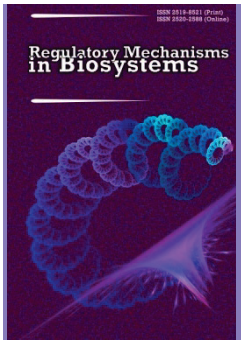

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Features of *Proteus mirabilis* clinical isolates and genetic relations inside the group

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Proteus mirabilis, a bacterium causing urinary tract infections and exhibiting multidrug resistance, poses challenges in treatment and infection control. Molecular typing methods aid in understanding genetic diversity and relationships among isolates. Repetitive Intergenic Consensus ERIC-PCR and Random Amplified Polymorphic DNA (RAPD) are considered some of the rapid and simple genetic tests. The aim of the current study was to determine the differential ability of RAPD and ERIC-PCR in biotyping *P. mirabilis* isolated from clinical samples. This study was conducted in Al-Diwaniyah Governorate, Iraq, between 2023 and 2024. A total of 189 samples were collected from wounds, burns, stools, and urine from patients hospitalized in several hospitals in the city of Diwaniyah (Iraq). From these samples, twenty isolates of *P. mirabilis* were isolated and initially diagnosed using biochemical tests. Then the diagnosis was confirmed using the VITEK system 2. Bacterial resistance to antibiotics was assessed using the VITEK system 2, then an examination of the bacteria's ability to produce biofilm was performed, following which the genomic DNA was extracted from the bacteria, and the ERIC and RAPD tests were performed. The differential ability of RAPD and ERIC-PCR was evaluated based on the Simpson's Index of Diversity (SID). The results revealed high multidrug resistance prevalence (55%) while a smaller number of isolates displayed resistance to either one (15%) or two (25%) antibiotic classes. There was only one susceptible isolate (5%). Biofilm-forming capabilities showed negative biofilm formation (30%), weakened positive (25%), moderate positive (30%), finally strong positive was exhibited in 3 isolates (15%). ERIC-PCR demonstrated high differentiation ability between bacterial isolates in the current study compared to RAPD-PCR. Notably, significant associations were found between biofilm formation categories with ERIC-PCR clusters, and between sample sources with ERIC-PCR clusters. This highlights ERIC-PCR's potential for epidemiological investigations and infection control of *P. mirabilis*.

Keywords: *Proteus mirabilis*; virulence factors; urinary tract infections; ERIC; RAPD and phylogenetic tree.

Introduction

Proteus mirabilis are Gram-negative rod-shaped bacteria. *Proteus mirabilis* has gained considerable attention due to its association with urinary tract infections and other clinical conditions and as a diverse opportunistic pathogen. The emergence and spread of antibiotic-resistant bacteria poses a major threat to global public health (Alonso et al., 2017).

In the area of bacterial pathogenesis, knowledge of the genetic range and relatedness of isolates is crucial.This know-how aids in epidemiological investigations, outbreak tracking, and the improvement of powerful interventions. Molecular typing techniques provide precious means for characterizing bacterial traces and discerning their genetic relationships (Vashisht et al., 2023).

RAPD-PCR and ERIC-PCR are extensively used molecular typing techniques. RAPD makes use of short, arbitrary primers to generate random DNA fragments, which can be then analyzed to show polymorphisms amongst isolates. ERIC-PCR, on the other hand, targets repetitive DNA sequences in the bacterial genome, amplifying intervening areas to supply strain-precise banding patterns (Finger et al., 2006).

Proteus mirabilis is a significant cause of UTIs, in particular in catheterized patients and people with compromised immune systems.The bacterium possesses an array of virulence factors, which includes fimbriae, flagella, and urease, which contributes to its potential to colonize the urinary tract and avoid the host defenses. Moreover, *P. mirabilis* is famous for its propensity to form biofilms, thus improving its endurance and resistance to antibiotics (Flores-Mireles et al., 2015).

The differential ability of molecular typing strategies is a vital issue in epidemiological investigations. An approach with high differential ability can efficiently distinguish among closely associated strains, permitting specific monitoring of transmission and identity of outbreak sources. Conversely, a method with low differential ability can also additionally fail to distinguish among epidemiologically significant isolates, leading to erroneous conclusions (Van Looveren et al., 1999).

