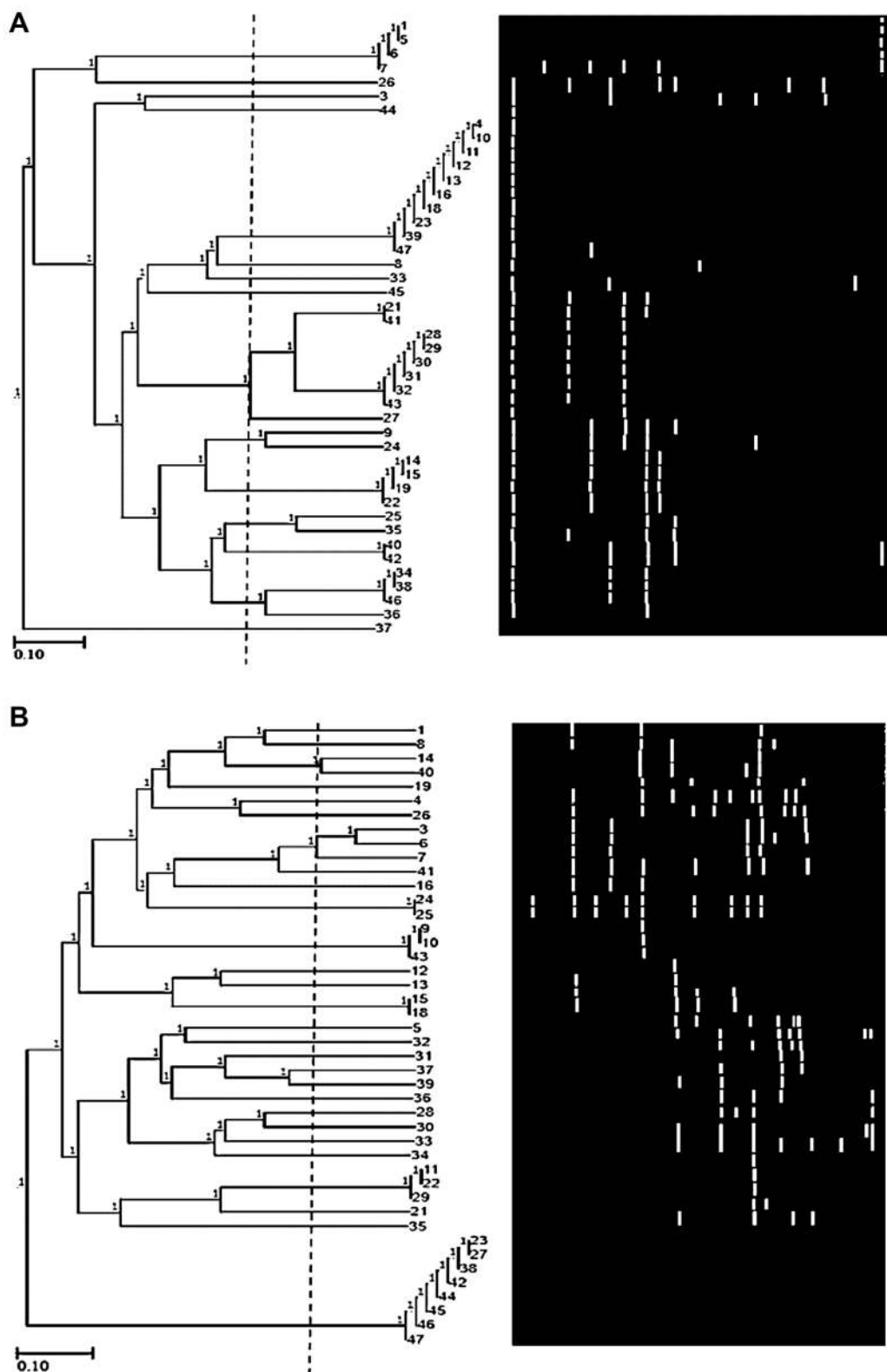


Figure 3 Dendrogram of 44 *P. aeruginosa* isolates using the UPGMA algorithm and Dice coefficient for tDNA-PCR typing (A) and ERIC-PCR typing (B). Patterns are schematically represented.

