Original Article

Prevalence of *AcrAB-TolC* Efflux Pump among *Proteus mirabilis* Isolated from Cystitis Patients in Hilla City, Iraq

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Abstract

Background: *Proteus mirabilis* is a prevalent profiteer pathogen that causes the Spartan human ailment. It has been identified as a urinary tract infection (UTI) etiological agent that adheres to uroepithelial cells and the catheter surface. The most important efflux system in *P. mirabilis*, acrAB-to1C, is elaborated not merely in antimicrobial conflict but also in ferocity. **Objectives:** The current research conducted to look into the antibiotic sensitivity profile and *acrAB-to1C* efflux pump genes occurrence among *P. mirabilis* isolated from UTI patients. **Materials and Methods:** 515 urine cases were gathered from UTI patients, who visited urology consultancy clinics of three main hospitals and private clinic labs in Hilla City, Iraq. All mid-stream urine samples were cultured on diagnostic agars for *P. mirabilis* primary recognition and finally confirmed by16S *rRNA* gene PCR-sequencing. Antibiotic sensitivity was performed according to CLSI (2021), and then PCR detection of *acrAB-to1C* efflux pumps genes was observed. **Results:** The occurrence of *P. mirabilis* in the studied cases was 10.5% with 100% swarming isolates. The recovered isolates were extremely resistant to cefotaxime (100%), doxycycline (88.6%), minocycline (75.7%), and amoxicillin-clavulanic acid (67.1%). In contrast, the isolates showed nonresistance to pipracillin-tazabactam (0%), meropeneme (0%) and tinier resistance to amikacine (1.4%), imipeneme (2.9%), levofloxacine (4.2), and ofloxacin (5.7%), MDR rate was 96%. Molecular investigation using PCR, revealed *acrA, acrB,* and *tolC* genes with a prevalence of 51.4%, 61.4%, and 51.4%, respectively. **Conclusion:** These findings emphasize the significant title role of acrAB-to1C efflux impel in increasing antibiotic tolerance in *P. mirabilis* with a high incidence of MDR, so the future insights may need to focus on efflux pumps inhibitor-antibiotic combination treatment as a preventive device.

Keywords: *16S rRNA*, AcrAB-TolC, antibiotic resistance,efflux pumps, *P. mirabilis*, UTI

Introduction

The g=gram-negative bacillus *Proteus* is an item of the Enterobacterial family of the Enterobacteriaceae, which has been known for a long time. According to a reassembled phylogenetic tree grounded on joint central proteins, ribosomal protein, and four multi-locus sequences, *Proteus* belongs to the new Morganellaceae family.[1] *P. mirabilis* is well-known in microbiology and medical labs for two distinct characteristics: urease production and efficient swarming motility; it is significant in medicine and often causes serious bacteriological infections in majority of hospitals in patients' ear, wound, and urinary tract, and other infections.[2]

Urinary tract infections (UTIs) are the most frequent clinical indication of *Proteus* infection, which remain over and over again persistent and difficult to treat; however, *P. mirabilis* causes 80%–90% of *Proteus* infections, which are classified as community-acquired illnesses,[3] with 90% being from UTI.[4] It causes complex UTIs or UTIs in patients who are long-term catheterized, ranking third after *Escherichia coli* and *Klebsiella pneumonia*.[5] It is quite serious because it causes catheter obstruction, kidney and bladder stone formation, cystitis, bacteremia, and acute and chronic pyelonephritis.[6,7]

The suitable treatments for UTIs include choosing antibiotics that are efficient, existing, and high

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performing; however, in the past ten years, there has been a noteworthy rise in the number of antibiotics to which bacteria have developed resistance.[8,9] Efflux pumps are one of the greatest effective corporate adaptive antibiotic resistance mechanisms that have been discovered, allowing antibiotics, organic solvents, toxic heavy metals, and other elements to be expelled directly, from the bacterial cell's cytosol or periplasm to the outside.[10]

