Phenotypic and Molecular detection of Virulence factors in *Proteus mirabilis* isolated from different clinical sources

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Abstract

The objective of this work is to detection some the phenotypic and virulence genes characteristics of *P. mirabilis* isolates from human. During the period from Jun to August 2014. Nineteen *P. mirabilis* isolates were isolated from patients were suffering different infections and admitted to the Al-Diwaniya city hospitals. Isolates were identified by traditional biochemical tests, and then confirmed by Vitek- 2 system. Various virulence factors including Urease, protease, swarming activity, adhesive factors haemolysin, biofilm formation, and siderophores production were determined. These isolates of *P. mirabilis* consisted of 7 isolates (36.9%) obtained from children with diarrhea, 5 isolates (26.3%) obtained from wounds/pus, 5 isolates (26.3%) obtained from otitis media and 2 isolates (10.5%) obtained from burns. Results were showed that all isolates (100%) posses *UreC* gene, *ZapA* gene, *flaA* gene and *mrp* gene. So, these results show that all isolates (100%) have the ability to produce Urease, protease, haemolysin and Haemaglutination (colonization factor antigen-CFA), as well as swarming activity. Whereas 89.5% and 10.52% of the isolates gave positive results for biofilm formation and siderophores production respectively.

Introduction

Proteus spp. are widely distributed in nature. They can be found in polluted water, soil and manure, where they play an important role in decomposing organic matter of animal origin (56, 39, and 76). *Proteus* is a genus of Gram-negative bacteria; many of which cause infections in humans (43), and about 90% of these infections are caused by *P. mirabilis* (17) and is mostly found in people with compromised immune

system (19). It is an opportunistic pathogen that is able to cause severe invasive diseases (59) in critically ill and immunocompromised patients (38). It is an important source of hospital-acquired infections (22). *P. mirabilis* may, for instance, the main cause of urinary tract infections (50, 53, 59, 11), respiratory tract and wounds infections (62), burns and digestive tract infections (75), Ear infection and Otitis media (4). *P. mirabilis* has a wide array of cell associated factors and it can secreted many factors, some of which have been closely linked with disease producing potential called virulence factors (18, 63), such as swarming, fimbriae, urease, hemolysin, iron acquisition systems (29), protease and Lipopolysaccharides (LPS)(71). This pathogen have developed several pathogenic factors including which listed above which enable them to colonize, survive and grow in the host organism (18, 66). Furthermore, *P. mirabilis* is well known for its ability to form biofilms that colonize the airways, and thereby resists antibiotic treatment (40, 66). Recently, new genomes were sequenced and many studies of this bacterium will help in the discovery of new factors in the future (11). Therefore, this study is carried out to evaluating the current (phenotypic and genotypic) findings of some virulence factors of *P. mirabilis* isolated from different clinical sources.

Materials and Methods

Patients and Specimens

During the period from Jun to August 2014, total numbers of samples (clinical specimens) were collected from those patients who sent to Al-Diwaniya city hospitals and suffered from different infections. The collection process has been conducted according to (44).

Identification of Bacterial Isolates

The isolates were identified according to (23) by using traditional microscopic examination (Gram's stain), colony morphological features

on MacConkey agar and blood agar, and standard biochemical tests. All isolates were further confirmed as *P. mirabilis* by Vitek-2 identification system (BioMerieux, Marcy L'E toile, France). This system was used according to the manufacturer's instructions and (26).

Detection of the virulence factors of *P. mirabilis*

1-Hemolysin production

Hemolysin production can be detected using the blood agar media (20). The cultured blood agar plates were checked after 24 hours incubation at 37 ºC for the presence of hemolysis (α orβ) or no hemolysis (γ).

2-Urease production

The isolates were inoculated into Christensen medium and incubated at 37 °C for 24 hours.Then the diameter of the formed pink zone was assessed (72).

3-Colonization Factor Antigen (CFA)

A-Detection of CFA/I

The colonization factor antigen type I of the study isolates were determined according to (70) through RBC agglutination test. After culturing the organism on Tryptic soy agar and incubating it for 24 hours at 37C˚, the agglutination of RBC group A with bacteria occurs in presence of D-mannose.

B- Detection of CFA/II

This factor was determined according to (9), it is the same procedure as for determination of CFA/I except of using chicken blood rather than human blood.