Comparative Analysis of Banding Genotypes Generated by Typing Systems

ERIC marker system showed superior values of the mean number of alleles per assay unit, the number of monomorphic and polymorphic bands per assay unit (21, 6, and 15, respectively) as shown in Table 4. Despite that, a higher percentage of polymorphism was obtained from RAPD analysis (76.85%) as compared to ERIC (71.4%). Moreover, ERIC showed higher values of mean genotypic gene diversity (1.41), EMR (10.71), and AEI (15) followed by RAPD marker system accounting 0.97, 10.44, and 13.67, respectively. The Ribotyping system showed the lowest values of marker parameters; mean number of alleles per assay unit (12.67), number of monomorphic (3.33), and polymorphic bands (9.33) per assay unit, mean genotypic gene diversity (0.38), EMR (6.9). tDNA marker system showed the lowest mean percentage of polymorphism per assay (68.7%) and AEI (11).

Discriminatory Power and Concordance Among Typing Systems

Concerning the discriminatory power, Table 5 shows the discriminatory indices (DIs) of the typing methods using

each primer and those of the combination of the methods. The DIs ranged from 0.622 to 0.975. Three primers (P-640, Ribo-2, ERIC) gave DI above the recommended value, 0.95. The highest DI value (0.975) was obtained by using a P-640 primer. The DIs for D-10514 and OP-13 primers were 0.949 and 0.94, respectively. The lowest DI was 0.622 of D-14306 primer, showing poor discriminatory power (Table 5).

The combination of different typing methods noticeably increases the DI values. ERIC highly increases the DI of all typing methods, DI of 1 was achieved with OP-O2 +P-640 pair. Also, all ribotyping primers and tDNA increased the DI with the combined typing methods. D-14306 primer that gave the lowest DI alone, revealed higher DI in all combination pairs.

The highest concordance was found between RAPD typing using P-640 primer and ribotyping using Ribo-2 primer calculated by Rand's coefficient ($R=0.948$) (Table 6). Both ERIC and ribotyping using Ribo-2 primer gave high concordance with all typing methods ($R=0.607$ – 0.939 and 0.627 – 0.948 , respectively). Only D-14306 and Ribo-1-types which were moderately predicted by AP3 and ERIC using Wallace coefficient ($W=0.427$ and 0.536) with a 95% confidence level (CI), respectively. The other types of all typing methods were poorly predicted by either of these methods (Table 7).

For combinations of typing methods, it was found that concordance highly increased as indicated by the Rand coefficient for all combined pairs. Complete concordance was found between the ERIC+ OPA-O2 pair using the ERIC+P-640 pair ($R=1$). Increased concordance ($R=0.998$) was found using tDNA+D-10514 and tDNA+Ribo-2 with tDNA+ERIC, $R=0.979$ using Ribo-1+OPA-O2 with Ribo-1+P-640, $R=0.998$ in Ribo-2+D-10514 with Ribo-2+ERIC and $R=0.993$ in Ribo-3+D-10514 with Ribo-3+P-640 and Ribo-3+P-640 with Ribo-3+ERIC (data not shown).

Additionally, ERIC+OPA-O2 and ERIC+P-640 pairs were able to predict the types produced by all other combination pairs with ERIC ($W=1$). ERIC+Ribo-1 could be completely predicted by either ERIC+OP-13 or ERIC+tDNA ($W=1$) (Table 8). Moreover, tDNA+D-10514 and tDNA+Ribo-1 were predicted completely with tDNA+ERIC. Ribo-2+D-14306 was highly predicted by Ribo-2+OPA-O2 and Ribo-2+AP3 with $W=0.797$ and 0.855 , respectively. Besides, Ribo-3+D-14306 was best predicted by Ribo-3+AP3 and Ribo-3+ERIC with $W=0.802$ and 0.728 , respectively (data not shown).