Energy is required for efflux pumps, which are divided into two types: primary efflux pumps that draw their energy from ATP hydrolysis and secondary efflux pumps that draw their energy from chemical gradients. They are chiefly divided into the superfamilies listed below: "Small Multidrug Resistance (SMR.) superfamily, Multidrug and Toxic Composite Extrusion (MATE.) superfamily, Resistance Nodulation and Cell Division (RND.) superfamily, Major Facilitator Superfamily (MFS.), and ATP Binding Cassette (ABC.) superfamily."[11]

RND superfamily, utmost a clinically significant group, are found in all gram-negative bacteria, acrAB-to1C pump being the main RND efflux system in *Salmonellae, E. coli,* and other Enterobacteriaceae.[12] It is tripartite complex, comprising (i) an outer-membrane channel protein (OMP), (ii) an inner-membrane transporter (IMP), and (iii) a membrane fusions protein (MFP).[13]

It was demonstrated that a high level of acrAB–to1C pump confers resistance contrary to a wide range of antimicrobial classes, such as macrolidies, β_lactams, aminocoumarins, rifamycin's, quinolones, oxazolidonies, and tetracyclines, nonetheless coloring-agents, anti-cancer drugs, bile salts, cleaners, and solvents.[14] "Deletion of the gene encoding any one of these three proteins from the chromosome increases susceptibility to many antibiotics. AcrAB-tolC efflux activity can also preserve resistance acquisition by plasmid transfer in the presence of antibiotics with other modes of action."[15]

Prokaryotic *16SrRNA* genes, which translate a small subunit of rRNA, have been extensively utilized in taxonomic classification and phylogenetic associations.[16] Genotypic bacterial analysis begins with the nucleotide sequence analysis of the relevant gene(s) through the PCR then followed by a comparison of those sequences with sequences found in the database. This is made possible by the statement that all bacterial species embrace the 16SrRNA gene, which has hyper-variable sections that are cooperative for identifying species and extremely conserved areas on which to develop universal primers.[17]

Identification of new and existing resistance mechanisms is crucial for lowering the cost of medical care associated with bacterial infections. Hence, this study was done to find the role of acrAB-to1C efflux impels in the reduced *P. mirabilis* susceptibility.

Materials and Methods

Study design

This is a cross-sectional study involved 515 urine specimens which were collected from UTI patients (376 specimens from female patients and 128 specimens of male patients with age ranging from ≤10 to ≤60), who attended Al Hillah Teaching Hospital, Margan hospital, Alamam Al sadeq Hospital and private clinic labs in Babylon city, for the period between January and June 2022. Mid-stream urine was collected in a disinfected screwcap container.

Bacterial identification

Every single specimen is directly inoculated on the MacConke y agar and the non-lactose fermented isolates were identified by a series of biochemical tests (such as oxidase, catalase, urease, etc.) and inoculated onto the UTI Chromogenic agar. For swarming phenomenon test, suspected isolates were cultured on blood agar plates then incubated at 37°C for 24 h.[16,18]

DNA extraction

The Favoregen Genomicall y DNA Extractions Kit was used to separate gnomically DNA from Suspected *P. mirabilis* isolates following the manufacturer's protocol.

PCR technique

Wholly strains were subjected to molecular characterization by the detection of particular genes, *16SrRNA acrA, acrB,* and *tolC*, respectively, using specific primer pairs that are itemized in Table 1. Amplification of DNA was carried out in a final volume of 25 µL consisted of upstream primer (10 pmol) 1 µL, downstream primer (10 pmol) 1 µL, DNA template (50–500 ng) 4 µL, Nuclease-free water

Table 1: Sequence of Primer pairs used in the study for identification of *P. mirabilis* and efflux pump genes

Primer	Sequence (5′–3′)	Product (bp)	Annealing (°C)	Ref.
16SrRNA	27F: AGAGTTTGATCCTGGCTCAG 1495R: CTACGGCTACCTTGTTACGA	1500	53ºC	Loy *et al*.[21]
acrA	F: ATCACCTTTCGCACTGTCGT R:CGACAAACAGGCCCAACAAG	256	58.3ºC	Ali and. Al-Dahmoshi[22]
acrB	F:CATAAACACGCCCTGGTCCT R:GCTACCCGTAAGTCGATGGG	432	60.3ºC	
TolC	F: CGATCGTGATGCTGCCTTTG R: GGTTGCGTTTTTCGGCTTCT	596	58.3ºC	

