Relationship between Antibiotic Resistance, Biofilm-Formation and Virulence Factors among Multidrug-Resistant Proteus Mirabilis Isolated From Uti Patients in Wasit Province, Iraq

Zahra Ali Hadi ALjeeliz[1], Rana H. Raheema[2]*, Sabah Fadhil Abood[3]
[1, 2, 3]Department of Medical Microbiology, Faculty of Medicine, University of Wasit, Iraq
Department of Medicine, Faculty of Medicine, University of Wasit, Iraq
Email: e-mail: rraheema@uowasit.edu.iq

Abstract
One of the most common bacterial illnesses worldwide is urinary tract infections (UTIs). The pathogenesis of Proteus mirabilis depends on its capacity to express virulence factors, such as adhesion molecules, biofilms, proteases, urease, siderophores, and toxins. This species also causes a variety of infections in the urinary tract, burns, and wounds. They also exhibit resistance to several classes of antibiotics. To characterize Proteus mirabilis isolates from UTI patients phenotypically and genotypically for determinants of antibiotic resistance as well as the existence of virulence genes. Out of 228 urine specimens collected from a patient with suspected UTIs, 40 (17.5%) Proteus mirabilis isolates were identified. The isolates were first identified as related to culture, microscopic examination, and biochemical tests. DNA was extracted from these 40 isolates, the sample's nucleic acid purity ranged from (1.8-2) while its concentration was between (50 - 360 mg/ul). Whole Proteus mirabilis isolates were examined for their resistance against 15 antibiotics belonging to different classes. Meropenem, Amikacin, Ciprofloxacin, Norfloxacin, and Levofloxacin had the lowest rates of resistance discovered in this study, this may be attributable to the poor usage of these antibiotics in Al-Kut hospitals. Meropenem was in the first place it was the most effective and sensitive drug against Proteus mirabilis isolates in the current study. In a phenotypic analysis of ESBLs, it was discovered that 8 (20.5%) of the clinical isolates of the proteus mirabilis were ESBL produced by confirmatory testing whereas 39 (97.5%) were ESBL producers by screening testing. To detect the formation of biofilm, all isolates of Proteus mirabilis were screened by microtiter plate (MTP) and modified Congo red agar (MCRA) methods. In the MCRA method, the results showed that 38 (95%) were black isolates of Proteus mirabilis strong producer biofilm and 2 (5%) were pink isolates which non-producers biofilm. In the MTP method observed 20(50%) non-producers, 5(12.5%) isolates of Proteus mirabilis produced weak biofilm, 9(22.5%) isolates as moderate biofilm and 6 (15%) isolates strong biofilm. In addition, a molecular study using ureC showed that all isolates possess the ureC gene with a molecular weight of 317bp. luxS gene was also detected by PCR and the results showed that (95%) isolates possess this gene by the appearance of the amplicon with a molecular weight of 283bp. ZapA and pm1 genes are present in all (100%) of isolates, while pet gene are not present in all Proteus mirabilis isolates. mrpA gene was detected in Proteus mirabilis isolates by PCR technique and the results showed that (97.5%) of the isolates possessed the gene. Also, the results of this experiment revealed positive amplification for all isolates (95%) mrpI gene in Proteus mirabilis isolates. The current study has shown the great emergence of biofilm-forming and MDR in Proteus mirabilis isolates and harbours a variety of virulence genes.

Keywords: Proteus mirabilis, antimicrobial resistance, extended-spectrum β-lactamases; biofilm formation and virulence genes.

1. Introduction

A prevalent health issue in nosocomial and community settings is urinary tract infection (UTI). According to the National Ambulatory Medical Care Survey, one million of UTI alone visits to hospital emergency rooms and close to seven million outpatient department (OPD) visits, leading to roughly 100,000 admissions [1]. A frequent pathogen that causes severe UTIs is *Proteus mirabilis* [2]. The virulence factors expressed by *Proteus mirabilis* are many. Quorum sensing molecules, adhesion proteins, efflux pumps, lipopolysaccharides, and urease enzyme are a few examples of such elements [3]. This pathogen is categorized as an opportunistic pathogen that affects immune-compromised individuals with a variety of disorders and causes nosocomial infections and infections of the urinary system [4].