C- Detection of CFA/III This factor was determined according to (70), it is the same procedure as for determination of CFA/I except of using tannic acid instead of D- mannose.

4-Biofilm formation

Culturing conditions corresponded to those described in (73); all isolates were cultivated in microtitre tissue culture plates and incubated at 37°C for 24 hours in brain heart infusion broth (BHI) and in BHI Supplied with 4% glucose (BHI-g). Wells of the microtitre plates were then washed three times and the biofilm layer was fixed by air-drying. The fixed biofilm layer was stained with crystal violet and the biofilm positivity was assessed quantitatively based on assessment of OD at 595nm. All isolates were examined in triplicate.

5-Extracellular protease

This method was carried out by using M9 media prepared according to (37). After sterilization in the autoclave and cooling to 50 ^{o}C, 0.25 mg/L glucose (sterilized by filtration) was added, and then, the media was supported by 1% gelatin. After the inoculation of this media with bacterial isolates and incubation for (24 - 48) hours at 37 ^{o}C, 3 ml of Trichloroacetic acid (5%) was added to precipitate the protein. The positive result was read by observing a transparent area around the colony (61).

6-Siderophores production

The bacterial isolates were examined for growth in the presence of 2, 2-dipyridin after incubates at 37^{o}C for 24 hour. Growth on M9 media in the presence of 2, 2-dipyridin indicates a positive result (69).

DNA extraction

Ten isolates only were used for the molecular detection depending on speed and strength of phenotypic results tests of many virulence factors. These isolates consisted of all clinical sources. Genomic DNA was extracted using the Sigma's GenElute™ Bacte-rial Genomic DNA Kit (Sigma-Aldrich, France), according to manu-facturer instructions. DNA

concentration was measured by biophotometer system (Eppendorf, Germany).

Polymerase chain reaction

Polymerase chain reaction was used to amplify the entire sequences of the genes studied in this research. The specific primers (Bioneer, Korea) used for the amplification of these genes were shown in table (1). Amplification reaction mixture was carried out in a 25 µl volume depending the manufacturer's instructions (Promega, USA) using 12.5 µl Master mix, 5 µl DNA template, 2.5µl of 10 pmol/µl of up stream primers, 2.5µl of 10 pmol/µl of down stream primers and 2.5µl PCR grad water. The PCR reactions involve three steps as shown in table (2).

Table (1): The primer sets used for PCR assay in this study

Type	Gene name		Oligo sequence (5'-3')	Product size (bp)	Reference
Virulence factors	*ZapA*	F	ACCGCAGGAAAACATATAGCCC	540	72
		R	GCGACTATCTTCCGCATAATCA		
	UreC	F	CCGGAACAGAAGTTGTCGCTGGA	533	74
		R	GGGCTCTCCTACCGACTTGATC		
	mrp	F	ACACCTGCCCATATGGAAGATACTGGTACA	550	79
		R	AAGTGATGAAGCTTAGTGATGGTGATGGTGAT GAGAGTAAGTCACC		
	flaA	F	AGGATAAATGGCCACATTG	417	6
		R	CGGCATTGTTAATCGCTTTT		

Table (2): Programs of PCR thermocycling conditions

Gene name	Temperature (˚C)/ Time					Cycle number
	Initial denaturation	Cycling condition			Final extension	
		denaturation	annealing	extension		
ZapA	95/1 min	94/30 sec	53/1 min	72/1 min	72/5 min	35
UreC	94/3 min	94/1 min	63/30 sec	72/1 min	72/7 min	30
mrp	94/3 min	94/1 min	40/1 min	72/1 min	72/5 min	30
flaA	95/3 min	95/30 sec	54.2/30 sec	72/30 sec	72/5 min	30

Agarose gel electrophoresis

The products were separated in 1.5% agarose gel in TBE buffer (pH 8), stained with ethidium bromide, and photographed in ultraviolet light (13). The electrophoresis result noticed by using gel documentation system.

Results & Discussion

Isolation and identification

19 isolates were identified as *P. mirabilis* consisted of 7 isolates (36.9%) obtained from children with diarrhea, 5 isolates (26.3%) obtained from wounds/pus, 5 isolates (26.3%) obtained from otitis media and 2 isolates (10.5%) obtained from burns (Table-3).