(ddH2O) 6.5 µL, and Accustart™ Taq PCR SuperMix 12.5 µL (Promega/USA). The PCR products (5 µL) were evaluated in a 1.5% (w/v) agarose gel by electrophoresis with 1× TBE buffer and envisaged by staining using red safe dye. Product size was located by contrast with a 100 bp DNA ladder.[19] UV transilluminator was situated to notice the DNA-bands, and then gel was photographed with a digital camera. The PCR amplicons were scanned in a Macrogen_Corporation laboratory in Korea[16] using an automated DNA sequencer called the ABI3730XL. The results were collected and analyzed employing a clever software. By means of the BLAST analysis, the sequence identity to GenBank accessions was determined.

Antimicrobial susceptibility testing

Kirby-Bauer disk diffusion method was followed to examine the antibiotic of the gotten strains by activating them for 18 h and 37°C in brain heart infusion broth and then adjusted to 0·5 McFarland's standard (1.5×10^8 CFU/mL) and dispersed on Mueller Hinton agar by means of cotton swabs. Twenty antimicrobial agents were elaborated. The incubation lasted for 18–24 h at 37°C, and the diameter of the inhibitory region in mm was assessed as labeled by CLSI.[20]

Ethical approval

This study was directed in agreement by means of the ethical values that have been originated in the Declaration of Helsinki and attained from the general directorate of health and Laboratory of the Hospitals and Scientific Committee from the College of sciences/University of Babylon, Hilla City, Iraq. "It was carried out with patients verbal and analytical approval before sample was taken. The study protocol, the subject data, and permission formula were reviewed and approved by a local ethics committee according to the project no.: m 220101 (including the number and the date in 26-1-2022) to get this approval."

Results

515 clinical specimens from UTI patients were gathered; among those 504 samples (97.9%) gave positive culture [Figure 1], and most of the cases age were 21–40 year (41.7%) trailed by 41–59 year (28.3%). Majority and minority of the patients' percentages were 73.5% female patients and 26.5% male patients, respectively, as revealed in Table 2.

Seventy *P. mirabilis* strains were recovered from 504 positive clinical samples (10.5%), with 100% swarming phenomenon [Figure 2]. The molecular identification via PCR technique of the *16s rRNA* gene yielded 100% confirmation of the strains identified. As shown in Figure 3, all *P. mirabilis* strains produced the similar band size (1500 bp), which was the product size of the primers used for identification. By using the automatic DNA sequencer ABI3730XL, the PCR products of ten randomly selected isolates were analyzed in Macroegen Corporation, Korea by Sanger sequencing. Four strains' retrieved sequences that shared a common ancestor