Table 4 Comparative Analysis of Banding Genotypes Generated by the Four PCR-Typing Techniques

Components	RAPD	Ribotyping	tDNA	ERIC
Number of assay units	6	3	1	1
Total amplicons	770	359	120	149
Total No. of alleles	108	38	16	21
Mean No. of alleles per assay unit	18	12.67	16	21
Number of monomorphic bands per assay unit	4.33	3.33	5	6
No of polymorphic bands per assay unit	13.83	9.33	11.00	15.00
Mean (%) polymorphism per assay	76.85	75	68.7	71.4
Mean genotypic gene diversity	0.97	0.38	0.68	1.41
Effective multiplex ratio	10.44	6.9	7.56	10.71
Assay efficiency index	13.67	12.67	11.00	15.00

Table 5 The Discriminatory Power of the Typing Methods, Apart and in Combination, Evaluated with 44 *P. aeruginosa* Strains

Methods	ERIC Combinations				Ribotyping Combinations						tDNA Combinations	
					Ribo-1		Ribo-2		Ribo-3			
	No. of Types	DI	No. of Types	DI	No. of Types	DI	No. of Types	DI	No. of Types	DI	No. of Types	DI
D-14306	13	0.622	38	0.989	22	0.93	38	0.983	22	0.92	32	0.968
D-10514	27	0.949	41	0.996	35	0.98	43	0.999	39	0.994	42	0.997
OP-13	25	0.94	43	0.999	37	0.989	40	0.993	32	0.979	40	0.995
OP-O2	18	0.911	44	1	32	0.983	40	0.995	32	0.983	39	0.995
P-640	31	0.975	44	1	38	0.992	42	0.998	41	0.997	40	0.996
AP3	26	0.908	40	0.989	33	0.971	40	0.993	37	0.988	39	0.992
Ribo-1	8	0.697	36	0.976	31	0.978						
Ribo-2	33	0.969	43	0.999	42	0.998						
Ribo-3	13	0.811	41	0.996	35	0.988						
tDNA	21	0.921	43	0.999								
ERIC	32	0.964										

Discussion

Several techniques have been used to evaluate the genetic diversity of *P. aeruginosa* including PFGE which is the genotypic characteristics gold standard tool, primed PCR, ribotyping, multilocus sequence typing (MLST), and ERIC-PCR. PCR-based typing represents a simple and fast method for typing as compared to PFGE. Their discrimination power may be equivalent to PFGE.²⁷

To the best of our knowledge, this is the first study that represents the most extensive evaluation of RAPD, ribotyping, tDNA, and ERIC to assess the relative strengths and weaknesses of each method or their combinations in discrimination of *P. aeruginosa* isolates from UTIs. In this study, we analyzed 44 *P. aeruginosa* isolates to quantify the performance characteristics and congruence between the studied methods.

RAPD is used to detect the genetic diversity by using a short oligonucleotide random sequence to arbitrarily amplify segments of target DNA.³ Six primers of RAPD were used for the molecular characterization of *P. aeruginosa* isolates. All primers gave ≥ 10 alleles indicating good resolving power. It was observed that primers, D-10514 and OPA-02, gave the higher PM% (86.7% and 83.3%, respectively) with the allele range of 150–2000 and 300–2500 bp, respectively. The mean number of alleles, number of polymorphic and monomorphic bands per unit for the RAPD typing method were 18, 13.83, and 4.33, respectively besides a high EMR of 10.68. In addition, the mean (%) polymorphism was 76.85. The monomorphic