Both techniques (RAPD and ERIC-PCR) have their advantages and disadvantages, as the use of both techniques can be customized for a specific target. Many researchers have investigated the differential ability of RAPD and ERIC-PCR. However, the results have been relatively inconsistent (Hafiane et al., 2011). K of target organism DNA sequence is not necessary for using RAPD. Depending on the locations that are complementary to the primer's sequence, a single short primer can amplify a DNA sequence. As a result, a PCR product will not be generated and the amplified product will have a different pattern if a mutation occurred in the template DNA that was previously complementary to the primer (Mbwana et al., 2006). This study aims to evaluate the differential ability of RAPD and ERIC-PCR in biotyping *P. mirabilis* isolated from clinical samples using SID.

Materials and methods

Sample collection and bacterial isolation. A total of 189 samples were collected from stools, urine, wounds and burns from patients hospitalized in Al-Diwaniyah Teaching Hospital, Al-Hamza General Hospital, and Women and Children's Hospital in Al-Diwaniyah Governorate, Iraq as shown in Table 1. Samples were transported from the hospital using sterile containers for urine and stools. As for burns and wounds, transport media containing cotton swabs was used. The samples were then transferred to the laboratory, and all samples were cultured on MacConkey Agar and Blood Agar media. Isolates were identified using phenotypic

and biochemical tests performed according to (Al-Mousawi et al., 2018). *P. mirabilis* isolates were then characterized using the Vitek-2 Compact system (Biomerieux, France).

Table 1
Distribution of clinical samples that were collected in the current study according to the source from which they were collected

Sample type	Count
Urine	82
Wounds	21
Stools	78
Burns	8

Antibiotic susceptibility testing was done using the Vitek-2 System in keeping with the manufacturer's instructions. The antibiotics tested were: imipenem, amoxicillin, ampicillin, amikacin, piperacillin / tazobactam, gentamicin, cefazolin, ciprofloxacin, cefoxitin, levofloxacin, ceftazidime, tigecycline, ceftriaxone, nitrofurantoin, cefepime, and tri-methoprim / sulfamethoxazole. This assay was done according to (Gherardi et al., 2012).

Biofilm formation assay. Biofilm formation was evaluated using the microtiter plate method. The bacteria were cultured on Nutrient Broth 370 for 48 hours, then they were loaded onto the microplate with a 1/10 dilution of the suspension using fresh media. Three replicates were made for each sample as well as the control, then incubation took place, after which it was poured gently, then the microplate was washed using a buffer, then a dye was added. Crystal Violet was then removed from the dye using a water tab, then left for up to an hour at room temperature, then examined using a subwavelength 570 nm by ELISA. This assay was done according to (Stepanović et al., 2007).

DNA extraction. Bacterial DNA was extracted using the AMB DNA purification kit (Canada) following the manufacturer's protocol. The extracted DNA was quantified using a Nanodrop spectrophotometer and stored at –20 °C until further use the genomic DNA extraction procedure was performed according to the instructions of the kit manufacturer.

DNA amplification by PCR. PCR amplification was carried out for both ERIC-PCR and RAPD-PCR. The primers were used for ERIC-PCR (5'-ATGTAAGCTCCTGGGGATTCAC-3'). For RAPD-PCR, the primer 1254 (5'-CCGCAGCCAA-3') was used. The PCR reaction mixture was prepared with the following components and volumes: 10 µL of 2X Taq PCR Master Mix, 1 µL of forward primer (10 pmol), 1 µL of reverse primer (10 pmol), 5 µL of DNA template, and 3 µl of nuclease-free water. This resulted in a total reaction volume of 20 µL. PCR amplification was performed in a thermal cycler programme with the following conditions: an initial denaturation step at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 52 °C for ERIC-PCR or 36 °C for RAPD-PCR for 1 minute, and extension at 72 °C for 2 minutes. A final extension step was performed at 72 °C for 10 minutes.

Gel electrophoresis. PCR products were separated by agarose gel electrophoresis (1.5% agarose in 1X TBE buffer) at 100 V for 1 hour. The gels were stained with ethidium bromide and visualized under UV light that is done according to (Törün et al., 2019).

Construction of genetic tree. The banding patterns obtained from ERIC-PCR and RAPD-PCR were analyzed using GelAnalyzer software (Applied Maths, Belgium). A dendrogram representing the genetic relationships among the isolates was constructed based on the Dice coefficient and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm that done according to Ugarte et al. (2006).