A public health issue is the spread of Gram-negative, multidrug-resistant (MDR) bacteria [5]. In recent years, *Proteus mirabilis* is the multidrug-resistant Enterobacteriaceae with the greatest extended-spectrum beta-lactamases (ESBL) and carbapenemase synthesis implicated in UTIs, Antibiotic resistance is caused by bacteria building biofilms due to limited antibiotic penetration and

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expression of resistant genes, as a result of these factors, indwelling medical devices (IMDs) are the most sensitive to biofilm-producing microbial colonizers [6]. Biofilms are a structured layer of bacterial colonies adhering to biotic or abiotic surfaces enclosed within a self-produced exopolysaccharide matrix. It aids bacteria's capacity to resist host defensive mechanisms and is involved in a variety of bacterial infections [7, 8]. So, in the case of *Proteus mirabilis* biofilm production is a significant resistance mechanism because it enhances resistance gene transfer, renders bacterial colonies antibiotic-resistant, increases antibiotic metabolism, and boosts efflux pump expression [9]. Determining the spread of *Proteus mirabilis* from UTI patients in Kut City, Wasit Province, Iraq and characterizing it at the molecular level through analyses of its biofilm formation, antimicrobial resistance genes, and virulence genes are the goals of this project, which was presented. The following steps were carried out to achieve these aims: *Proteus mirabilis* strains from patient-associated urinary tract infections were isolated and identified, determination the antibiotic susceptibility profile of these bacteria, investigation of ESBLs production and biofilim formation phenotypically and molecular detection of genes responsible for production of biofilm and some virulence factor by using PCR technique.

2. Material and Methods

In a cross sectional study a total of two hundred and twenty eight urine specimens were collected from patient with different ages admitted to: Al-Zahraa teaching hospital, Al-Karama teaching hospital, Al-Kut hospital for Gynecology obstetrics and pediatrics and from private clinics in wasit province, Iraq from both sex male and female during a period from 15th of September 2021 to 1st of February 2022, isolated bacteria have been recognized base on morphological, biochemical tests, API 20E kit andVitek2 for some isolates. Antibiotic Susceptibility testing this test has been conducted by the Kirby-Bauer process on the Muller Hinton agar [10] and the results have been interpreted based on the [11]. Then phenotypic detection of ESBLs' by disk diffusion technique (screening test) has been used to phenotypically detect ESBL production, and the double disc synergy test (DDST) has been used to validate it [12] by Screening test and Confirmatory test.

Biofilm production: Clinical microbiologists have utilized a variety of techniques to identify and quantify the production of biofilms by Modified Congo red agar (CRA) method in this study and Microtiter plate method.

Molecular detection

DNA extraction and estimation of the concentration and purity of extracted DNA:

Concentration of the DNA of the isolates were estimated by use the Nanodrop through putting 1µl of obtained DNA in instrument for the detection of the concentration in ng/µm and the purity has been obtained through the noticing of OD 260/280 ratio for checking DNA isolates contamination with the protein.

PCR Technique

Primer's preparation:

Primers were supplied by the manufacturer (Integrated DNA Technologies, USA) as lyophilized powder in Eppendorf tubes (1.5 ml)[13]. Then optimization of the primer, several trials of thermal cycles were done using Mastermix 20x to optimize the PCR reactions to obtain annealing temperature for the primers. For those annealing temperatures were gradient (53, 55, 57, 59, 61, 62) ℃ [14]. The sequences of primers as illustrated in Table (1).

Table (1): Primer's sequence of genes.