bands among strains illustrated the conserved regions in the genome.³³ In contrast, Pethannan et al found that all RADP primers used for typing *Pseudomonas* species produced polymorphic bands and no monomorphic bands were detected.³⁴ Comparison between the six RAPD markers revealed that no individual marker achieved both high typeability and discrimination. When measuring the efficacy of the markers according to DI, P-640 primer was the best marker as it showed the highest DI value (0.975) where it classified the 44 isolates into 31 genotypes with ISD=70.45%. While when assessing these markers according to typeability, D-14306 and OP-13 markers recorded 97.7% as only one isolate was non-typeable by each marker. Evaluating the efficacy of the markers in measuring the similarities between isolates illustrated that the D-14306 marker was the best as its ISD=29.54%. Moreover, it classified the isolates into 7 clusters at 70% cutoff, 13 clusters at 97% cutoff, and 4 clusters enclosed 79.54% of isolates at 100% cutoff.

Three ribotyping markers were included in the present study. On using Ribo-1 primer, 23 (52.27%) isolates have the same genotype No. 1 while the other 20 isolates were distributed over seven ribotypes with DI=0.697. These results indicate that these isolates may originate from the same source, besides that it demonstrated the constancy of the ISRs of these isolates.³⁵ This may be also explained by the limited number of bands in each ribotype that did not give sufficient differentiation. However, Satpathy et al showed that Ribo-1 classified *P. aeruginosa* isolates into

four ribotypes with $DI=0.91$.²⁵ For the Ribo-2 primer, genotype No. 2 was predominant with seven (15.90%) isolates while for Ribo-3, out of the 13 genotypes generated, genotypes No. 1 and 3 consist of 15 (34.09%) and 10 (22.72%) isolates, respectively. Wolska and Szwedra also reported that ribotyping of 62 *P. aeruginosa* isolates resulted in 9 groups with 80% of isolates being in two groups.²⁶ Spacov et al reported that Ribo-3 generated 8 types with 79% of isolates classified as one type.¹⁰ rRNA genetic loci of prokaryotes have three types of genes (16S, 23S, and 5S) that are separated by spacer regions with diverse lengths and sequences in the genus and species. The spacer region between 16S and 23S can be used for the identification of bacteria by detection of the polymorphism of PCR products of this region.³⁶ The genome of many bacteria has multiple copies (alleles) of the rRNA operons. Within different operons, the size of the spacer region may vary which is related to the number and type of tRNA genes (tRNA_{Agly}, tRNA_{Aile}, tRNA_{Aala}) found in some spacer regions. ISRs show wider genetic variation as they are subjected to lower evolutionary pressure.³⁷ The comparison between the three primers revealed that although Ribo2 gave $PM\%=70$, its efficiency in the other parameters was better than Ribo-1 and Ribo-3 primers. Typeability, ISD, H_g , and EMR for Ribo-2 were 97.7%, 75%, 0.53, and 9.80, respectively and it gave 20 alleles ranging between 200–2000 bp that gave rise to 33 ribotypes. In contrast, Wolska and Szwedra²⁶ showed that Ribo-2 classified 62 *P. aeruginosa* isolates into 9 types with 1–3 bands ranging from 220–900bp. The mean number of alleles, number of polymorphic and monomorphic bands per unit for the ribotyping technique were 12.67, 9.33, and 3.33, respectively, with EMR 6.9. In addition, the mean (%) polymorphism was 75. AZIMIRAD et al showed that PCR-ribotyping gave high discrimination of isolates as PFGE.³⁸ Accordingly, our results revealed the high discriminatory power of Ribo-2 ($DI=0.969$). Collier et al reported that PCR-ribotyping was more discriminating than AP-PCR in differentiating 49 *P. aeruginosa* isolates.³⁹ Martin et al illustrated that using ribotyping was of little discrimination power with strains of *P. aeruginosa*.⁴⁰

tDNA marker classified the isolates into 21 genotypes with genotype No.3 being the most pervasive one seen in 22.72% of isolates. A study conducted by Spacov et al¹⁰ using tDNA showed that tDNA had clustered 33 *P. aeruginosa* into 8 genotypes with genotype No.1 comprising about 79% of isolates. The number of alleles,

polymorphic bands, monomorphic bands, and EMR were 16, 11, 5, and 7.56, respectively. In addition, $PM\%$ was 68.7 and DI was 0.921 where % typeability was 97.7%. Investigating the similarities between isolates showed that at 100% similarity among 7 clusters was found comprising 31 isolates.

