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Biofilm formation, Antibiotic resistance and Detection of mannose-resistant Proteus-like (MR/P) fimbriae genes in *Proteus mirabilis* isolated from UTI.

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Abstract: *P. mirabilis* is among the most frequently recovered etiologic agents from urinary tract infections. This pathogen can generate a biofilm as one of its important virulence factors and there is increasing evidence for the role of bacterial biofilm in antibiotics resistance. This study was aimed to determine the correlation between antibiotic resistance patterns and the biofilm formation capacity of clinical *P. mirabilis* isolates, also detection some (MR/P) fimbriae genes. The antibiotics susceptibilities of *P. mirabilis* strains isolated from different UTI by a disc-diffusion method were investigated. Biofilm formation was determined by a crystal violet binding assay. Uniplex and multiplex PCR were used for genes detection. Out of 529 urine samples, *P. mirabilis* was isolated from 85 (43.58%) samples. Of total 85 uropathogenic strains of *P. mirabilis*, 34 (40%) were strong biofilm producers, 21 (24.7%) were moderate biofilm producers, 18 (21.17%) were weak biofilm producers and 12 (14.11%) were biofilm non producers. The highest numbers of the isolates were susceptible to Amoxicillin followed by Piperacillin, Cephalexin, Ceftazidime, Cefotaxime and Cefixime. The most of isolates showed moderate resistance to Cefepime and Ceftriaxone. Also the highest numbers of the strains were susceptible to Aztreonam. The results of this study revealed the dominance of ureC gene among P. mirabilis isolates (88.23%). Also the results demonstrated the presence of fimbrial genes *mrp*A, *and mrp*H in 81.17 % and 72.94% of the isolates respectively. Present study concluded that there was higher resistance rate to antimicrobial agents among biofilm producing isolates of *P. mirabilis* than that in biofilm non producing strains, also we found a significant correlation between the presence of fimbrial genes (*mrp*A *and mrp*H) and biofilm formation.
Key words : *P. mirabilis,* biofilm, antibiotic resistance,(MR/P) fimbriae genes.

Introduction

Among the most common community-acquired and nosocomial infections are those of the urinary tract, *Proteus mirabilis* is the most frequent etiological agent, most frequently in patients with indwelling catheters or structural abnormalities of the urinary tract [1,2]. Colonization of the bladder and kidney obviously contributes to the ability of uropathogenes to establish infection in the urinary tract. Several observations support a role for fimbriae in colonization by *P. mirabilis* and the adherence to renal and uroepithelial cells by these bacteria have been shown to be mediated by fimbriae [3]. Mannose-resistant Proteus-like (MR/P) fimbriae are required to maximally colonize the bladder and kidneys and are important for hemagglutination, biofilm formation[4]. *P. mirabilis* adheres to living tissue or nonliving surfaces by forming a slimy layer known as a biofilm. The

biofilm protects these bacteria from the host defense system and from antibiotics; often leading to repeated UTI infection [5]. Many of previous studies revealed that the establishment of a biofilm enhances virulence and antibiotic resistance [6,7].

Urease is very important in *P. mirabilis* pathogenesis. This enzyme catalyzes the formation of kidney and bladder stones or to encrust or obstruct indwelling urinary. The urea-inducible urease gene cluster (ureRDABCEFG) encodes a multimeric nickel-metalloenzyme that hydrolysing urea to ammonia and carbon dioxide, thereby increasing the pH and facilitating the precipitation of polyvalent ions in urine (stone formation) [8].

The aim of this study was to quantitatively evaluate biofilm formation among *P. mirabilis* isolated from urinary tract infections from one of Baghdad hospitals. In addition, study the antibiotic susceptibility patterns and the presence of some virulence genes such as *ure*C, *mrp*A and *mrp*H genes in these isolates and the role of *mrp* genes biofilm formation.

Materials and methods

Collection of specimen

Totally 200 clinical samples (urine samples) were collected from inpatients which admitted at Al Kindy Teaching Hospital, Baghda, Iraq.