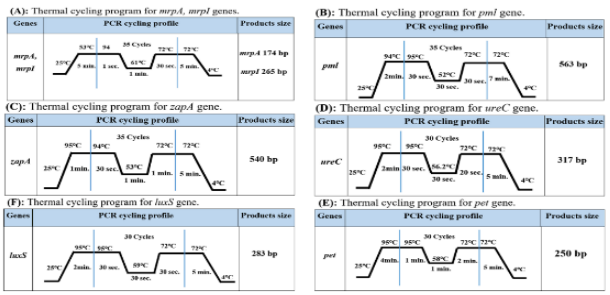
primer type	Sequence 5'- 3'	Size (bp)	Reference
mrpA	F: GGTGGCCGTAGTAATTCTCGT	174	This study
	R: ACCCGCATCGGTGATAACAA		
mrpI	F: GGCGTTTAAAAGGTGGGCTT	265	
	R: TCTCTGCCTAAATCCGCCAG		
pml	F: GGATCATCTATAATGAAACTG	563	[15]
	R: CTGATAATCAACTTGGAAGTT		
zapA	F: CCGCAGGAAAACATATAGCCC	540	[16]
	R: GCGACTATCTTCCGCATAATCA		
ureC	F: GTTATTCGTGATGGTATGGG	317	[17]
	R: ATAAAGGTGGTTACGCCAGA		
pet	F:TGACTCTGCATGGATTGAGC	250	[18]
	R: GACGCATCACTCAGTACAGT		
luxS	F: GTATGTCTGCACCTGCGGTA	283	[19]
	R:GCTGCCTTCCATGCATTAGC		

Programmable thermal controller

The mrpA, mrpI, zapA, luxS, pet, pml, and ureC genes was detected using the PCR cycle program settings in this experiment.

Thermocycling Conditions of PCR

Table (2): Thermal cycling program of mrpA I, zapA, luxS, pet, pml, and ureC genes.



Gel Electrophoresis and Documentation :PCR products and the DNA ladder were loaded in well of (1.5-2) % agarose gel with 1 ul Ethidium Bromide and the electrophoresis runs at (70 or 100) volts. Agarose was removed from tank visualized under U.V light to measure the DNA bands with DNA ladder according to [20].

Statistical Analysis: Statistical analysis of data was performed using SAS (Statistical Analysis System - version 9.1). Two-way ANOVA and Least significant differences (LSD) post hoc test were performed to assess significant differences among means, $P < 0.05$

is considered statistically significant [21].

3. Results and Discussion

The results of identification revealed the detection of 40 (17.5%) isolates belong to Proteus mirabilis out of 228 urine samples collected from patient suspected UTIs. The isolates were first determined by the swarming phenomenon on blood agar, the cultures, distinctive smell, and the pale appearance of bacteria (non-lactose fermenting) on MacConkey agar and convex, round, and smooth colonies with a distinct fishy smell [22]. Additionally, through microscopic analysis of the bacteria, which were gram-negative and looked as straight rods when stained with gram stain. The suspected Proteus mirabilis isolates were subjected to a number of conventional biochemical tests to determine their characteristics. All of the isolates tested positive for catalase, urease, citrate, and motility, but they all tested negative for indole and the oxidase test. These results coincide with mentioned [23–26]. Whole Proteus mirabilis isolates were examined for their resistance against 15 antibiotics belonging to different classes. As shown in Figure (1).