Statistical analysis included calculation of Simpson's Index of Diversity (SID), which was calculated to evaluate the differential ability of ERIC-PCR and RAPD-PCR. SID values range from 0 to 1, with higher values indicating greater the differential ability. The SID was calculated using the following formula: $SID = 1 - [\Sigma n_j(n_j - 1)] / [N(N - 1)]$, where n_j is the number of isolates belonging to the *j*th type, and N is the total number of isolates (Dibia et al., 2023).

Results

Bacterial diagnosis. The initial identification of bacterial isolates involved phenotypic and biochemical characterization, Subsequent confirmation and further differentiation were accomplished using the Vitek-2 Compact system. Following these diagnostic procedures, out of 189 clinical specimens collected, 20 isolates were definitively identified as *P. mirabilis*. This corresponds to a prevalence rate of 10%.

The distribution of the 20 confirmed P. mirabilis isolates across different clinical sample types. The majority of isolates (14 out of 20) were obtained from urine samples, Additionally, a smaller number of isolates was recovered from wounds (1 isolate), stools (3 isolates), and burns (2 isolates). The presence of *P. mirabilis* in these diverse sample types highlights its opportunistic nature and ability to colonize various sites within the human host.

Antibiotic sensitivity. Table 2 illustrates the distribution of *P. mirabilis* isolates based on their resistance to different antibiotics. The table shows the antibiotics tested, their respective groups, and the specific isolates that exhibited resistance. For instance, amikacin, an aminoglycoside, was resisted by isolate 20, while amoxicillin and ampicillin, both penicillins, were resisted by multiple isolates, including isolates 1, 2, and 20. Various cephalosporins such as cefazolin, cefepime, cefoxitin, ceftriaxone, and ceftazidime met resistance from different isolates, with cefazolin resisted by isolates 1 through 7, 16, 17, and 18. Ciprofloxacin and levofloxacin, both fluoroquinolones, were resisted by isolates 1 and 2. Imipenem, a carbapenem, showed resistance across a wide range of isolates, including isolates 2 through 8, 10, 12, 13, 17, 18, and 20. Additionally, all isolates showed resistance to nitrofurantoin and tigecycline, which belong to the nitrofuran and tetracycline groups, respectively. Piperacillin/tazobactam was resisted by isolate 6, and trimethoprim/sulfamethoxazole, a sulfonamide, was resisted by isolates 3, 4, 5, 6, 10, and 14.

Table 2
The antibiotics used in the current study with the groups to which they belong and the isolates that showed resistance to those antibiotics