ERIC-PCR typed *P. aeruginosa* isolates into 32 genotypes. Similar results were reported by Stehling et al²⁷ where ERIC-PCR classified 62 *P. aeruginosa* isolates into 25 genotypes. The number of alleles, polymorphic bands, monomorphic bands, and EMR were 21, 15, 6, and 10.71, respectively. The H_g , Mean PM (%) and DI were 1.41, 71.4, and 0.964, respectively. In contrast to our results, previous studies reported that ERIC-PCR showed the least discrimination power.^{27,41} Typeability was 81.8% where 8 isolates were untypable. Other studies reported the inability of ERIC-PCR to type some isolates of *Plesiomonas shigelloides*,⁴² and *E. coli*.^{43,44} At 100% similarity, ERIC gave five clusters with 18 isolates.

Calculation of DI of each typing method revealed that RAPD marker (P-640) has the highest power in discriminating isolates followed by Ribo-2 and ERIC. Higher discriminatory power does not always represent accurate epidemiologic relatedness. The effectiveness of a molecular typing method is a measure of the capability to form significant clustering and discrimination of the unrelated strains.⁴⁵ Azimirad et al construct a correlation between RAPD, PFGE, and ribotyping in *C. difficile* isolates, they showed that the same discrimination power was obtained by PFGE and RAPD.³⁸

In general, various methods were used to characterize *P. aeruginosa* strains. A vital point is how the use of a single method or combination of methods could provide information as DI , typeability, or ISD of the isolates. The comparison between typing methods permits the user to retain a convenient typing method and dismiss an inadequate one.⁴⁶

By comparing the four PCR-based methods used in this study, ERIC was the best marker used in most of the studied parameters indicating its potency in detecting genetic variation between isolates and its high polymorphic and discrimination power. The second best marker that can be used in discrimination was RAPD (P-640) followed by ribotyping (Ribo-2) then tDNA. Similar results were reported.⁴¹ Azimirad et al reported that RAPD and ERIC were the best markers in discrimination between *C. difficile* isolates followed by ribotyping.³⁸ Besides, ribotyping using Ribo-2 primer was able to

Table 6 Concordance Among Typing Methods According to Rand's Coefficient

Typing Method	Rand's Coefficient									
	D-14306	D-10514	OP-13	OPA-O2	P-640	AP3	Ribo-1	Ribo-2	Ribo-3	tDNA
D-14306										
D-10514	0.586									
OP-13	0.621	0.893								
OP-O2	0.628	0.871	0.872							
P-640	0.615	0.928	0.919	0.894						
AP3	0.648	0.874	0.856	0.836	0.891					
Ribo-1	0.479	0.699	0.660	0.642	0.690	0.677				
Ribo-2	0.627	0.921	0.924	0.891	0.948	0.892	0.685			
Ribo-3	0.593	0.773	0.793	0.756	0.792	0.742	0.598	0.795		
tDNA	0.606	0.876	0.871	0.842	0.904	0.846	0.662	0.894	0.755	
ERIC	0.607	0.922	0.906	0.875	0.939	0.893	0.709	0.936	0.783	0.887

differentiate 75% of isolates followed by ERIC (72.72%) and P-640 primer (70%). Oleńska and Malek found that ERIC-PCR can differentiate 61% of 77 rhizobial strains.³⁰ Moreover, ERIC was the best one in detecting the conserved regions in the genome followed by tDNA, RAPD, and ribotyping as the number of monomorphic bands per assay unit for them were 6, 5, 4.33, and 3.33, respectively.