Isolation and identification of *P. mirabilis*

The collected urine samples were processed for isolation of clinical isolates by streaking on MacConkey agar (Himedia) and Blood agar. After the incubation period (37ºC for 24 hrs), Isolates were identified by standard biochemical methods [9], and identification was further confirmed by using a Vitek-2 system (bioMérieux).

Biofilm formation

The crystal violet binding assay was used to determine biofilm formation by *P. mirabilis* strains [10]. Bacterial cells were inoculated into brain–heart infusion broth medium and subsequently incubated at 37°C overnight. The overnight culture was diluted 1:100 and the wells of a microtiter plate were filled with diluted inoculum. Then, the plates were incubated for 48 h at 37°C. Following this, the wells were washed with distilled water, dried, and then stained with 1% crystal violet for 45 min at room temperature. Finally, after washing the wells again and waiting for them to dry, bound crystal violet in each well was solubilized by ethanol–acetic acid (90:10) solution and the crystal violet in solution from each well was determined using a spectrophotometer at 540 nm. The experiments were performed in triplicate. Strains having an optical density (OD) ≥0.1, were identified as biofilm producers and classified into 3 categories as follows:

$0.1 \leq OD < 0.4$ Weak Biofilm Former (WBF)
$0.4 \leq OD < 0.8$ Intermediate Biofilm Former (IBF)
$OD \geq 0.8$ Strong Biofilm Former (SBF).

Antimicrobial susceptibility testing

Disk diffusion susceptibility testing was performed on Mueller Hinton agar for the following antibiotics (Mast, UK) with their concentrations given in parenthesis: Aztreonam (30 mg), Ceftriaxone (30 mg), Cefotaxime (30 mg), Ceftazidime (30 mg), Amoxicillin (30 mg), Cephalexin (30mg), Cefixime (30 mg), Cefepime (30 mg), Piperacillin (100 mg). *Pseudomonas aeroginosa* ATCC 27853 were used as quality control organisms. The results were analyzed and interpreted according to NCCLS criteria [11].

DNA amplification by PCR

Genomic DNA of the isolates was used as a template to amplify the ureC, mrpA and mrpH genes. DNA extraction was conducted by Promega kit as the instructions of company. The primers used in this study were synthesized by Bioneer and are described in Table 1.

Table 1. Primers sequences and sizes of amplified fragments employed in the PCR reactions

Genes	Primer Sequence (5′-3′)	Product size (bp)	Reference
***ure*C F** ***ure*C R**	CCGGAACAGAAGTTGTCGCTGGA GGGCTCTCCTACCGACTTGATC	**533**	**12**
***mrp*A F** ***mrp*A R**	GAGCCATTCAATTAGGAATAATCCA AGCTCTGTACTTCCTTGTACAGA	**648**	**13**
***mrp*H F** ***mrp*H R**	CCTTGTTATGGTTGGCCTGT AGCCAGATGCGATAACCAAC	**444**	**13**

The cycle used for amplification ureC gene was heating at 94°C for 3 minutes, 30 cycles of the reaction, which consisted of denaturation at 94°C for one minute, annealing at 63°C for 30 seconds, and extension at 72°C for one minute. The third step was the final extension for seven minutes [12]. The cycle used for amplification mrpA (major subunit of MR/P fimbriae) was 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, with a final cycle at 72°C for 7 min [14]. The P. mirabilis isolates were screened for the presence of mrpH (pilin of MR/P fimbriae) by using the same conditions of mrpA gene.

Multiplex PCR was carried out by the simultaneous addition of primer pairs for *ure*C, *mrp*A and *mrp*H genes in the same reaction mixture. The cycle used for multiplex PCR was 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, with a final cycle at 72°C for 7 min.

All assays were done at least in duplicate. A negative control without the addition of DNA was included in the reactions. PCR products were electrophoresed in 0.8 % (w/v) agarose gels at 80 V and stained with ethidium bromide for 45 min.