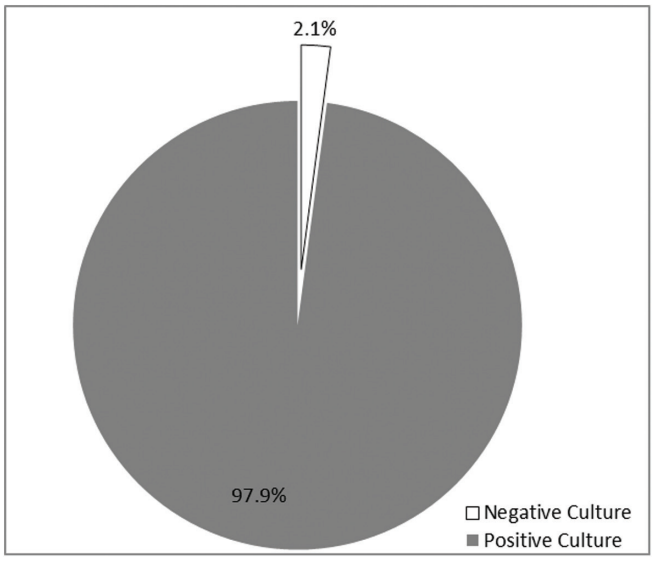


Figure 1: Percentage number of UTI culture

Table 2: Distribution of UTI patients' samples according to age and gender

Age range	Patients No. (%)	Gender No. (%)	
		Male	Female
1–10	30 (5.8)	18(60)	12(40)
11–20	26 (5.1)	15(57.7)	11(42.3)
21–40	215 (41.7)	40(18.6)	175(81.4)
41–59	146 (28.3)	35(24)	111(76)
≥60	98 (19)	26(26.5)	72(73.5)
Total	515 (100)	134(26)	381(74)

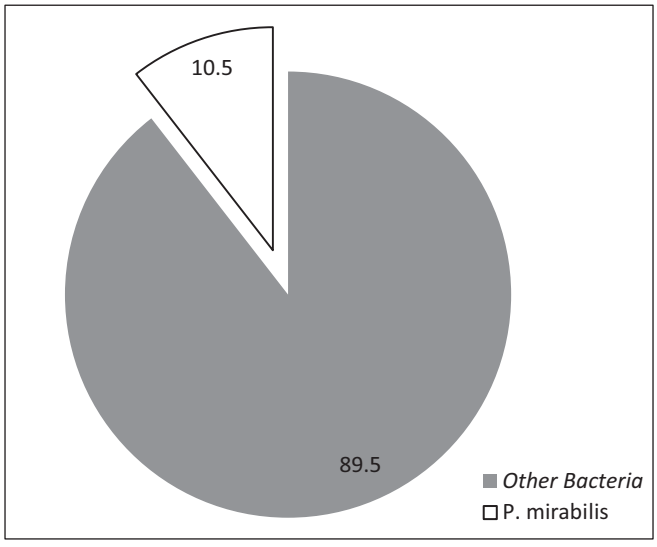


Figure 2: The percentage of bacterial isolates recovered from the urine samples

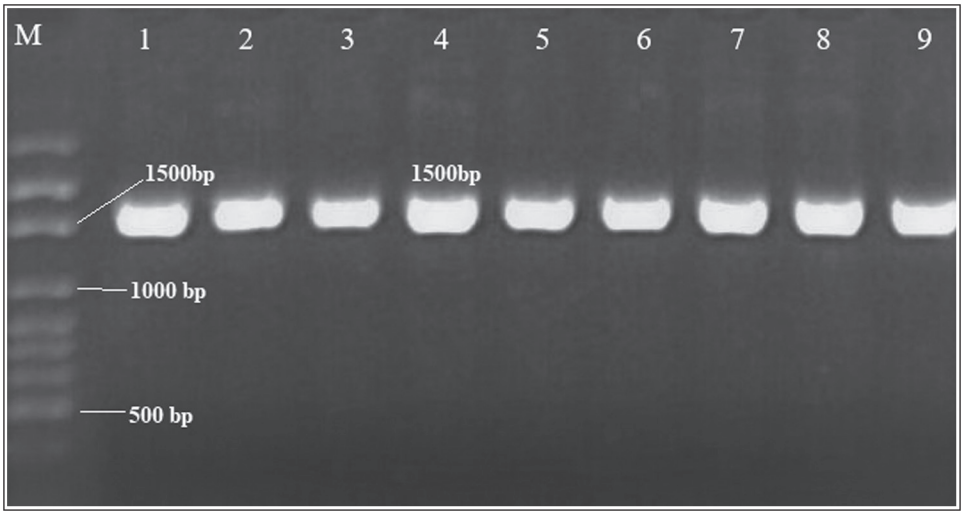
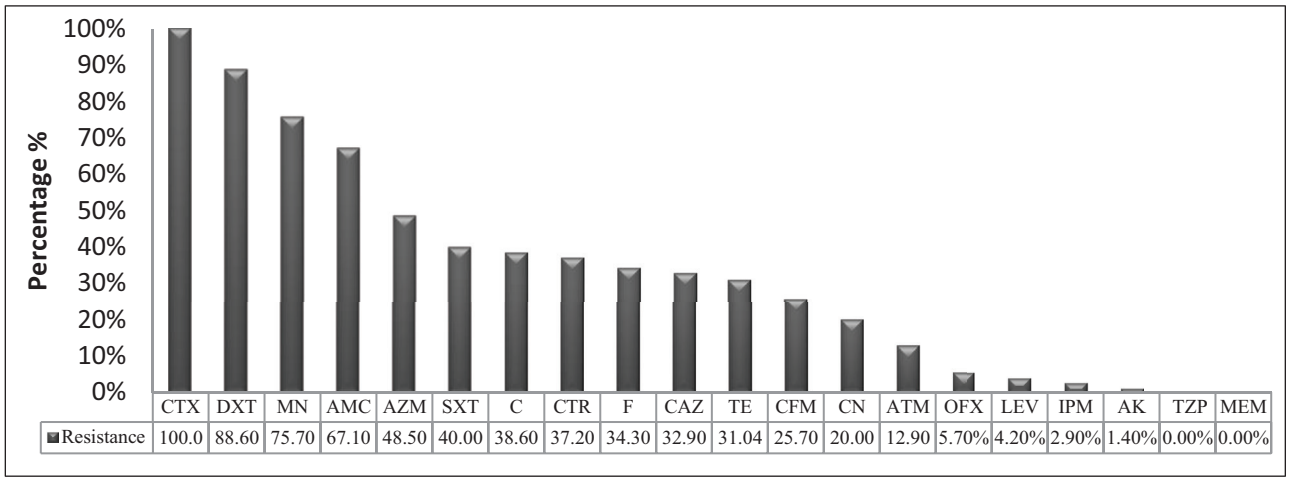