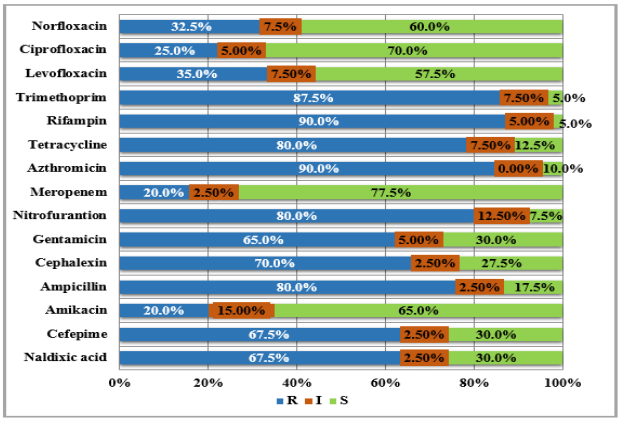


Figure (1): Frequency of antibiotics resistance profiles of Proteus mirabilis

Meropenem, Amikacin, Ciprofloxacin, Norfloxacin, and Levofloxacin had the lowest rates of resistance discovered in this study, this may be attributable to the poor usage of these antibiotics in Al-Kut hospitals. Meropenem was in the first place it was the most effective and sensitive drug against Proteus mirabilis isolates in the current study. The occurrence of resistance within Proteus mirabilis to several antibiotics is an emerging problem, extremely complex and made UTI management progressively more costly and challenging. Which limited the choices for selecting the appropriate drug for the treatment of Proteus mirabilis the high incidence of Proteus mirabilis infections, has been known widely for its contribution to the worldwide dissemination of multidrug resistance (MDR). In the present study demonstrated that all Proteus mirabilis isolates (100%) were MDR showing resistance to a minimum of three classes of the antibiotics tested.

Phenotypes of ESBLs: Forty Proteus mirabilis clinical isolates were investigated phenotypically (screen and confirmatory tests) for ESBL production. Resistance of Proteus mirabilis to third generation cephalosporins and monobactams were as the following: cefotaxime: 82.5%, ceftazidime: 72.5 %, ceftriaxone: 77.5%, and aztreonam: 50 %. By screening test 97.5% of Proteus mirabilis clinical isolates were ESBL producers, while 20.5% were ESBL producers by confirmatory test. The excessive and improper use of these drugs in the empirical treatment of UTIs is responsible for the rise in the prevalence of ESBL-producing Proteus mirabilis, which is also spread by a variety of variables, including geography, population density, hygiene, and antibiotic use.

Biofilm analysis in Proteus mirabilis :The results showed that 38 (95%) were black isolates of Proteus mirabilis strong producer biofilm and 2 (5%) were pink isolates of Proteus mirabilis non producers' biofilm, whereas observed that orange colonies in (24) hr. and became black after (48) hours.

In MTP method for detection of biofilm production, Considering Microtiter plate method as gold standard [27]. The results demonstrated that isolates of Proteus mirabilis biofilm production is 20(50%) non producer, 5(12.5%) isolates of Proteus mirabilis produce weak biofilm, 9(22.5%) isolate as moderate biofilm and 6 (15%) isolates strong biofilm.These results resemble to study reported by [28], who found the percentage of biofilm production by MTP method were strong biofilm producers 8 (16.6%), 17 (33.3%) were moderate and 33(46.06%) isolates were considered as non or weak biofilm producers. Results of biofilms producing Proteus mirabilis by MTP VS. MCRA is shown in Table (3).

Table (3): Distribution of Biofilms Producing Proteus mirabilis by MCRA vs MTP

	MTP		
MCRA	-	+	
-	2	0	2 (5.0%)
+	18	20	38 (95.0%)
	20 (50.0%)	20 (50.0%)	40
Weighted Kappa		0.100	
Sensitivity		100.00	
Specificity		10.00	

Test	Total No	No of infec d	McNemar test value (Paired proportions)	P-value
MTP	40	20(50%)	6.44	0.01
MCRA	40	38(95%)		

The McNemar test is used for paired proportions. Results revealed that the degree of agreement between two tests is a slight (0.10), therefore we can conclude that Congo red test is efficient for detect the positive cases (sensitivity 100%) but not efficient for detect of the negative cases (specificity 10%). Moreover, the McNemar test showed that the differences in the positive percentage between the two test was significant (P=0.01), so to increase specificity of biofilm forming, we must combine this method modified congo red agar and microtiter plate assay as a gold standard with the others.

Among biofilm producers, 20(50%) were MDR, and the non-biofilm producer, 20(50%) were MDR. The

development of biofilms and MDR Proteus mirabilis were not shown to be significantly correlated, and multidrug-resistant isolates did not exhibit a tendency to create more biofilm.