Previous studies reported the usefulness of ERIC-PCR in detecting the difference in number and distribution of repetitive alleles in isolates as *P. aeruginosa* isolates^{47,48} *E. coli*⁴⁹ and *Leptospira* isolates⁴⁵ with high typeability %, DI and ISD. ERIC-PCR is cheaper, easier to perform, reliable, rapid in addition to its high discrimination power. All these factors facilitate its use in the epidemiology of *P. aeruginosa* in Australia and Brazil.^{50,51}

All permutations of combinations of two types were analyzed and all combinations resulted in increased DI. The trend toward using more than one type for analysis was previously used by Clark et al who reported higher DI using combinations of more than one typing method than using either alone.⁵² Wolska and Szweida²⁶ and Stehling et al²⁷ proposed that the combination of ribotyping with other PCR-typing methods or traditional techniques can give extreme discrimination of *P. aeruginosa*. Combining ERIC with RAPD primers (OPA-02 and P-640) increased the DI to 1 as 44 types were obtained. Moreover, typeability increased to 100% as the isolates that could not be typed by one method were typed by the combined one.

The congruence between typing methods indicates the probability that pairs of isolates that are assigned to the same type by one typing method are also typed as identical by the other. The congruence between typing methods

determined by Rand's coefficient revealed that D-15014 and OP-13-types were best predicted by P-640, Ribo-2, and ERIC. While P-640-types were best predicted by Ribo-2 and ERIC. AP3-, Ribo-1-, Ribo-2-, Ribo-3- and tDNA-types were best predicted by ERIC. Evaluation of the congruence among combinations of typing methods by Rand's coefficient indicated a high increase in the coefficient of all markers where R=1 in ERIC+OPA-02 using ERIC+P-640.

Evaluation of the congruence between typing methods using the Wallace coefficient showed that only D-14306- types and AP3; Ribo-1-types and ERIC showed moderate congruence (0.427 and 0.536, respectively) with 95% CI. The other typing methods showed no or poor congruence suggesting that they grouped the tested isolates in different ways. The Wallace coefficient values among combinations of ERIC with typing methods were very high indicating complete congruence (W=1). Clark et al found complete congruent and higher Wallace coefficient values when the method of a higher resolution was used.⁵² Worth mentioning that ERIC +OPA-02 and ERIC+P-640 combinations were the highest resolution methods (DI=1) and showed complete congruence with all other combinations when used as the secondary methods. ERIC+ Ribo-1 combination gave high to complete congruence when used as the primary method. Following our results, Faria et al found that combining two typing methods (PFGE type-spa type combination) increased the predictive power of each method for determining the SCCmec type in *Staphylococcus aureus*.¹⁵

