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Molecular Typing of the Uropathogenic*Proteus mirabilis*by using ERIC-PCR, RAPD-PCR, and PCR Detection of *rbsA*, *ureC*, and *zapA*VirulenceGenes

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ABSTRACT

The current work addresses the molecular profiling of ten clinical strains isolated from patients withurinary tract infections (UTIs) using three molecular typing tools: ERIC1b-PCR, RAPD640-PCR, and PCR detection of the three virulence genes *rbsA*, *ureC*, and *zapA*.Tenuropathogen clinical strains were isolated from patients with confirmed UTIs after culturing of urine samples on blood agar and trypticase soybean agar according to the colonial andcell morphological features. Results of VITEK2 system, positive catalase test, negative oxidase test, positive urease test, characteristic swarming, and distinctive fishy odorverified that all tenuropathogen clinical strains under investigation were affiliated to *Proteusmirabilis*. ERIC1b-PCR and RAPD 640-PCR could discriminate the ten strains into three groups and six groups (clades), respectively according to the DNA banding pattern specific to each profile.The frequency of occurrence of the three virulence genes *rbsA*, *ureC*, and *zapA* in all *P.mirabilis* strains under investigation was 100% as deduced from the obtained banding pattern of PCR partial amplification with 467, 533, and 350bp, respectively using gene specific primers. The present data would underpin the use of RAPD640-PCR for powerful discrimination of *P.mirabilis* clinical strains.

Keywords: *Proteus mirabilis*; UTIs; ERIC1b-PCR; RAPD640-PCR; *rbsA*; *ureC*; *zapA*

INTRODUCTION

Among the most prevalent infectious diseases worldwide both in hospitals and community are urinary tract infections (UTIs)that ultimately lead to inflammations in the ureter, urethra, kidneys, and urinary bladder(1,2) UTIs have high morbidity rates and extreme expenditures associated with medications targeting the spectrum of antimicrobial resistance(3,4)

*E.coli*and*Klebsiellapneumoniae*arearethe most top two uropathogens clinically implicated in chronic and severe bacteriuria worldwide(5). However, a low frequency of bacteriruriaoccurrence was correlated with other species like *Proteus* spp., *Entrobacter* spp.,*Acinetobacter* spp., *Citrobacter* spp., *Pseudomonas aeruginosa*,*Enterococcus faecalis*and *Staphylococcus aureus*(6,7).

The Gram-negativebacterium*Proteus mirabilis* is anuropathogen especially isolated from patients with urinary tract disorders like urolithiasis andlong-term urinary catheterization(8). As a rule of thumb, the pathogenicity of the uropathogen*P.mirabilis* is prompted by its virulence factors that would help in the environmental adaptation and the host invasion. The virulence factors encountered in the *P.mirabilis* UTIs are adhesions, motility, toxins, quorum-sensing (biofilm formation), enzymes, nutrients acquisition, and immune invasion .Urease, one of the virulence factors, does play a major role in the pathogenesis of *P. mirabilis in* UTIs.The urease production by the uropathogen*P. mirabilis* would result in the hydrolysis of urea with the concurrent liberation of ammonia and shifting in the urine pH to the alkaline side(9). As a

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consequence, the crystallization of magnesium and calcium in the alkaline environment of the urine would occur alongside with the blocking of the catheter lumen. This would in turntriggeracute urinary retention, bacteriuria, and other unwanted consequences like pyelonephritis, stone formation, and bacteremia(10).

The *rsbA* gene, another virulence factor with essential role in swarming regulation, does encode a sensory protein namely rbsA(11).Moreover, the *rbsA*proteincould stimulate the quorum-sensing and the formation of extracellular polysaccharides (12). The extracellular metalloprotease of 54 kDa (*zapA*) isacrucialvirulencekeyfactorinthe pathogenesis of the uropathogen*P.mirabilis.*The*zapA*secreted by the uropathogen*P.mirabilis*does degrade the host immunoglobulins (Igs) and cleave the antimicrobial peptides (human β-defensin 1 (hBD1) and LL-37) involved in the innate immune response(13).