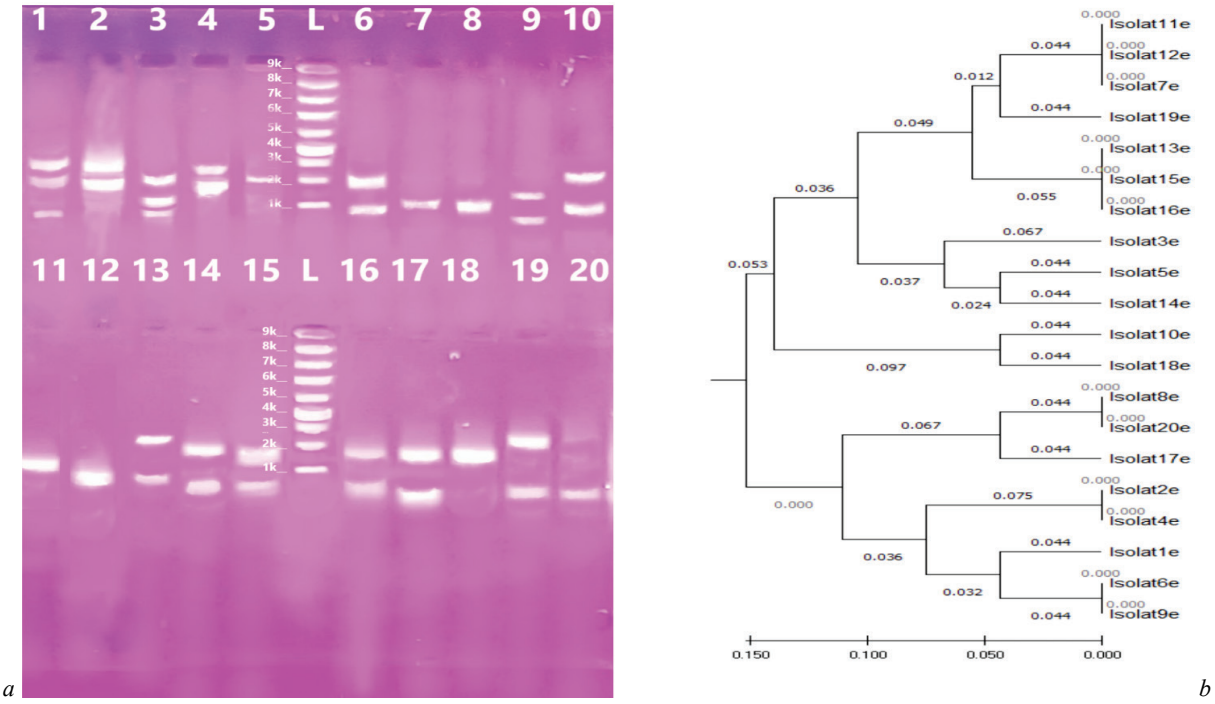
Antibiotic	Group	Resistant strains
Amikacin	Aminoglycosides	Isolate 20
Amoxicillin	Penicillins	Isolates 1, 2, 5, 6, 7, 12, 15, 16, 17, 18, 20
Ampicillin	Penicillins	Isolates 1, 2, 9, 11, 12, 13, 15, 18, 19, 20
Cefazolin	Cephalosporins	Isolates 1, 2, 3, 4, 5, 6, 7, 16, 17, 18
Cefepime	Cephalosporins	Isolate 10
Cefoxitin	Cephalosporins	Isolates 14, 15, 16, 17, 18
Ceftriaxone	Cephalosporins	Isolates 3, 4, 9, 12, 13, 15, 17, 18, 19
Ceftazidime	Cephalosporins	Isolates 10, 11, 12
Ciprofloxacin	Fluoroquinolones	Isolates 1, 2
Imipenem	Carbapenems	Isolates 2, 3, 4, 5, 6, 7, 8, 10, 12, 13, 17, 18, 20
Levofloxacin	Fluoroquinolones	Isolates 1, 2
Nitrofurantoin	Nitrofurans	All isolates
Piperacillin/ tazobactam	Penicillins	Isolate 6
Trimethoprim/ sulfamethoxazole	Sulfonamides	Isolates 3, 4, 5, 6, 10, 14
Tigecycline	Tetracyclines	All isolates

The antibiotic resistance profiles of the 20 P. mirabilis isolates included in the study. The isolates were categorized based on their resistance to different classes of antibiotics. Multidrug Resistance (MDR): a significant proportion of the isolates (11 out of 20, or 55%) exhibited MDR, indicating resistance to three or more antibiotic classes. Resistance to 1 or 2 categories: a smaller number of isolates displayed resistance to either one (3 isolates, 15%) or two (5 isolates, 25%) antibiotic classes. Susceptible Isolates: only one isolate (5%) showed no resistance to any of the tested antibiotics.

Biofilm formation. The distribution of biofilm formation capabilities among the 20 *P. mirabilis* isolates obtained from clinical samples. Negative: these isolates did not exhibit any biofilm formation (6 isolates, 30%). Weakened positive: these isolates demonstrated weak biofilm-forming ability (5 isolates, 25%). Moderate positive: these isolates formed moderate biofilms (6 isolates, 30%). Strong positive: These isolates exhibited strong biofilm-forming capacity (3 isolates, 15%).

RAPD-PCR was performed on the *P. mirabilis* isolates to assess their genetic diversity. The resulting dendrogram, constructed using the Dice coefficient and UPGMA, is shown in Figure 1. The distribution of *P. mirabilis* isolates across different clusters was identified through RAPD-PCR analysis. The isolates were grouped into three distinct clusters based on similarities in their RAPD-PCR banding patterns, reflecting

their genetic relatedness. Cluster 1: this cluster contained 10 out of 20 isolates, suggesting it represents a dominant genotype within the *P. mirabilis* population studied. Cluster 2: this cluster included only 2 isolates, indicating that it represents a less prevalent genotype. Cluster 3: this cluster contained 8 isolates, suggesting that it represents a moderately prevalent genotype. SID provides a quantitative measure of the differential ability of RAPD-PCR in differentiating the isolates. The SID value of 0.611 indicates a moderately high level of the differential ability, suggesting that RAPD-PCR was able to distinguish between most strains within the *P. mirabilis* population. However, the relatively low number of clusters and the presence of a dominant cluster (Cluster 1) suggest that the differential ability of RAPD-PCR might be limited in some cases. The value of SID ranges from 0 to 1. A value closer to 1 indicates greater diversity.