Table 7 Wallace's Coefficient Values Among Typing Methods

Typing Method	AW Coefficient (95% CI)												
	RAPD						Ribotyping			tDNA	ERIC		
	D-14306	D-10514	OP-13	OP-O2	P-640	AP3	Ribo-1	Ribo-2	Ribo-3				
D-14306	0.000 (0.000-0.041)	0.000 (0.000-0.118)	0.019 (0.000-0.099)	0.040 (0.000-0.118)	0.000 (0.000-0.040)	0.071 (0.000-0.225)	0.000 (0.000-0.173)	0.017 (0.000-0.083)	0.028 (0.000-0.138)	0.005 (0.000-0.087)	0.000 (0.000-0.043)		
D-10514	0.000 (0.000-0.322)	0.017 (0.000-0.188)	0.000 (0.000-0.133)	0.017 (0.000-0.145)	0.082 (0.000-0.297)	0.312 (0.000-0.734)	0.000 (0.000-0.121)	0.000 (0.000-0.191)	0.000 (0.000-0.207)	0.000 (0.000-0.167)	0.049 (0.000-0.207)		
OP-13	0.181 (0.000-0.594)	0.010 (0.000-0.113)	0.095 (0.000-0.294)	0.010 (0.000-0.154)	0.000 (0.000-0.154)	0.000 (0.000-0.215)	0.095 (0.000-0.278)	0.199 (0.000-0.423)	0.000 (0.000-0.096)	0.009 (0.000-0.152)	0.000 (0.000-0.096)		
OP-O2	0.253 (0.000-0.589)	0.023 (0.000-0.118)	0.063 (0.000-0.203)	0.004 (0.000-0.137)	0.004 (0.000-0.137)	0.000 (0.000-0.195)	0.030 (0.000-0.138)	0.002 (0.000-0.159)	0.000 (0.000-0.079)	0.000 (0.000-0.103)	0.000 (0.000-0.079)		
P-640	0.000 (0.000-0.470)	0.085 (0.000-0.298)	0.025 (0.000-0.228)	0.085 (0.000-0.298)	0.082 (0.000-0.351)	0.103 (0.000-0.406)	0.054 (0.000-0.251)	0.000 (0.000-0.230)	0.000 (0.000-0.178)	0.095 (0.000-0.306)	0.000 (0.000-0.178)		
AP3	0.427 (0.000-0.879)	0.003 (0.000-0.120)	0.000 (0.000-0.101)	0.003 (0.000-0.120)	0.021 (0.000-0.119)	0.125 (0.000-0.497)	0.051 (0.000-0.185)	0.000 (0.000-0.161)	0.082 (0.000-0.255)	0.014 (0.000-0.154)	0.082 (0.000-0.255)		
Ribo-1	0.000 (0.000-0.253)	0.000 (0.000-0.055)	0.000 (0.000-0.052)	0.000 (0.000-0.055)	0.006 (0.000-0.050)	0.029 (0.000-0.146)	0.001 (0.000-0.051)	0.000 (0.000-0.098)	0.046 (0.000-0.142)	0.000 (0.000-0.069)	0.046 (0.000-0.142)		
Ribo-2	0.334 (0.000-0.807)	0.092 (0.000-0.328)	0.193 (0.000-0.493)	0.092 (0.000-0.328)	0.045 (0.000-0.222)	0.165 (0.000-0.475)	0.010 (0.000-0.230)	0.064 (0.000-0.370)	0.000 (0.000-0.158)	0.000 (0.000-0.188)	0.000 (0.000-0.158)		
Ribo-3	0.074 (0.000-0.365)	0.001 (0.000-0.085)	0.055 (0.000-0.144)	0.001 (0.000-0.085)	0.000 (0.000-0.051)	0.000 (0.000-0.085)	0.009 (0.000-0.083)	0.000 (0.000-0.070)	0.000 (0.000-0.059)	0.000 (0.000-0.070)	0.000 (0.000-0.059)		
tDNA	0.035 (0.000-0.397)	0.000 (0.000-0.113)	0.007 (0.000-0.121)	0.000 (0.000-0.113)	0.029 (0.000-0.132)	0.016 (0.000-0.174)	0.000 (0.000-0.254)	0.000 (0.000-0.149)	0.000 (0.000-0.092)	0.000 (0.000-0.158)	0.000 (0.000-0.092)		
ERIC	0.000 (0.000-0.405)	0.000 (0.000-0.137)	0.000 (0.000-0.155)	0.000 (0.000-0.137)	0.000 (0.000-0.128)	0.223 (0.000-0.565)	0.536 (0.102-0.969)	0.000 (0.000-0.150)	0.000 (0.000-0.239)	0.000 (0.000-0.158)	0.000 (0.000-0.158)		

Table 8 Wallace's Coefficient Values Among Combinations of ERIC with Other Typing Methods