It is worth mentioning that therepetitive element-based PCR (Rep-PCR) and random amplified polymorphic –polymerase chain reaction (RAPD-PCR) have been extensivelyencountered in molecular typing of the closet bacterial members at the strain level (intaspecific level). Enterobacterial repetitive intergenic consensus –polymerase chain reaction (ERIC-PCR), a type of Rep-PCR, amplifies highly conserved central inverted repeat localized in non-coding transcribed loci in the bacterial chromosome using ERIC primers; generating a specific DNA fingerprint related to each strain. Whereas, RAPD-PCR involves the usage of random oligonucleotides primers that haphazardly amplifies specific regions of the genome with the generation of unique DNA banding pattern related to each strain under investigation. ERIC-PCR and RAPD-PCR are more advantageous over the parallel molecular typing methods regarding the high discrimination power in the differentiation of the closely related bacterial strains, uncomplicatedness, rapidness, cheapness, reliability, and high throughputs genotyping method(14,15).

From epidemiological point of view, it is indispensable to unravel the types of the clinical uropathogen strains prevailing in every country to combat the antibiotic resistance phenomenon and reduce the unwanted extra costs encountered in medications as well. In the context of the aforementioned, the objective of the current work is to type on a molecular level the uropathogen*P.mirabilis* clinical strains isolated from patients with UTIsfrom Al DiwaniyahTeachingHospital, Iraq using three molecular typing tools: ERIC1b-PCR, RAPD640-PCR, and PCR detection of *rsbA*, *ureC*, and *zapA* virulence genes.

PATIENTS AND METHODS

Patients selection

This study was carried out on 105 patients that visit Urology center in Al Diwaniyah Teaching Hospital, Iraq during the period from December 2019 to March 2020 who were diagnosed with Urinary Tract Infection by the urologist. The diagnosis of UTI was confirmed through taking detailed history thorough physical examination and complementary investigations includes at least urine analysis and ultrasound examination for urinary system. Patients who currently on antibiotics treatment were excluded from the study.

Urine samples

Mid-stream urine samples were collected in sterile containers from patients with clinical signs and symptoms of urinary tract infections (UTIs); for catherized patients; urine sample was taken

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directly from urinary catheter after disregard the initial urine drops.Urine analysis was done to reveal the presence of white blood cells. The remainder of the urine samplewas sent to the Microbiology laboratory in the college of Medicine-Al-Qadisiyah University for immediate culturing and isolation of pathogen by microbiologist.

Media

Trypticase soybean agar (TSA), MacConky agar and blood agar were used for the routine isolation and culturing of bacteria from the urine samples. Trypticase soybean broth (TSB) was used for the performing of overnight cultures of the clinical isolatesfollowed by the addition of glycerol to a final concentration of 15% for the long-term preservation of the clinical strains at -80 ^{o}C.

Isolation and identification of bacteria from urine samples

The urine samples were cultured on TSA plates. The bacterial isolates were identified based on the morphological examinations (colony morphology with distinctive odor on blood agar and cell morphology by Gram-stain), catalase test, oxidase test,urease test, and VITEK-2 system as reported previously (16). From 150 urine samples only 10 *Proteus mirabilis* were obtained.

Genomic DNA isolation

Genomic DNA isolation from the bacterial isolates was carried out using the Genomic Bacterial DNA isolation kit (Anatolia, Turkey) according to the manufacturer's instructions. The integrity of the isolated genomic DNA was checked on 1% agarose gel electrophoresis followed by staining with ethidium bromide and visualization by UV-Transilluminator [Sambrook et al. 1998). However, the concentration of the isolated genomic DNA was evaluated using Nano-drop Spectrophotometer (Applied Biosystem, USA).

ERIC-PCR and RAPD–PCR techniques

ERIC1R-PCR and RAPD640-PCR were employed in this study for fingerprinting of the bacterial isolatesunder investigation using the ERIC-1R primer: 5'-ATGTAAGCTCCTGGGGATTCAC-3'(17)and RAPD-640: 5'- CGTGGGGCCT-3' (18) respectively. The primers were synthesized by Integrated DNA Technology, USA. Briefly, the PCR mixture for ERIC-PCR and RAPD-PCR each contained 12.5 µL of PCR master mix (iNTRON, Korea), 3 µLof(0.3 µM) of primer, 9.5µL of distilled water, and 2.0 µL (100 ng) of template DNA. All reaction mixtures were put in the thermocyclcer (Master Cycler® ep RealplexEppendorf, USA). The PCR cycling conditions were set to be as follow: an initial denaturation step at 94 ºC for 2 min, 45 cycles eachcycle 94 ºC for 1 min, 25 ºC for 1 min, 72 ºC for 5 min, and a final extension at 72 ºC for 8 min. The ERIC1R and RAPD640 DNA fragments were electrophoresed on 1.5% agarose gel electrophoresis alongside with100 bpDNA ladder(abm, Canada)at 75 Volt for 45 min using DNA submarine unit (Cleaver Scientific, UK). The visualization of the DNA bands was achieved by ethidium bromide staining followed by exposure to UV-Transilluminator (Cleaver Scientific, UK). The obtained ERIC1R and RAPD 640 DNA fragments were analyzed optically and their molecular weight were estimated. A DNA standard curve was established using 100 bp DNA ladder (abm, Canada). The pattern of ERIC 1R and RAPD 640 fragments was displayed in the binary scoring system (Eftekhar and Nouri 2015).