Data analysis

SPSS version 16.0 statistical software package was used for statistical analysis. Chi-square test was applied. *P*-value < 0.05 was considered statistically significant.

Results

Out of 529 midstream urine samples 195 (36.86%) samples showed significant growth ($\geq 10^5$ cfu/ml). *P.mirabilis* was isolated from 85 (43.58 %) samples. Among 85 *P.mirabilis* isolates 34 (40%) were found to be strong biofilm producers, 21 (24.7%) were moderate biofilm producers, 18 (21.17%) were weak biofilm producers and 12 (14.11%) were biofilm non producers.

Antibiotic susceptibility pattern of the uropathogenic *P.mirabilis* isolates

Of total 85 *P.mirabilis* isolates, the highest numbers of the isolates were susceptible to Amoxicillin (91.6 – 100%) followed by Piperacillin, Cephalexin, Ceftazidime, Cefotaxime and Cefixime. The most of isolates showed moderate resistance to Cefepime and Ceftriaxone (25-67.6%). Similarly highest numbers of the strains were susceptible to Aztreonam (27.7- 33.3%) (Table 1).

Antibiotic resistance pattern of *P.mirabilis* among biofilm producers and biofilm non producers

The antibiotic resistance among biofilm producing *P.mirabilis* was found significantly higher than that of biofilm non producing *P.mirabilis* ($p < 0.05$). The correlation between biofilm production and antibiotic resistance was found statistically significant ($p < 0.05$) in most of the antibiotics (Piperacillin, Cephalexin, Ceftazidime, Cefotaxime, Cefixime, Cefepime and Ceftriaxone.) but the correlation was not found to be significant in case of Aztreonam and Amoxicillin (Table 2). Our results revealed that the antibiotic resistance of isolates, which unable to form biofilm, was lower in contrast with those strong producers especially with the antibiotics: Piperacillin, Cephalexin, Ceftazidime, and Ceftriaxone.

Table 2. Antibiotic resistance pattern of *P.mirabilis* among biofilm producers and non producers along with the antibiotic susceptibility patterns .

Antibiotic	Strong producers N = 34 No (%)	Moderate producers N = 21 No (%)	Weak producers N = 18 No (%)	Non producers N = 12 No (%)
Aztreonam	11 (32.4)	7 (33.3)	5 (27.7)	4 (33.3)
Amoxicillin	34 (100)	19 (90.5)	17 (94.4)	11 (91.6)
Piperacillin	30 (88.2)	17 (80.9)	14 (77.7)	7 (58.3)
Cephalexin	31 (91.1)	18 (85.7)	15 (83.3)	8 (66.6)
Cefepime	21 (61.8)	11 (52.4)	7 (38.8)	5 (41.6)
Ceftazidime	24 (70.6)	15 (71.4)	11 (61.1)	4 (33.3)
Ceftriaxone	23 (67.6)	12 (57.1)	12 (66.6)	3 (25.0)
Cefotaxime	22 (64.7)	18 (85.7)	11 (61.1)	7 (58.3)
Cefixime	25 (73.5)	15 (71.4)	10 (55.5)	6 (50.0)

In order to detect *u*reC, *mrp*A, *and mrp*H genes in *P.mirabilis* isolates, Uniplex and multiplex PCR was performed using designed specific primers and genomic DNA from each *P.mirabilis* isolate. *P.mirabilis u*reC, *mrp*A, *and mrp*H genes amplified by PCR exhibited the predicted sizes (533, 648 and 444 bp, respectively) .These results show in Figures (1), (2) and (3) respectively, and these results confirmed by the detection of the genes by multiplex PCR (Figure 4).

PCR amplification of urease and fimbrial genes indicated that the most isolates carried those genes. The results of this study revealed the dominance of *u*reC gene among *P.mirabilis* strains isolated from urinary tract infections, and prevalence of this gene in these isolates was 88.23%. Also the results demonstrated the presence of fimbrial genes *mrp*A, *and mrp*H in 81.17 % and 72.94% of the isolates respectively.