a *b*

Fig. 1. RAPD technique: *a* – gel electrophoresis for PCR product of RAPD technique for 20 detected isolates of *P. mirabilis* that were isolated from clinical samples; *b* – UPMG phylogenetic tree for 20 isolated *P. mirabilis* depending on RAPD PCR

ERIC-PCR was also employed for molecular typing of the *P. mirabilis* isolates. The dendrogram generated from ERIC-PCR analysis is presented in (Fig. 2). The distribution of *P. mirabilis* isolates across different clusters was identified through ERIC-PCR analysis. The isolates were grouped into four distinct clusters based on similarities in their ERIC-PCR banding patterns, reflecting their genetic relatedness. Cluster 1: this cluster contained the highest number of isolates (8 out of 20), suggesting it represents a dominant genotype within the *P. mirabilis* population studied. Cluster 2: this cluster included 6 isolates, indicating it represents a moderately prevalent genotype. Cluster 3 and Cluster 4: these clusters contained fewer isolates (2 and 4, respectively), suggesting they represent less common genotypes. The Simpson's Index of Diversity (SID) provides a quantitative measure of the differential ability of ERIC-PCR in differentiating the isolates. The SID value of 0.737 demonstrates a relatively high level of the differential ability, suggesting that ERIC-PCR was able to efficiently distinguish between closely related strains within the *P. mirabilis* population.

The differential ability of ERIC and RAPD. We conducted an analysis of the genetic diversity and characteristics of 20 *P. mirabilis* isolates obtained from clinical samples that utilizes two molecular typing methods, ERIC-PCR and RAPD-PCR, to categorize these isolates into distinct clusters based on similarities in their genotype. Furthermore, it explores the relationships between these genetic clusters and virulence factors of the bacteria such as biofilm formation, antibiotic resistance, and the source of the isolates.

Comparing the diversity indices calculated for ERIC and RAPD, it is evident that ERIC shows a higher diversity index than RAPD. This indicates that the bacterial strains characterized using ERIC typing display greater diversity compared to those characterized using RAPD.

The comprehensive analysis of the relationships between the different characteristics of *P. mirabilis* isolates, as determined by the ERIC-PCR and RAPD-PCR typing methods as shown in Table 3 is divided into three main sections. First, biofilm formation, where the relationship between biofilm formation (classified as negative, weak positive, moderate positive, or strong positive) and the genetic combinations was determined by ERIC-PCR and RAPD-PCR. Secondly, antibiotic resistance, where the association between antibiotic resistance profiles (classified as negative, single-class resistance, two-class resistance, or multidrug resistance, MDR) and genetic groups identified by ERIC-PCR and RAPD-PCR was studied. Third, the source of the samples, where the relationship between the source of the clinical samples (urine, wounds, stool, or burns) and the genetic groups identified by ERIC-PCR and RAPD-PCR was explored. The statistical analysis using the SID test reveals a higher diversity index in ERIC (0.737) more than RAPD (0.611).

Discussion

Among the samples collected, *P. mirabilis* was found in 20 samples, or about 10% of the total number of samples collected. The presence of *P. mirabilis* in samples can be explained by its ability to colonize a variety of habitats, such as soil, water, and the intestine or urinary tract of mammals (Yuan et al., 2021). This may be due to a number of factors, including poor sanitation or wrong societal habits (Drzewiecka, 2016).

Opinions differed in previous studies about the prevalence of the aforementioned bacteria in clinical samples in general and urinary tract infection samples in particular. Elhoshi et al. (2023) obtained 55% of the isolates of *P. mirabilis* from urine, while the remaining samples were obtained from catheters and wounds. While some studies are consistent with the results of the current study (Al-Naqshbandi et al., 2019) found that *P. mirabilis* causes 4.5% of all infections in their study.