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PCR detection of *rsbA*, *ureC*, and *zapA* genes

The presence of the three virulencegenes*rbsA*, *ureC*, and *zapA*wereunraveled in the bacterial isolates under investigation by PCRpartial amplification of each gene using three gene specific primers sets. The following three gene specific primer sets F-*rbsA*:5'-TTGAAGGACGCGATCAGACC-3'/ R-*rbsA*: 5'-ACTCTGCTGTCCTGTGGGTA-3', F-*UreC*: 5'-GTTATTCGTGATGGTATGGG-3'/R-*UreC*:5'-ATAAAGGTGGTTACGCCAGA-3', and F-*ZapA*: 5'-ACCGCAGGAAAACATATAGCCC-3'/R-*ZapA*:5'-GCGACTATCTTCCGCATAATCA-3' were used to detect the presence of *rbsA*, *UreC*, and *ZapA* genes in each bacterial isolate, respectively.The primers were synthesized by Integrated DNA Technology, USA. For each bacterial isolate under investigation, three polymerase chain reactions were directed separately using the aforementioned primer sets. Each PCR reaction mixture contained 30 ng of genomic DNA, 0.3µM of each forward and reverse primer, 12.5µL of PCR Master mix (2X) (iNTRON, Korea), and nuclease free water to a final volume of reaction mixture 25µL. The thermocycler(Master Cycler® ep Realplex Eppendorf, USA)was programmed as follow: 95 ^{o}C, 5 min for initial denaturation, 30 cycles each cycle: 94 ^{o}C, 45 sec for denaturation, 58 ^{o}C (for *rbsA*), 56 ^{o}C (for *UreC*), 59 ^{o}C (for *ZapA*), 45 sec for annealing, 72^{o}C, 30 sec for extension, and 72 ^{o}C, 10 min for final extension. After the PCR termination, all PCR products were checked by running on 1% agarose gel electrophoresis alongside with 100 bp DNA ladder (abm, Canada). The visualization of the PCR products was done using the UV-Transillumiantor(Cleaver Scientific, UK). The expected lengths of PCR products were 467, 533, and 350bp resulting from the partial amplification of *rbsA*, *UreC*, and *ZapA*genes, respectively.

RESULTS

Identity of the uropathogen clinical strains

A tenuropathogen clinical strains was isolated from urine samples collected from patients with UTIs; admitted in the Al Diwaniyah Teaching Hospital, Iraq during the period from December 2019 to March 2020. The tenuropathogen clinical strains were negative for catalase and oxidase tests. However, all strains under investigation showed strong urease positive test.All ten strains displayed swarming motility on blood agar with distinctive fishy odor. The tentative identification conferred that all strains under investigation assigned to *Proteus sp.* Moreover,the biochemical pattern derived from the VITEK-2 system did confirm that all strains under investigation affiliated to the bacterium *Proteusmirabilis*. The ten*P.mirabilis* clinical strains were nominated serially from mir1 to mir 10.

Molecular profile with ERIC-PCR and RAPD-PCR

The tenuropathogen clinical strains were discriminated based on ERIC1R-PCR and RAPD640-PCR DNA banding pattern as shown in Fig1. Generally, ERIC1R-PCR showed a very small number of DNA bands (1-3)allover the ten clinical strains under investigation. In contrast, RAPD640-PCR did display a high number of DNA bands when compared to the profile of ERIC1R-PCR (Fig1). The number of DNA banding pattern of RAPD640-PCR ranged from 2-8 allover the ten uropathogen clinical strains.The molecular weight of each DNA band both from ERIC1R-PCR and RAPD640-PCR were determined by the aid of DNA standard curve (Fig 2,Table 1 and Table 2).For ERIC1R-PCR DNA banding pattern, the obtained molecular weights were 3000, 1500, and 50 bp (Fig 2 and Table 1). For RAPD640-PCR DNA banding pattern, the obtained molecular weights (Fig 2 and Table 2) were 3000, 1500, 1000, 800, 700, 300, and 200 bp. The ERIC1R-PCR could discriminate the ten uropathogen clinical strains into three groups with three different ERIC profiles namely P1 (3000 and 50 bp) for mir 1, mir 2, mir 3, mir 4, mir