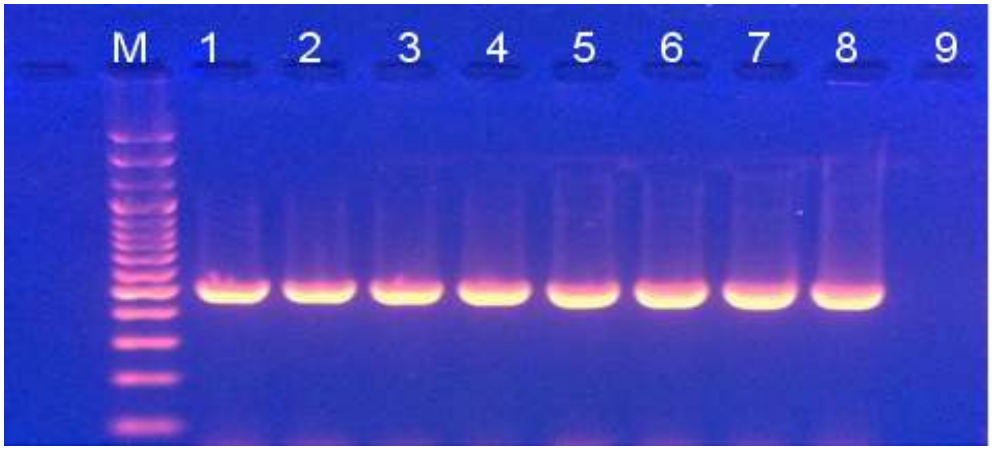

Figure 1. Electrophoresis of the amplified products of *ure*C (533bp) genes by a singleplex PCR in a 2% agarose gel. Lane 1-8; positive result of gene detection in *P. mirabilis* isolates. Lane 9, Negative control (PCR product without the DNA template). Lane M, 100 bp DNA ladder.

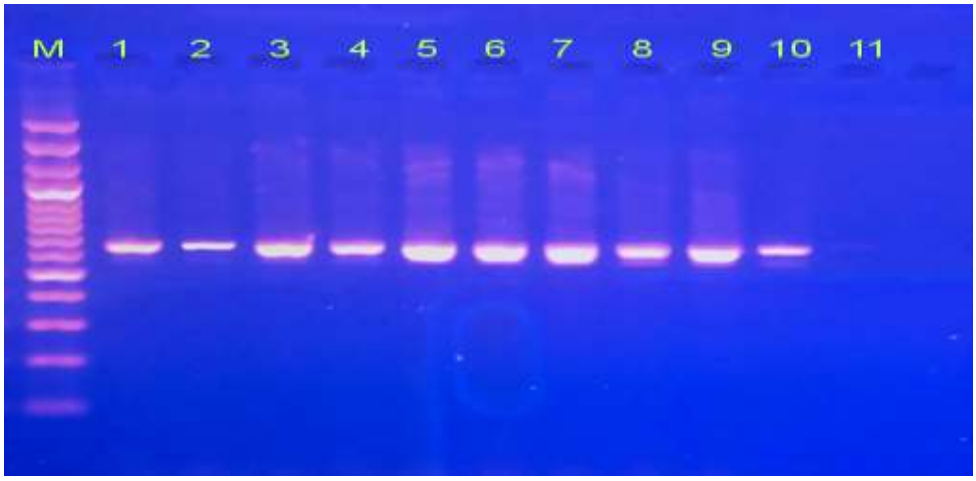

Figure 2. Electrophoresis of the amplified products of *mrp*A (648BP) genes by a singleplex PCR in a 2% agarose gel. Lane 1-10; positive result of gene detection in *P. mirabilis* isolates. Lane 11, Negative control (PCR product without the DNA template). Lane M, 100 bp DNA ladder.