Antibiotic susceptibility testing revealed a high prevalence of multidrug resistance (MDR) among the isolates (55%), with only one isolate (5%) exhibiting no resistance. Biofilm formation assays demonstrated varying degrees of biofilm-forming capabilities, with 30% of isolates sho-

wing no biofilm formation and 15% exhibiting strong biofilm formation. Such results may be due to inherent resistance mechanisms in *P. mirabilis*, such as beta-lactamase production, efflux pumps, and altered membrane permeability (Munita et al., 2016). Additionally, the overuse and misuse of antibiotics in clinical settings and agriculture could contribute to the development of resistance (Davies et al., 2010; Jadooa et al., 2022). Prior research aligns with current findings on the susceptibility of *P. mirabilis* to ertapenem and resistance to nitrofurantoin (Miranda et al., 2014) finding that 99.5% of isolates from urine cultures were susceptible to ertapenem and 0% to nitrofurantoin. However, conflicting results may arise due to differences in antibiotic use patterns, geographic locations, and genetic diversity of bacterial populations.

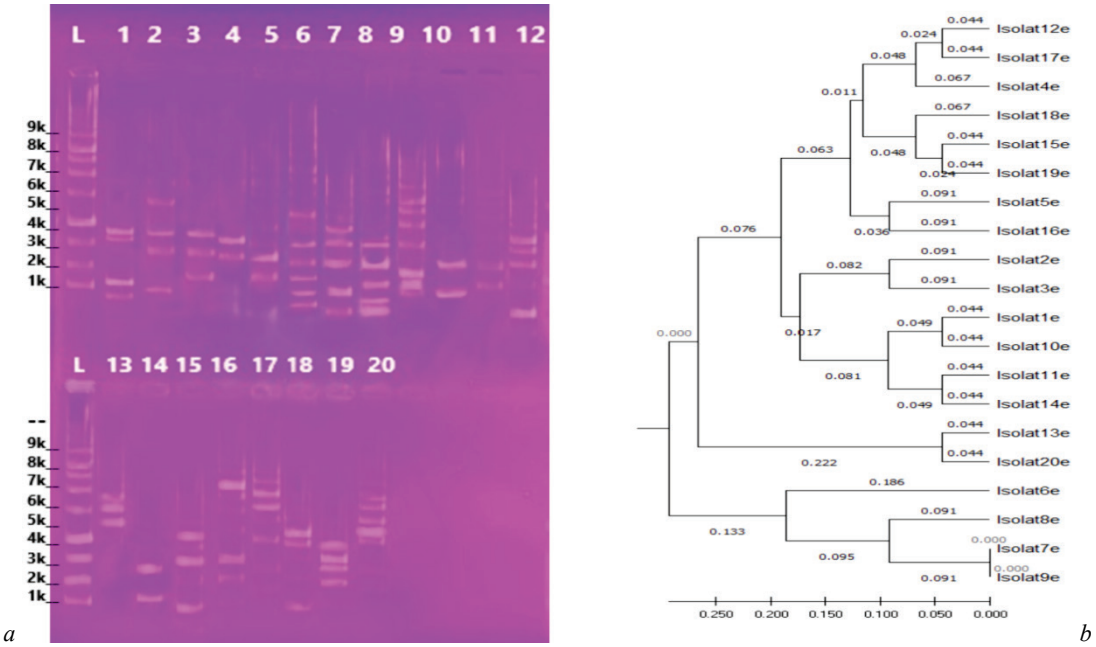


Fig. 2. ERIC technique: *a* – gel electrophoresis for PCR product of ERIC technique detected in 20 *P. mirabilis* isolates that were isolated from clinical samples; *b* – UPMG Phylogenetic tree for 20 isolated *P. mirabilis* depending on ERIC PCR

Table 3
Simpson's Index of Diversity of 20 isolates of *P. mirabilis* collected from clinical samples according to ERIC-PCR and RAPD-PCR genotypes and their relationship with biofilm formation and antibiotic resistance groups and sources of isolates