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6, and mir 9, P2(3000, 1500, and 50 bp) for mir 5 and mir 8, and P3(50 bp) for mir 7 and mir 10 (Table 1). Whilst, the RAPD640-PCR could discriminate the ten uropathogen clinical strains into six groups with six different RAPD profiles namely P1(1500, 1000, 800, 700,and 300bp) for mir 1, mir 2, mir 6, andmir 10, P2 (3000 and 1500 bp) for mir 7, P3 (1500, 1000, 800, 700, 300, and 200 bp) for mir 3, P4 (3000, 1500, 1000, 800, 700, 400, and 300 bp) for mir 9, P5 (1500, 1000, and 300 bp) for mir 4 and mir 8, and P6 (1500, 1000, and 200 bp) for mir 5 (Table 2). The DNA fragment derived from ERIC1R-PCR and RAPD640-PCR were displayed in the scoring binary matrix as shown in Table 3 and Table 4.

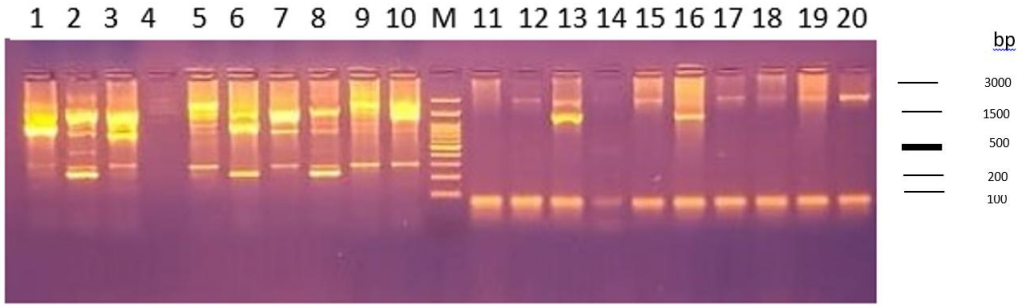

Fig1: Agarose gel electrophoresis (1.5%) showing the banding pattern of ERIC1R-PCR and RAPD640-PCR for the ten clinical strains using ERIC-1R and RAPD-640 primers, respectively. Lanes (1-10): RAPD640-PCR DNA banding pattern for the ten *P.mirabilis* clinical strains namely mir1 to mir10 serially. M: 100 bp DNA ladder. Lanes (11-20): ERIC1R-PCR DNA banding pattern for the ten *P.mirabilis* clinical strains namely mir1 to mir10 serially.

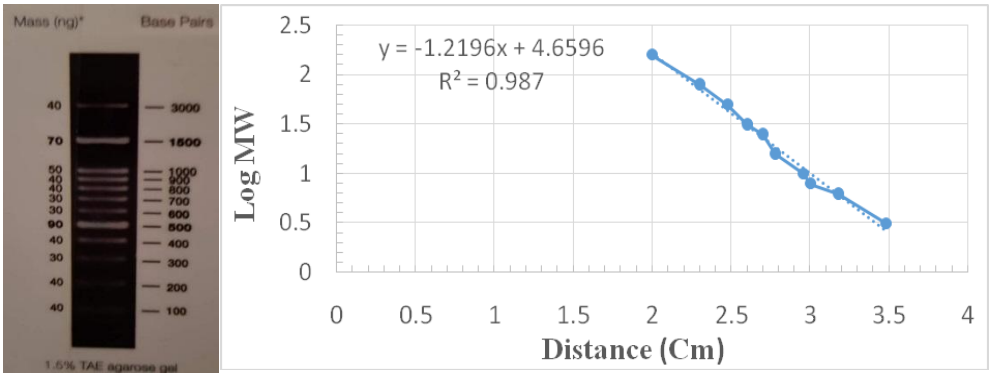

Fig 2: A DNA standard curve using 100 bp DNA ladder (abm, Canada). The R^2 value was approaching to 1.0 that reflects the little difference between the predicted values and the experimental values. MW: molecular weight of DNA ladder bands.