Typing Method	AW Coefficient (95% CI)										
	ERIC+ D-14306	ERIC+ D-10514	ERIC+ OP-13	ERIC+ OP-O2	ERIC+ P-640	ERIC+ AP3	ERIC+ Ribo-1	ERIC+ Ribo-2	ERIC+ Ribo-3	ERIC+ tDNA	
ERIC+ D-14306	0.000 (0.000-0.287)	0.000 (0.000-0.286)	0.000 (0.000-0.000)	0.000 (0.000-0.000)	0.000 (0.000-0.000)	0.293 (0.000-0.668)	0.385 (0.018-0.752)	0.000 (0.000-0.286)	0.297 (0.000-0.670)	0.000 (0.000-0.286)	
ERIC+ D-10514	0.000 (0.000-0.484)	0.000 (0.000-0.479)	0.000 (0.000-0.000)	0.000 (0.000-0.000)	0.000 (0.000-0.000)	0.242 (0.000-0.781)	0.744 (0.488-1.000)	0.000 (0.000-0.479)	0.000 (0.000-0.481)	0.249 (0.000-0.783)	
ERIC+ OP-13	0.000 (0.000-1.000)	0.000 (0.000-1.000)	0.000 (0.000-0.000)	0.000 (0.000-0.000)	0.000 (0.000-0.000)	0.000 (0.000-1.000)	1.000 (1.000-1.000)	0.000 (0.000-1.000)	0.000 (0.000-1.000)	0.000 (0.000-1.000)	
ERIC+ OP-O2	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	
ERIC+ P-640	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	
ERIC+ AP3	0.293 (0.000-0.734)	0.096 (0.000-0.420)	0.000 (0.000-0.253)	0.000 (0.000-0.000)	0.000 (0.000-0.000)	0.253 (0.000-0.593)	0.590 (0.104-1.000)	0.000 (0.000-0.253)	0.096 (0.000-0.420)	0.000 (0.000-0.253)	
ERIC+ Ribo-1	0.165 (0.000-0.422)	0.127 (0.000-0.386)	0.042 (0.000-0.228)	0.000 (0.000-0.000)	0.000 (0.000-0.000)	0.253 (0.000-0.593)	0.042 (0.000-0.228)	0.042 (0.000-0.228)	0.170 (0.000-0.426)	0.042 (0.000-0.228)	
ERIC+ Ribo-2	0.000 (0.000-1.000)	0.000 (0.000-1.000)	0.000 (0.000-1.000)	0.000 (0.000-0.000)	0.000 (0.000-0.000)	0.000 (0.000-1.000)	1.000 (1.000-1.000)	0.000 (0.000-1.000)	0.000 (0.000-1.000)	0.000 (0.000-1.000)	
ERIC+ Ribo-3	0.747 (0.495-1.000)	0.000 (0.000-0.481)	0.000 (0.000-0.479)	0.000 (0.000-0.000)	0.000 (0.000-0.000)	0.242 (0.000-0.781)	1.000 (1.000-1.000)	0.000 (0.000-0.479)	0.000 (0.000-1.000)	0.000 (0.000-0.479)	
ERIC+ tDNA	0.000 (0.000-1.000)	1.000 (1.000-1.000)	0.000 (0.000-1.000)	0.000 (0.000-0.000)	0.000 (0.000-0.000)	0.000 (0.000-1.000)	1.000 (1.000-1.000)	0.000 (0.000-1.000)	0.000 (0.000-1.000)	0.000 (0.000-1.000)	

Conclusion

Based on the results obtained, the genetic diversity of 44 isolates of *P. aeruginosa* were analyzed using primers of RAPD, ribotyping, tDNA, and ERIC. All the PCR-typing methods are useful for epidemiological typing of *P. aeruginosa* isolates as they are simple and fast methods. ERIC-PCR and RAPD-PCR showed the highest performance in discriminating isolates. The usage of two or more typing methods in combination may result in a better evaluation of the genetic diversity of isolates. The performance characteristics of ERIC and its combinations make them the most suitable typing methods for epidemiological analysis. So, we suggest their use to study the genetic diversity of *P. aeruginosa*. The application of two or more typing methods in combination may result in a better discrimination of isolates.

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Disclosure

The authors report no conflicts of interest in this work.

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