Table (3): Distribution of *P. mirabilis* among various clinical sources

Type of samples	No of samples (%)
Stool	7 (36.9)
Wounds/Pus	5(26.3)
Otitis media	5(26.3)
burns	2(10.5)
Total	**19 (100)**

The results of this study are identical with those obtained by (32) who have been isolated *proteus* species from different clinical sources (except urine) at (30%) from stool, (30%) from otitis media where as (20%) and (10%) from burns and wounds respectively. In addition, (21) and (77) also have been isolated *proteus* spp and *P. mirabilis* respectively, from wounds and pus/abscess respectively at the rate of (21.1%, 20.1%) respectively.

These isolates were diagnosed as *P. mirabilis* depending on morphological and cultural properties on blood agar, MacConkey agar and nutrient agar as well as conventional biochemical tests as mentioned in table (4) and confirmed by the Vitek-2 identification system.

Table (4): Morphological and biochemical tests of *P. mirabilis* isolated from different clinical sources

Test	Gram-negative bacilli	Indole	Methyl red	Vogas-Proskaur	Citrate utilization	Motility	Urease	TSI (K/A+ G)	H_2S production	Oxidase	Catalase
Result	+	-	+	-	-	+ swarming migration	+	+	+	-	+

TSI, triple sugar iron; A, acid; G, gas; K, alkaline

Virulence factors of *P. mirabilis*

The phenotypic and genotypic characters were tested for all *P. mirabilis* isolates in this study in order for detection of the virulence factors. The universal primers were used for screening the presence of (*UreC*, *ZapA* and *mrp*) genes, but local primer were examined of *flaA* gene. The expected size fragments those genes were 533 bp, 540 bp, 550 bp and 417 bp respectively.

The results of the PCR for the isolates of *P. mirabilis* showed that all isolates were positive for the presence of *UreC* gene (Figure-1 and Table-5).

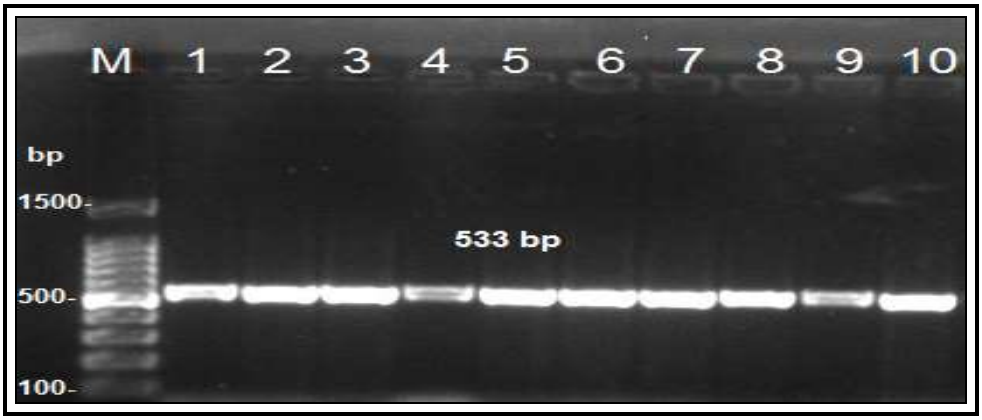

Figure (1): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *P. mirabilis* isolates and amplified using *UreC* gene primers. Lane (M), DNA molecular size marker (100-1500 bp ladder). Lanes 1-10 the isolates showed positive results with *UreC* gene.

As they all were phenotypically positive (100%) for extra cellular urease. The results are in agreement with the results of (72, 21, 5, 7, 8, 12, 1, 3, and 6), who found that (100%) of *P. mirabilis* isolates showed strong production of urease.