Table 1: ERIC1R-PCR profile and molecular weight of DNA bands for the ten uropathogen*P.mirabilis* clinical strains

DNA band size (bp)	*P.mirabilis* strains with DNA band size	ERIC1R-PCR profile no in relation to the strain
3000, 50	mir 1, mir 2, mir 3, mir 4, mir6, mir 9	P1
3000, 1500, 50	mir 5, mir 8	P2
50	mir 7, mir 10	P3

Table 2: RAPD640-PCR profile and molecular weight of DNA bands for the ten uropathogen*P.mirabilis* clinical strains

DNA band size (bp)	*P.mirabilis* strains with DNA band size	RAPD640-PCR profile no in relation to the strain
1500, 1000, 800, 700, 300	mir 1, mir 2, mir 6, mir10	P1

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3000,1500	mir7,	P2
1500, 1000, 800, 700, 300, 200	mir 3	P3
3000, 1500, 1000, 800, 700, 400, 300	mir 9	P4
1500, 1000, 300	mir 4, mir 8	P5
1500, 1000, 200	mir 5	P6

Table 3: Binary scoring matrix for the ERIC1R-PCR fragments for the ten uropathogen*P.mirabilis* clinical strains

***P.mirabilis*Strain number**										
Size of DNA fragment (bp)	mir 1	mir 2	mir 3	mir 4	mir 5	mir 6	mir 7	mir 8	mir 9	mir 10
3000	1	1	1	1	1	1	0	1	1	0
1500	0	0	0	0	1	0	0	1	0	0
50	1	1	1	1	1	1	1	1	1	1

0: DNA band is present & 1: DNA band is absent

Table 4: Binary scoring matrix for the RAPD640-PCR fragments for the tenuropathogen*P.mirabilis* clinical strains

***P.mirabilis*Strain number**										
Size of DNA fragment (bp)	mir 1	mir 2	mir 3	mir 4	mir 5	mir 6	mir 7	mir 8	mir 9	mir 10
3000	0	0	0	0	0	0	1	0	1	0
1500	1	1	1	1	1	1	1	1	1	1
1000	1	1	1	1	1	1		1	1	1
800	1	1	1	0	0	1	0	0	1	1
700	1	1	1	0	0	1	0	0	1	1
400	0	0	0	0	0	0	0	0	1	0
300	0	0	1	1	0	0	0	1	1	0
200	0	0	1	0	1	0	0	0	0	0

0: DNA band is present & 1: DNA band is absent

Molecular profile for *rbsA*, *UreC*, and *ZapA*

With regard to the presence of the three virulence genes *rbsA*, *UreC*, and *ZapA*, the tenuropathogen*P.mirabilis* clinical strains do carry the three virulence genes (Fig 3 and Fig 4). The PCR banding pattern resulting from the partial amplification of *rbsA*, *UreC*, and *ZapA*using gene specific primers showed positive PCR products with the expected lengths of 467, 533, and 350bp, respectivelyfrom all strains under investigation.