Figure 3: The results of *P. mirabilis* isolates' *16SrRNA* gene am ¸plifications were separated on a l.5% a ¸garose ¸gel, TBE l× at 80 volt for 60 min, and electrophoresis stained with red safe. DNA 100-bp marker

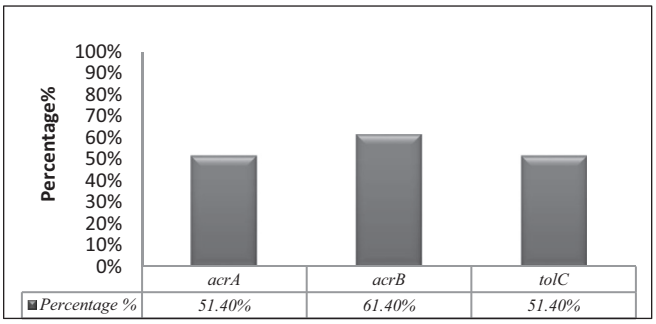


	CTX	DXT	MN	AMC	AZM	SXT	C	CTR	F	CAZ	TE	CFM	CN	ATM	OFX	LEV	IPM	AK	TZP	MEM
Resistance	100.0	88.60	75.70	67.10	48.50	40.00	38.60	37.20	34.30	32.90	31.04	25.70	20.00	12.90	5.70%	4.20%	2.90%	1.40%	0.00%	0.00%

Figure 4: Antibiotic resistance percentage of *P. mirabilis* to all antibiotic employed in current study

were assigned accession numbers OP245946, OP245947, OP245948, and OP245949 then placed in the Gen Bank.

In the present study, 20 antibiotics agents were used for resistance testing depending on CLSI,[23] and all the isolates were indicated to be nonresistant to Piperacillin-tazabactam and Meropenem with tinier resistance to Amikacin, Imipenem, Levofloxacin, and Ofloxacin, with prevalence (0%, 0%, 1.4%, 2.9%, 4.2%, and 5.7%, respectively). These were the supreme active drugs against *P. mirabilis* strains [Figure 4]. "While the resistance appears elevated to azithromycin, trimethoprim-sulfamethoxazole, chloramphenicol, ceftriaxone, nitrofurantion, ceftazidime, tetracycline, cefixime, gentamicin, and aztronam (48.5%, 40%, 38.6%, 37.2%, 34.3%, 32.9%, 31.4%, 25.7%, 20%, and 12.9%, respectively) ." from wholly antimicrobials experienced, the strains obtainable a superior resistance to cefotaxime, doxycycline, minocycline, and amoxicillin-clavulanic acid (100%, 88.6%, 75.7% and



	acrA	*acrB*	*tolC*
Percentage %	*51.40%*	*61.40%*	*51.40%*

Figure 5: Percentage of *acrA, acrB* and *tolC* genes appearance in *P. mirabilis*