Table (5): Phenotypic (n=19) and genotypic (n=10) detection of virulence factors to the *P. mirabilis*

virulence factors	Phenotypic No. (%)	Virulence genes	
		Gene name	No. (%)
Urease	19(100)	*Ure C*	10(100)
Extracellular protease	19(100)	*Zap A*	10(100)
Swarming- Flagellin	19(100)	*fla A*	10(100)
Haemoglutination (CFA)	19(100)	*mrp*	10(100)
Haemolysin	17(89.5)	----	
Biofilm	17(89.5)	----	
Siderophores	2(10.52)	----	

Urease is one of the most important factors in *P. mirabilis* pathogenesis. *In vitro* (on basic urea agar), urease hydrolyzing urea to alkaline ammonia and carbon dioxide, thereby increasing the pH and will be changing the color of phenol red indicator to pink (25). But *In vivo* (human body) this enzyme catalyzes the formation of kidney and bladder stones or to encrust or obstruct indwelling urinary (18). The urea-inducible urease gene cluster (ureRDABCEFG) encodes a multimeric nickel-metalloenzyme that hydrolyzing urea to alkaline ammonia and carbon dioxide, thereby increasing the pH and facilitating the precipitation of polyvalent ions in urine and this meaning stone formation. This pH alteration is important during *P. mirabilis* catheter colonization, facilitating the bacterial adherence and formation of biofilm incrustation (51, 18). Urease activity increased in the swarmer cells and is necessary for a *P. mirabilis* infection. While swarmer cells do up-regulate the production of urease, swarming is not needed to form stones or crystalline biofilms (34). Lastly

stone formation is considered a hallmark of *P. mirabilis* infection, providing a number of advantages for these bacteria such as: Protection from the host immune system, the host immune system protection, blockage of the ureters, ammonia toxicity to host cells and direct tissue damage. These facts can lead to formation a protective and nutrient-rich environmental niche for these microorganisms (11).

The ability of *P. mirabilis* to produce extracellular protease in M9 media (supported by 20% glucose and 1% gelatin) was investigated and it was found that all of the study isolates (100%) were able to produce extracellular protease after 24 hours of incubation which indicated by the presence of a clear hallo of transparent area around the colony after the addition of 3 ml of Trichloroacetic acid (5%). Also, the presence of *ZapA* gene has been tested in the same ten isolates which gave positive results in all 10 (100%) isolates (Figure-2 and table- 5). These results were similar to the results obtained by (18, 72, 7, 1, and 6), who referred to that (100%) of *P. mirabilis* produced protease, but these results were different from those results obtained by (21, 8, and 5) who reported the positivity of *P. mirabilis* isolates as (0 %, 41.9 %, 45.8%) respectively. Metalloproteases are a major group of proteolytic enzymes in *Proteus* isolates (14). The protease produced by *P. mirabilis* is a metalloprotease of the serralysin family of zinc proteases, encoded by *zapA* gene (78). These proteases which have the capacity to degrade host proteins releasing amino acid as nutrients and may degrade proteins such as IgA which are involved in host defence and may also be involved in host tissue damage (36). *P. mirabilis* that lost IgA protease activity , has normal urease production activity, hemolytic activity, swarmer cell differentiation, production of flagella, swarming motility, and biofilm formation (60**)**.

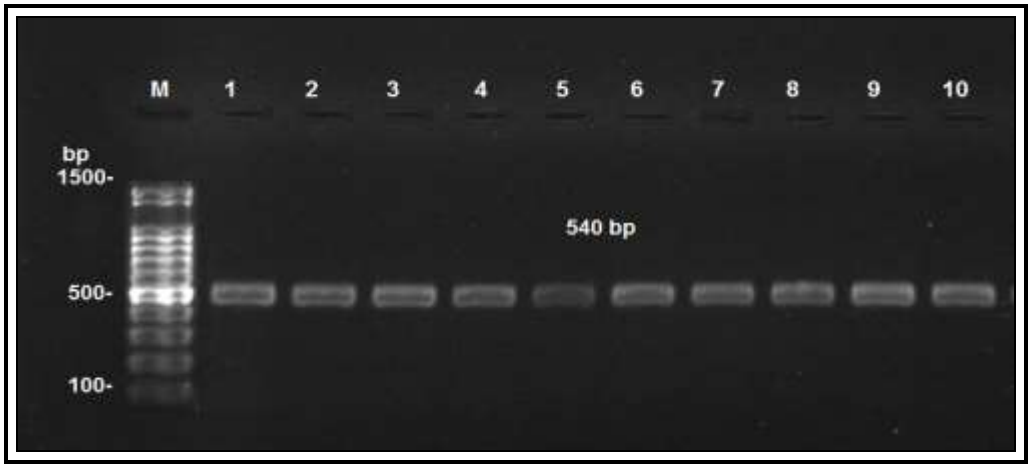

Figure (2): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *P. mirabilis* isolates and amplified using *ZapA* gene primers. Lane (M), DNA molecular size marker (100-1500 bp ladder). Lanes 1-10 the isolates showed positive results with *ZapA* gene.