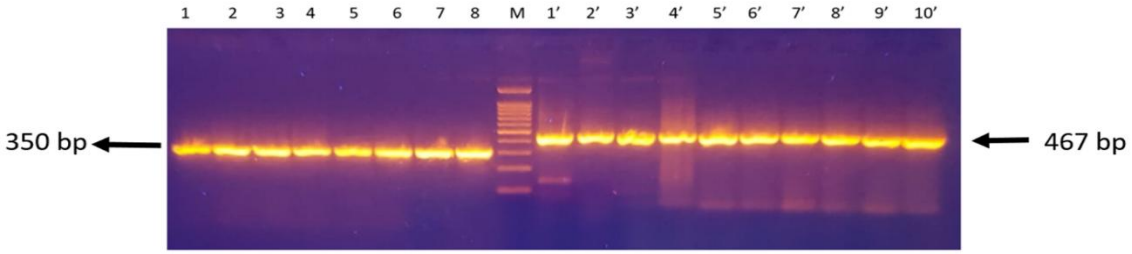

Fig 3: Agarose gel electrophoresis (1.5%) showing the PCR products of *rbsA* and *ZapA* genes after partial amplification using gene specific primers. M: 100 bp DNA ladder. Lanes (1-8): PCR products of *ZapA* gene partial amplification (350 bp) from eight*P.mirabilis*uropathogenclinical strains namely mir1 to mir 8 serially. Lanes (1'-10'): PCR products of *rbsA* gene partial amplification (467 bp) from ten*P.mirabilis*uropathogenclinical strains namely mir1 to mir10 serially.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 3137 – 3145
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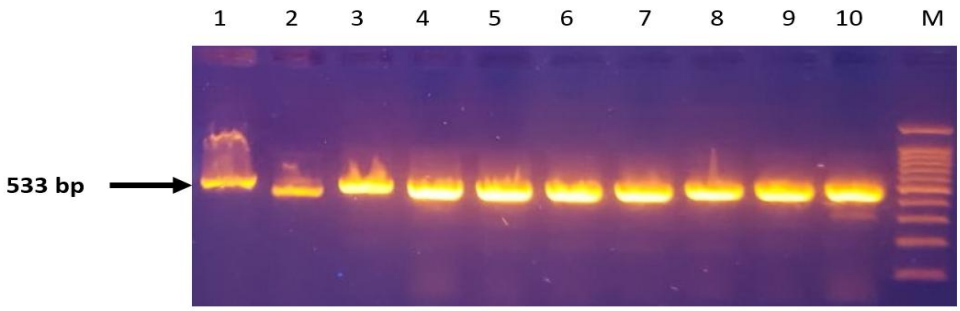

Fig 4: Agarose gel electrophoresis (1.5%) showing the PCR products of *UreC* gene after partial amplification using gene specific primers. M: 100 bp DNA ladder. Lanes (1-10): PCR products of *UreC* gene partial amplification (533 bp) from ten *P.mirabilis*uropathogen clinical strains namely mir1 to mir 10 serially.

DISCUSSION

P.mirabilis is a common uropathogen implicated in patients with urinary tract disorders especially those with long-term catheterization. Since UTIs are the most prevalent infectious diseases worldwide it is mandatory to unravel the molecular type of the prevalent uropathogen strains in every country from the epidemiological point of view. Previous reports highlighted the molecular epidemiology of UTIs due to *Proteus*spp using various tools like ribotyping, pulsed field gel electrophoresis (PFGE) and tandem-repeat microsatellite (19)However, ribotyping and PFGE are costy, time-consuming, and laborious. Meanwhile, other molecular tools like ERIC-PCR and RAPD-PCR were reported to be successful the clinical strains of *P. maribilis*(20)

In the present study, ERIC1R-PCR and RAPD640-PCR were employed to discriminate the ten uropathogen*P.mirabilis* clinical strains isolated from patients with UTIs; admitted in AlDiwaniyah Teaching Hospital, Iraq. ERIC1R-PCR showed a poor discriminative power for categorization of the ten uropathogen*P.mirabilis* clinical strains. ERIC1R-PCR did succeed to classify the ten strains under study into three groups (clades). Conversely, RAPD640-PCR showed high discriminative power as it did succeed to categorize the ten uropathagen*P.mirabilis* clinical strains into six profiles. To the best of authors' knowledge, the present work evaluates the discriminative power of RAPD640-PCR in differentiation of ten uropathogen clinical strains for the first time ever.The present finding is in a good agreement with previous reports stating the high discriminative power of a different sets of RAPD primers (e.g., OPZ20, OPX13, OPA11, OPZ08 and OPA19)other than RAPD640primer in the categorization of *P.mirabilis*clinical strains(20).The superior discriminative power displayed by RAPD640-PCR over that of ERIC1R-PCR could be attributed to the short length of the RAPD primer that would trigger randomization for binding elsewhere on the chromosome. The randomized binding would trigger a various number of PCR products with different molecular profilesamong the tested strains. In contrast, The ERIC1R primer binds to a consensus sequence on the chromosome among all members of enterobacteriaceae. The conservation in sequence would in turn reduce the chance of randomized binding on the chromosome elsewhere. Consequently, the generated DNA banding pattern of ERIC1R-PCR might be constant among various strains of *P.mirabilis* under strain. Conclusively, the present work would underpin that RAPD640-PCR is a powerful tool in discrimination of the uropathogen*P.mirabilis* clinical strains. However, increasing the number of tested strains is a mandatory task to better explore the efficacy of ERIC1R-PCR as a molecular typing tool in prospective studies (22-25).

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