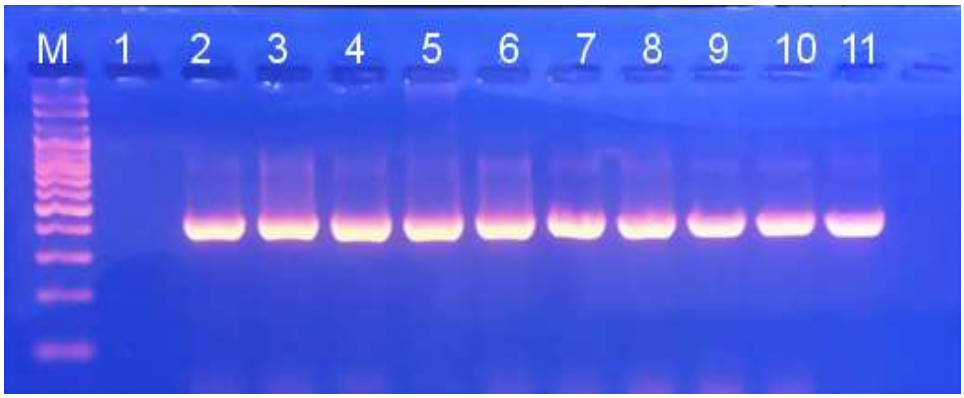

Figure 3. Electrophoresis of the amplified products of *mrp*H (444bp) genes by a singleplex PCR in a 2% agarose gel. Lane 2-11 ; positive result of gene detection in *P. mirabilis* isolates. Lane 1, Negative control (PCR product without the DNA template). Lane M, 100 bp DNA ladder.

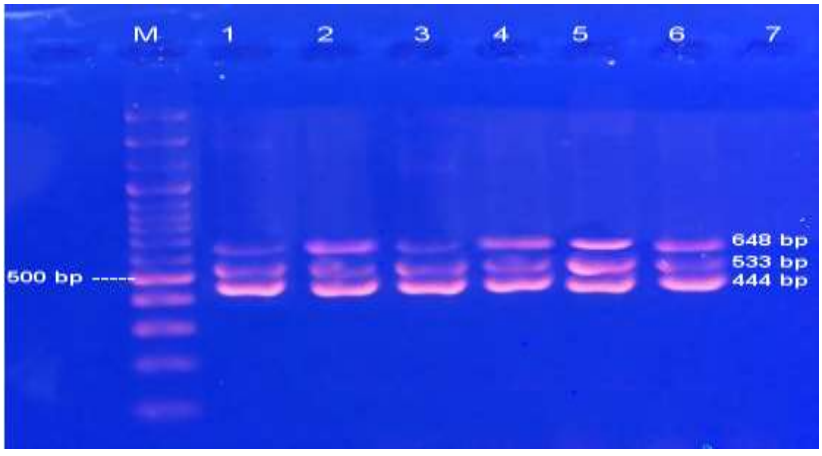

Figure 4. Electrophoretic banding patterns of the amplified products of three genes by multiplex PCR in a 2% agarose gel.. The amplified products of the genes : *ure*C (533bp), *mrp*A (648BP) and *mrp*H (444bp) were detected in six selected isolates of *P. mirabilis* (Lanes, 1-6). Lane 7, Negative control (PCR product without the DNA template). Lane M, 100 bp DNA ladder.

The correlation between these genes and biofilm formation by *P.mirabilis* was investigated in this study, hence the results indicated to significant correlation ($p < 0.05$) between the presence of fimbrial genes

(*mrp*A *and mrp*H) and bifilm formation, while there is no relationship between *u*reC gene and biofilm formation (Table 3).The gene *mrp*A was found in 100% of strong biofilm producers while the non producer isolates contained only 30.33%.

Table 3. Positive rates of genes in *P.mirabilis* according to biofilm production.