67.1%, respectively). The strains showed highest rate of MDR (96%).

This study estimated the occurrence of *acrA*, acrB in addition *tolC* genes of efflux impels in *P. mirabilis* strains as of UTI patients. The results of molecular investigation discovered the genes were distributed as follows: *acrA*

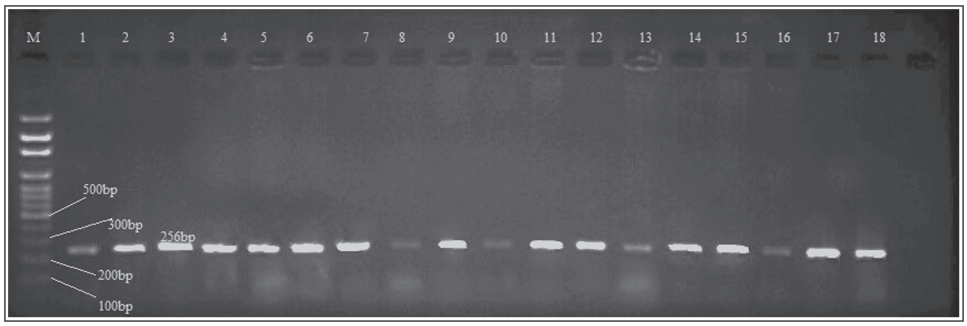


Figure 6: The results of the am plifications of *acrA* gene of *P. mirabilis* isolates were fractionated on l.5% agarose gel, TBE 1× at 80 volt for 60 min, electrophoresis stained with red safe. 100bp DNA marker

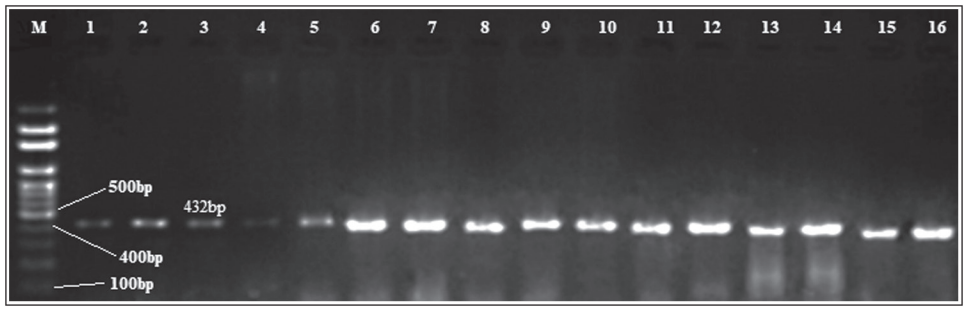


Figure 7: The results of the am plifications 0f *acrB* gene of *P.mirabi1is* isolates have been fractionated on l.5% a garose gel, TBE 1x at volta ge 80 volt for 60 min, electrophoresis stained with red safe. 100bp DNA marker