Molecular Analysis

The DNA extraction of forty isolates were extracted, purity and concentration were confirmed with Nanodrop. The purity of nucleic acid in the sample ranged between (1.8–2) while its concentration ranged from (50 – 360 mg/ul). purity DNA is a good indicator of the extraction process as well as confirming the absence of impurities that could impede the process [29].

Pathogenic microorganisms have developed several strategies for getting past the human defensive mechanism. Proteus mirabilis employs a variety of virulence factors to gain access to and colonize the host urinary system. Many bacterial species share a number of biological traits known as virulence factors. The detection of virulence genes and biofilm genes (ureC, mrpA, mrpI, pet, zapA, luxS, and pml) by Uniplex and multiplex PCR was performed using designed specific primers and genomic DNA from each Proteus mirabilis isolates. The ureC gene detected in all isolates and the high prevalence of ureC gene (100 %) among Proteus mirabilis isolates from Iraqi patients included in this study was in accordance with [17,24,30–32]. As shown in Figure (2).

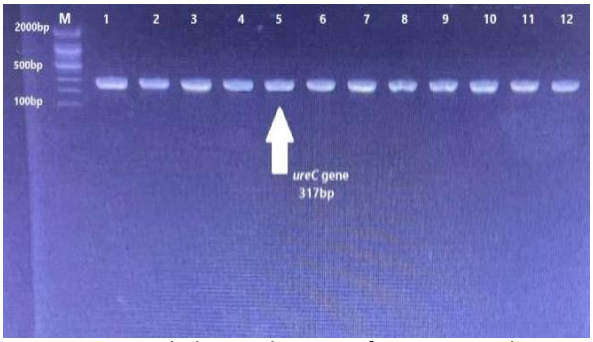


Figure (2): Gel electrophoresis of conventional PCR-amplified Proteus mirabilis ureC gene. Agarose 1.7%, 100 V/cm for 35 min, ethidium bromide dye staining, and UV transilluminator visualization. 2000-bp DNA ladder Lane (M) Amplicons of the ureC gene Lane (1–12)

According on the multiplex PCR approach, the incidence rate of the mrpA and mrpI genes associated to the virulence factors were found to be 97.5% and 95%, respectively. As shown in Figure (3). MrpA,I encode Mannose Resistance Proteus Like Fimbriae (MRL) Responsible for agglutination with human red blood cells in the presence of mannose sugar, which is important in bacterial adhesion to urinary tract epithelial cells lining [33].A study [15]. specified the incidence of the gene in 35% of the total isolates of Proteus mirabilis bacteria, while [34] indicated the presence of the gene in 100% of the total isolates, as shown by each of the study [35] and [36] reported the presence of the gene in 100% of the total isolates of Proteus mirabilis and showed that this gene is a trait carried on the chromosomal DNA of Proteus mirabilis bacteria.

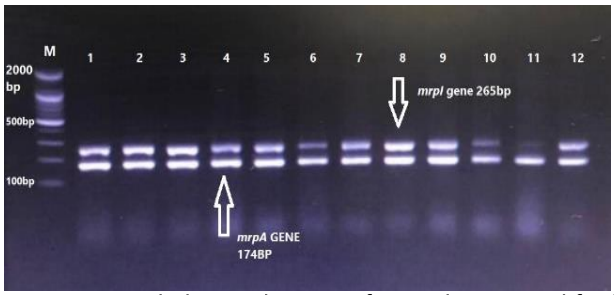


Figure (3): Gel electrophoresis of typical PCR-amplified Proteus mirabilis mrpA,I gene. Agarose 1.8%, 100 V/cm for 35 min, ethidium bromide dye staining, and UV transilluminator visualization. 2000 bp Lane (M) DNA stairway Lane (1–12): mrpA,I gene amplicons

The significant part that the potential poisons play in virulence has been described. These are *ZapA* metalloprotease and Proteus toxic agglutinin pta. By using PCR, the *pet* gene could not be found in *Proteus mirabilis* isolates shown in Figure (4), this finding is consistent with research by [37] referred to as the outer membrane autotransporter that facilitates cell aggregation and has a catalytic α-domain that has the ability to lyse kidney and bladder cells [38].