The molecular method was used to detect *flaA* gene. The results of this study revealed that all isolates (100%) possessed *flaA* gene (Figure 3 and Table 5). This finding is in agreement with other researches (48) and (6) that conducted in Iraq. They reported the presence of *flaA* gene in *Proteus* isolates (100%, 86.66%) respectively. While (12) found that (100%) of *P. mirabilis* isolates were carrying *FliL* gene which encodes for the flagellar basal body protein.

The biosynthesis of flagella is a key process in both motility and swarming and involves numerous genes on the *proteus* chromosome (55). The filamentous portion of the *P. mirabilis* flagellum is mainly composed of the flagellin protein FlaA, encoded by *flaA* (46).There are three copies of flagellin-determinant gene (*flaA*, *flaB*, *flaC*) that reside on the *P. mirabilis* genome with only one copy that is actively expressed (55). One of the most surprising findings was that all flagellum-related genes are located together within a single locus, which is highly unusual (58). Another interesting feature is the copy of multiple genes encoding flagellin, *flaA* and *flaB*, which are located in direct proximity to each other (52). Normally, the *flaA* allele is expressed while *flaB* is silent.

However, these genes can recombine, resulting in the formation of antigenically distinct flagella (45). Considering that flagellin is strongly antigenic, it has been postulated that this recombination could contribute to immune evasion during infection (16).

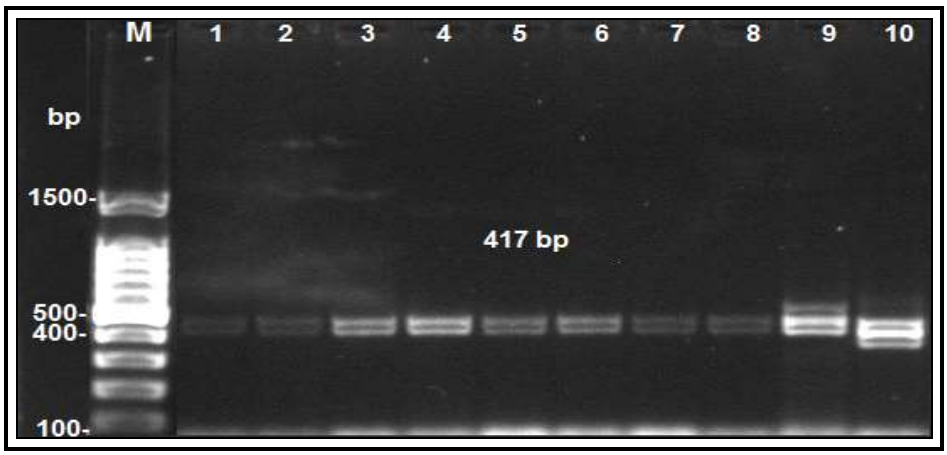

Figure (3): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *P. mirabilis* isolates and amplified using *flaA* gene primers. Lane (M), DNA molecular size marker (100-1500 bp ladder). Lanes 1-10 the isolates showed positive results with *flaA* gene.

Fimbriae are bacterial surface appendages used for adherence. Till now, only 5 fimbriae have been studied: mannose-resistant/*Proteus*-like (MR/P) fimbriae, *P. mirabilis* fimbriae (PMF), uroepithelial cell adhesion (UCA) (NAF), ambient-temperature fimbriae (ATF), and *P. mirabilis* P-like pili (PMP) (65, 52), and mannose-resistant/*Klebsiella*-like (MR/K) fimbriae (18, 80). The recent sequencing of the *P. mirabilis* genome revealed that there are 17 different fimbrial operons, spanning 5 different classes of fimbriae (58). Phenotypically, the results showed that all isolates (100%) agglutinate human red blood cells type A in the presence of mannose or tannic acid which indicates that all *P. mirabilis* isolates have CFA I and CFA III. Also the results of the present study show that all tested isolates (100%) have the colonization factor antigen II by using chicken blood (Table-5). In the previous studies a collection of *P. mirabilis* isolates was assessed for MR/P and MR/K (HA) activity, and 100% of isolates displayed both types of activity (54, 2). This finding is