***P.mirabilis* isolates**	**number of strains**	**Rate of gene (%)**		
		***ure*C**	***mrp*A**	***mrp*H**
Strong producers	34	91.17	100.00	97.05
Moderate producers	21	85.71	90.47	80.95
Weak producers	18	88.88	66.66	50.55
Non producers	12	83.33	30.33	25.00

Discussion

Urinary tract infection caused by bacteria is one of the common causes for seeking medical attention in the community [14]. In hospitals, *P.mirabilis* is the second most frequently isolated Enterobacteriaceae species after *Escherichia coli* from UTI [15]. The spreading of *Proteus mirabilis* resulted from the presence of many virulence factors such as cilia that help it in adhesion, flagella that helps it to move, in addition to its outer membrane and capsule [16]. The characteristics of *P. mirabilis* strains pose a common problem in the management of infections such as poor penetration of antimicrobials, horizontal transfer of resistance genes via plasmids, biofilms are regarded as responsible for most recurrent and persistent nosocomial infections [17, 18].

Our results indicated that 40% of *P.mirabilis* isolates were strong biofilm producers. By forming biofilms and undergoing morphological changes, uropathogens can persist and cause recurrent urinary tract infections [19]. In urology being one of the main fields in which biofilm can become a serious problem. Biofilm can be found in the urothelium, prostate stones, and implanted foreign bodies [20]. An important adaptation of the bacteria *Proteus* spp., to cause infections in the urinary system, is the ability to form a biofilm. A biofilm is a formation of communicating microorganisms that adhere to certain surfaces and to neighboring cells and are covered with an extracellural matrix [21].

The antibiotic resistance pattern of *P.mirabilis* strains isolated from our hospitals revealed that the majority of the isolates were resistance to the most of penicillins and cephalosporins used in this study and this created problem in the treatment of UTI caused by these bacteria. Increasing resistance toward broad-spectrum cephalosporins in clinical isolates of *P. mirabilis* is reported [22]. UTIs are becoming increasingly difficult to treat owing to the widespread emergence of an array of antibiotic resistance mechanisms especially beta-lactamase production and efflux pumps [23]. Previous studies showed a correlation between multiple drug resistance and biofilm production in clinical isolates [24, 25]. Increased antibiotic resistance is thought to be related to biofilm formation. This increased resistance is related to gene transfer within biofilms [26]. Bleich *et al.* (2015) reported that although antibiotics are chemical tools for elicit biofilm formation, they also have potential to stimulate biofilm formation [27]. Bacterial forming biofilm are difficult to eradicate due to the antimicrobial resistant phenotype that this structure confers being combined therapy recommended for the treatment of biofilm-associated infections [28]. Moreover, finding a relationship between biofilm forming *P. mirabilis* strains and MDR, suggests that gene transfer mechanisms within the biofilm environments are likely [29]. Also it was reported that horizontal gene transfer of virulence and antibiotic resistance genes may be facilitated by biofilm formation[7]**.** One of the local studies revealed that important role of antibiotic resistance in biofilm formation of some strains isolated from UTI in Baghdad hospitals [30]. High prevalence of *ure*C (88.23%) in the present study was agree with the previous study of Ali and Yousif (2015) who reported that PCR product was visible of *ure*C gene was (96.66%) [31]. The gene *ure*C is responsible for producing urease enzyme, it works on changing PH of urine to basic leading to deposition the calcium and magnesium phosphate in the biofilm formed which in its turn leads to the formation of Crystallin biofilm which is the more complex type biofilms for it protect the bacteria from antibiotics causing failure to the treatment with antibiotics [32].

It was obvious from the results that Mannose-resistant Proteus-like (MR/P) fimbriae genes were found in strong biofilm producing isolates at maximum rates.

MR/P fimbriae are expressed by most *P. mirabilis* cells infecting the urinary tract, dictate the localization of bacteria in the bladder, and contribute to biofilm formation [33]. The study of Schaffer *et al*., 2016 identified two virulence factors in *P. mirabilis* required for extracellular cluster development and stone formation, urease, which is required for urolithiasis, and mannose resistant Proteus-like fimbriae [34].

Conclusion

The present study indicated to the relationship between biofilm forming *P. mirabilis* strains and antibiotic resistance, this may be due to gene transfer mechanisms within the biofilm environments. Also the biofilm formation was correlated with the presence of (MR/P) fimbriae genes.

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