Category	Cluster No.			
	cluster 1	cluster 2	cluster 3	cluster 4
Biofilm and ERIC PCR				
Negative	2	2	0	2
Weakened positive	1	2	0	2
Moderate positive	3	2	1	0
Strong positive	2	0	1	0
Antibiotic resistance and ERIC PCR				
Negative	0	1	0	0
Resistant to 1 category	2	0	0	1
Resistant to 2 categories	1	2	1	1
Multiple Drug Resistance	5	3	1	2
Source of samples and ERIC PCR				
Urine	5	5	2	2
Wounds	1	0	0	0
Stool	0	1	0	2
Burns	2	0	0	0
Biofilm and RAPD PCR				
Negative	3	0	3	0
Weakened positive	2	1	2	0
Moderate positive	4	0	2	0
Strong positive	1	1	1	0
Antibiotic resistance and RAPD PCR				
Negative	1	0	0	0
Resistant to 1 category	1	0	2	0
Resistant to 2 categories	1	1	3	0
Multiple Drug Resistance	7	1	3	0
Source of samples and RAPD PCR				
Urine	8	1	5	0
Wounds	1	0	0	0
Stool	0	1	2	0
Burns	1	0	1	0

The data results of biofilm formation classes among the isolates indicate that 30% were negative while 25% of them weak positive, 30% showed moderate positive finally, 15% showed strong positive, which is partly consistent with a previous study (Kadhim et al., 2024) which found all *P. mirabilis* isolated from urine to have the ability to biofilm formation. The reason for the emergence of such results could be due to the difference in genetic and environmental factors of the isolates included in the current study, as in a previous study, the role of these factors and their effect on the formation of biofilm was indicated. Bacterial species often use biofilm formation as a survival mechanism in hostile environments, such as those encountered in host tissues such as the urinary tract (Rather et al., 2021). Similar patterns in biofilm formation among *P. mirabilis* isolates have been seen in previous research, with different proportions of negative, weakly positive, moderately positive, and strongly positive biofilm formation (Sun et al., 2020).

Both RAPD-PCR and ERIC-PCR successfully differentiated the *P. mirabilis* isolates into distinct clusters, with ERIC-PCR exhibiting a higher differential ability (SID = 0.737) compared to RAPD-PCR (SID = 0.611). This distinction in variety may be attributed to the inherent methodologies of ERIC and RAPD. ERIC normally targets repetitive DNA sequences dispersed at some point of the bacterial genome, probably shooting a much broader variety of genetic variations. RAPD on the alternative hand, is based on random primers that might not increase all genomic areas uniformly (Wilson et al., 2006).

This suggests that ERIC-PCR may be a more effective method for distinguishing closely related *P. mirabilis* strains, and this is consistent with a previous study (Wolska et al., 2008), which was aimed at evaluation of ERIC PCR for determining the diversity of clinical *Pseudomonas aeruginosa* isolates. The results of their study indicate that ERIC PCR analysis is a more discriminatory method than PCR ribotyping analysis and the traditional serotyping scheme while the current finding disagreed with (Abdel-Rhman et al., 2021), who found that RAPD-PCR gave the highest mean percent polymorphism per assay (76.85%) followed by ERIC-PCR. However, some studies have also reported comparable differential ability between the two methods, for example (Michelim et al., 2008) who found that both methods gave medium to high differential

ability in *P. mirabilis*. The results of Michelim's research obtained with RAPD, BOX-PCR and ERIC-PCR were in good agreement. The analysis of the relationships between different characteristics of *P. mirabilis* isolates, as determined by ERIC-PCR and RAPD-PCR typing methods, revealed a significant association between biofilm formation and ERIC-PCR clusters, but not with RAPD-PCR clusters. This suggests that ERIC-PCR may be more effective in differentiating isolates based on their biofilm-forming capabilities. Additionally, a significant association was found between the source of the clinical samples and ERIC-PCR clusters, but not with RAPD-PCR clusters, indicating that ERIC-PCR may be more effective in differentiating isolates based on their origin within the human host. While the Chi-square test indicated a marginally significant association between antibiotic resistance and RAPD-PCR clusters, the association with ERIC-PCR clusters was not statistically significant.

Conclusions

Proteus mirabilis showed a high prevalence among clinical samples, causing a large proportion of infections. *Proteus mirabilis* isolates also showed high levels of resistance to antibiotics. More than half of the isolates were able to produce biofilm. As for evolutionary tests, both RAPD-PCR and ERIC-PCR successfully differentiated the *P. mirabilis* isolates into distinct clusters, with ERIC-PCR exhibiting a higher differential ability compared to RAPD-PCR. The analysis of the relationships between different characteristics of *P. mirabilis* isolates revealed a significant association between biofilm formation and ERIC-PCR clusters, but not with RAPD-PCR clusters.

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The authors declare that there are no conflicts of interest.

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