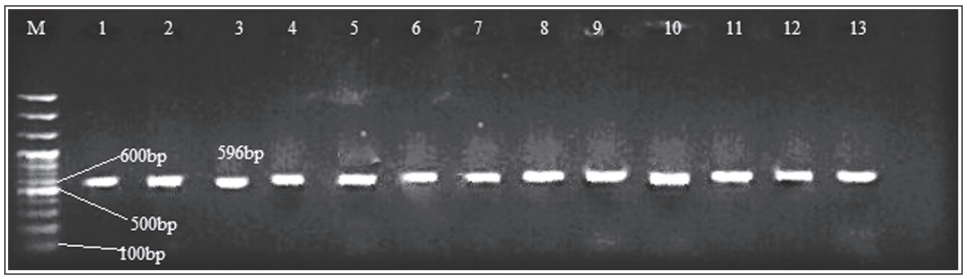


Figure 8: Results of the am plifications of *to1C* gene 0f *P.mirabi1is* isolates have been fractionated on l.5% a garos gel, TBE 1x at Voltage 80 volt for 60 min, electrophoresis stained with red safe. 100bp DNA marker

51.4%, *acrB* 61.4%, and *tolC* 51.4% [Figure 5]. Ratio of *acrA* to *acrB* gene was 43%, while the data for acrAB-tolC efflux pump were recorded as 23% in all strains. Figures 6–8 illuminates the finding of the genes am plicon on a volta ge of 80 volts for 60 min. The positive strains to *acrA, acrB* and *tolC* genes produced the similar band size (256 bp, 432 bp, and 596 bp, respectivel y), which was the product size of the primers used for identification.

Discussion

The most communal clinical appearances of *P. mirabilis* infection are UTIs, which frequently occur in female patients. One of the most pressing issues in public health and a top focus of microbiology research is the worldwide spread of antibiotic resistance among pathogenic bacteria. Efflux impels are one of the supreme dominant antibiotic resistance mechanisms. The study estimated the antibiotic sensitivity profile and commonness of *acrAB-tolC* efflux impel genes among *P.mirabilis*.

The current findings show that the majority of cases aged 21-40 and 41-59 years had a high female patients percenta ge (73.5%), and that parallel to the results of Al-Musawi and A1-Husseini, who found that the percentage of infected female patients aged 18-50 years was (89.81%).[24] These results are consistent with those of another study, which found a 40.9 percent increase in UTIs among patients aged 19 to 59, of which 81% were female patients and 19% were male patients.[25] The isolation rate of *P.mirabilis* from women was found to be higher than that of male patients.[26] The presence of previous UTI did influence substantially the further development of UTI in female patients,[27] which frequently occurs in female patients and leads to significant morbidity and mortality.

The isolation rate of *P.mirabilis* in the current study was 10.5%, which matched the rates found by Abbas and his team and Abed and Shareef, which were 12.6% and 12.7%, respectively, from patients with various clinical cases.[16,28] However, *Proteus* were established in very low numbers in clinical cases.[29] The information obtained from numerous studies showed that *P. mirabilis* as nosocomial, opportunistic pathogen and considered to be community-acquired infection and it is the third greatest rife etiological element of urinar y tract infections in addition to being found in 5 to 20 % of cases.[4]

Overall, *P.mirabilis* is typically found in the microflorae of the human intestine. They also live in other outside environments and are especially joint with hospitals, where patients and staff members may be the primary pathogenicity vectors. These bacteria are also metabolically involved in the production of urease and swarm, which is one of the successful causes for colonizing the urinary tract.[30]

Generally, antibiotic susceptibility profile in present study was showing an agreement with

other research including Mukhtar *et al*.[17] Strains that are resistant toward frequently used antibiotics represents risks and public_health threats toward the community. It was illuminated that gram-negative bacteria seem to show resistance to tetracycline , doxycycline, and minocycline.[31] In comparison with similar studies performed, our result got along with result by AL-Hamdani and AL-Hashimy, who indicated that the ma jority of *P.mirabilis* isolates were illustration high resistant to Cefotaxime 90% , whereas the maximum susceptibility was contrary to Imipenem (70%).[32] The current results are somewhat far from what was researched by Lv *et al*. who reported that *P. mirabilis* strains revealed resistance toward trimethoprim-sulfamethoxaezole 73.58%.[23] It was demonstrated that *P.mirabilis* was sensitive toward both imipeneme in addition meropeneme even though resistance to amoxicillin-clavulanic acid and ceftazidime.[33]

The current results were analogous in the direction of those obtained by Hussein *et al*.[5] in which the resistance ratio of Gentamicin was confirmed to be (20.6%) while not compatible with ceftriaxone (1.6%) and nitrofurantoin (88.9%), whereas it matched with Mirzaei *et al*.,[26] the ratio of Cefixime (22.5%) and Aztronam (15%) was established.