According to [39], the *Proteus mirabilis* negative *pta* gene displayed severe colonization defects in the spleen, kidneys, and urine in addition to decreased pathology. On the surface of bacteria, the autotransporter pta engages in serine protease activity [40]. The colonization of the kidney and bladder is aided by the *pta* protein. *Pta* has been shown to have additive effects in both in vitro and in vivo UTI trials, especially with regard to cystitis and perhaps interstitial nephritis [41].

Figure (4): Using traditional PCR, the 250 bp pet gene from Proteus mirabilis was amplified and then run on a gel. Agarose 1.6%, 100 V/cm for 45 min., ethidium bromide dye staining, and UV transilluminator visualization. 2000 bp Lane (M) DNA stairway lane (1–12): Pet gene amplicons.

Another type of potential toxins in this study, the zapA gene was found in 40 (100%) Proteus mirabilis isolates as illustrated in Figure (5). The outcome was consistent with the research done by [42] showed that all, 100% of investigate isolates contained this gene, Further more resemble to study in Al-Diwanyia city by [17] found that almost similar rate of gene prevalence in Proteus mirabilis strains isolated from patients with UTI. Also similar to study by [37] found percentage zapA gene 95% in Proteus mirabilis isolates.

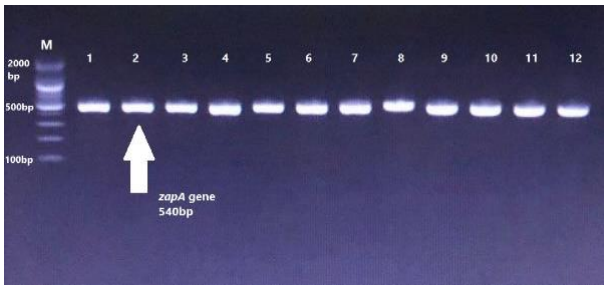


Figure (5): Using traditional PCR, the Proteus mirabilis zap A gene was amplified and run on gel electrophoresis. Agarose 1.5%, 100 V/cm for 35 min, ethidium bromide dye staining, and UV transilluminator visualization. Lane (1–12): Amplicons of the zap A gene Lane (M): 2000 bp DNA ladder

The luxS gene dissemination in forty isolates with (95%) as shown in Figure (6). The prevalence of the luxS gene in Proteus mirabilis isolates was shown to be 100% by the results of [37] and [43] when compared to those of other researchers. Also [44] found that luxS gene presence in (90%) Proteus mirabilis isolates.

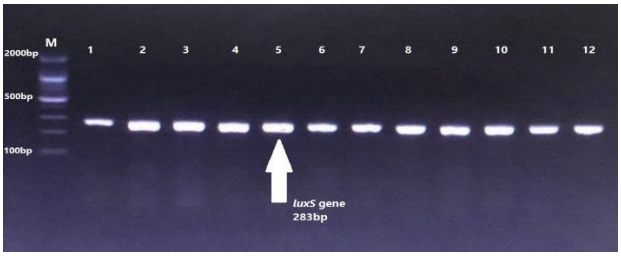


Figure (6): Using standard PCR, the luxS gene from Proteus mirabilis was amplified and run on gel electrophoresis. Agarose 1.7%, 100 V/cm for 35 min, ethidium bromide dye staining, and UV transilluminator visualization. 2000 bp Lane (M) DNA stairway Lane (1–12): LuxS gene amplicons.

In all Proteus mirabilis isolates, the pm1 gene was found, shown in Figure (7). The current analysis supported a recent report [37] that all isolates of Proteus mirabilis had the pml gene. Additionally, [34] found that 100% of the isolates of Proteus mirabilis could generate the pml gene. The results of the previous investigation were in disagreement with those of [15], which showed that 47% of the isolates of Proteus mirabilis bacterium had this gene. The previously reported prevalence rates most likely reflect variations in how virulence genes are distributed among various populations and geographical regions. The new study has certain limitations, including the fact that it only examined a small number of virulence genes.

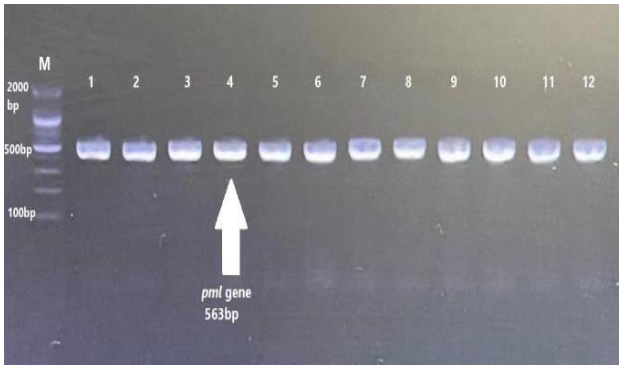


Figure (7): Using standard PCR, the pml gene from Proteus mirabilis was amplified and run on gel electrophoresis. Agarose 1.5%, 100 V/cm for 35 min, ethidium bromide dye staining, and UV transilluminator visualization. 2000 bp Lane (M) DNA stairway Lanes 1 through 12: pml gene amplicons.

The relationship between ESBL producer Proteus mirabilis isolates and virulence genes. As shown in Table (4).

Table (4): Association between ESBL producer Proteus mirabilis isolates and virulence genes

Virulence Gene	ESBL Producer (N=8) n%	Non-ESBL Producer (N=32) n%	P-Value
mrpA	8 (100.00)	31 (96.87)	0.613
mrpI	8 (100.00)	30 (93.75)	0.468
luxs	8 (100.00)	30 (93.75)	0.468
zapA	8 (100.00)	32 (100.00)	0.000
ureC	8 (100.00)	32 (100.00)	0.000
pml	8 (100.00)	32 (100.00)	0.000
N=Total number of isolated Proteus mirabilis.			

mrpA, mrpI and luxS showed non- significant with ESBL because the p-value > 0. 005.While zapA, ureC and pml showed significant because p-value < 0.005 Which indicates that there are complete statistically significant differences between the genes under study withe ESBL. among the isolated Proteus mirabilis, the association between virulence genes and biofilm development. According to Table (5).

Table (5): Expression of virulence genes is correlated with biofilm development in isolated Proteus mirabilis (N=40).

Virulence Gene	Biofilm Producer (N=20) n%	Biofilm non-Producer (N=20) n%	P-Value
mrpA	20 (100.00)	19 (96.87)	0.311
mrpI	20 (100.00)	18 (95.00)	0.147
luxS	20 (100.00)	18 (95.00)	0.147
zapA	20 (100.00)	20 (100.00)	0.000
ureC	20 (100.00)	20 (100.00)	0.000
pml	20 (100.00)	20 (100.00)	0.000
N=Total number of isolated Proteus mirabilis. n= Total number of virulence genes			

MrpA, mrpI and luxS revealed non- significant with Biofilm because the p-value > 0. 005.While, zapA, ureC and pml appeared significant because p-value < 0.005 Which indicates that there are complete statistically significant differences between the genes under study and Biofilm.

4. Conclusions

Proteus mirabilis was a significant pathogen responsible for a number of infections, particularly urinary tract infections (UTI) and all Proteus mirabilis isolates were multidrug resistance recovered from UTI. Also, to increase specificity of biofilm forming, we must combine this method modified congo red agar and microtiter plate assay as a gold standard with the others. No significant relationship was observed between biofilm formation with MDR Proteus mirabilis, indeed multidrug-resistant isolates

did not show a trend to being greater biofilm producers All Proteus mirabilis isolates characterized by high prevalence of virulence factors and Significant association found between ZapA, ureC and pml genes with ESBL producer Proteus mirabilis isolates ($p < 0.05$). Also, significant association biofilm formation and expression of virulence genes (zapA, ureC and pml genes) of Proteus mirabilis isolates ($p < 0.05$).

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