P.mirabilis showed resistance to chloramphenicol, which is a bacteriostatic' antimicrobia1 and the best rife broad-spectrum antibiotic. This was consistent with Mulani *et al*.[30] findings of chloramphenicol resistance (36%). In the current study, the distribution of resistance may be innately induced by a number of factors, including reduced membrane permeability, overexpression of efflux pumps, and the synthesis of enzymes that render drugs inactive. The further type of acquired resistance stayed affected by whichever horizontal transferal genes, mutations or by the production of biofilm that performances as a diffusion barrier's to diminish antibiotic entrance inside bacteria.[10]

For the purpose of screening antibiotic resistance as well as virulence genes in some bacterial pathogens, PCR is an efficient and specific epidemiological method.[34] The current study found that the *16S rRNA* gene had a robust discriminatory supremacy to identify insulated *P.mirabilis*, and that was consistent by means of the findings of Saleh *et al*.,[35] who used *16ṣrRNA* gene toward identify *Proteus*spp. insulated from UTI patients' and Mukhtar *et al*. who identify *P. mirabilis* on banknotes by this gene. Because it is present in all bacteria and is highly conserved across species , the *16SrRNA* gene has proven to be an appealing molecular target.[16,17]

The AcrAB–To1C tripartite efflux machinery stood the foremost pump in Enterobacteraceae as well as the furthermost vital efflux system in *E. coli , Klebsiella , P.mirabilis* besides *Salmonella typhimurium*, that elaborate not merely in antimicrobial resistance , but likewise in virulence.[36] Additionally, It is one of the utmost extensivel y studied efflux systems in gram-ne gative bacteria besides being a strategic factor in multidru g resistance to numerous antibiotics, comprising βlactams, tetracyclines, novobiocin, in addition to fluroquinolones.[37]

Overproduction of acrAB-tolC may a donate a peak level of resistance in *P.mirabilis* , which was trustworthy by way of increased MICs of minocycline as well chloramphenicol in this bacteria. Few previous studies have elucidated the role of acrAB-tolC pumps with resistance in *P. mirabilis*.[38]

Our findings are, in proportions, differed slightly from other investigators, such as those by Eaves *et al*. which displayed the correspondence in the middle of *acrA* plus *acrB* genes in *Salmonella enterica* and *E. coli* was nearby 94% plus 97%, respectivel y[39] and the study employed in Iran revealed that the occurrence of *acrA/ acrB* genes among *E. coli* strains were 82.9%/ 95.9% with 82.9% of isolates had both genes.[9]

Saeb and his colleagues[40] demonstrated that *P. mirabilis* had elevated acrAB-tolC adaptive resistance in some instances against antibiotics that had been initially rated as sensitive, these antibiotic as ceftriaxone, cefotaxime, cefaclor, and cefepime aztreonam, meropenem tetracycline, and chloramphenicol. Whereas an efflux pump can remove an antibiotic so that its level inside the cell is below a lethal threshold, this causes bacteria to develop antibiotic resistance. However, the omission of *acrA, acrB* and *to1C* genes outcomes in elevating the susceptibility toward antibiotics.

Conclusion

Pipracillin-tazabactam and meropenem, according to this study, are presently a worthwhile option for an experimental therapeutic approach toward UTLs

treatment; however, it is recommended to use these antibiotics with carefulness in order to avoid resistance. However, nitrofurantoin remains effective intended for the selective treatment of UTIs. Moreover, the frequency of MDR strains among isolates in this study was in height. The present study concluded that antibacterial tolerance in *P. mirabilis* is dependent upon increased efflux through AcrAB-tolC pump, so the future insights may need to focus on efflux pumps inhibitor-antibiotic combination treatment regimen.

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Conflict of interest
The authors declare they have no conflict of interest.

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