in agreement with other studies such as (32 and 48) who found that (100%) *Proteus* isolates were carrying colonization factor antigen (CFA) type (I, II, III) and (I, III) respectively. Also, they were in agreement with results of (71), who observed that every tested isolate of *P. mirabilis* was able to agglutinate fresh human erythrocyte and this reaction was not inhibited by D-mannose. While, (47) found that (73/116) (62.93%) of *P. mirabilis* isolates gave positive result of CFA and which divided into 8 (MR/P), 14(MR/K) and 51 (MR/P+ MR/K). Results amplification of region of the *mrpA* gene showed that all isolates (100%) were carrying this gene (Figure-4 and Table-5).

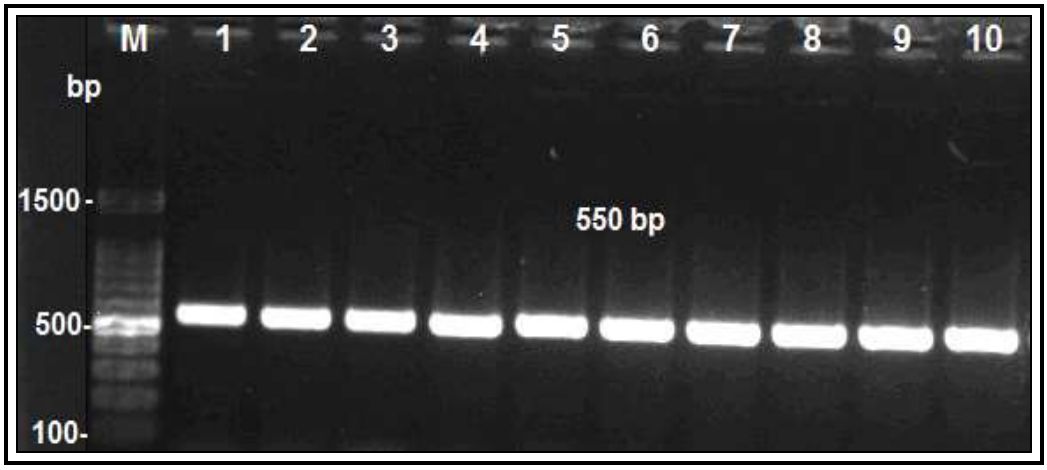

Figure (4): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *P. mirabilis* isolates and amplified using *mrp* gene primers. Lane (M), DNA molecular size marker (100-1500 bp ladder). Lanes 1-10 the isolates showed positive results with *mrp* gene.

These results were similar to the results obtained by (64, 12) and (48) who referred to that (100%) of *P. mirabilis* and *P. vulgaris* respectively, carrying this gene. Likewise, these results were similar to that results reported by (5) who found (95.8%) *P. mirabilis* isolates gave positive result to CFA in the presence of D-mannose. MR/P fimbriae are encoded by *mrp* operon containing 10 genes located on bacterial chromosome. The fimbrial genes are transcribed and MR/P fimbriae are synthesized when the promoter is in on orientation (42, 53). In the off orientation the

production of fimbriae are stopped. Expression of these fimbriae is increased under oxygen limitation (41). Initial studies revealed that sera from infected mice reacted strongly to MR/P fimbrial preparations, which indicates that these fimbriae are expressed *in vivo* (52). The expression of MR/P fimbriae appears to be highly induced during infection (42).

P. mirabilis isolates were checked to confirm their ability to produce extracellular hemolycin on blood agar and it was found (89.5%) of isolates have the ability to produce extracellular hemolycin.

These results in agreement with the results of (47, 3, and 6) who demonstrated that (87.07%, 88.89% and 89.8%) respectively, of *P. mirabilis* isolates exhibit hemolycin on blood agar plates, but (71, 7, 32, and 48) who found that all *proteus* isolates produce hemolycin on blood agar plates. Other studies (5 and 8) revealed that (66.7%, 47.7%) respectively, of *Proteus* isolates exhibit hemolycin on blood agar plates.

The function of hemolysin is to form pores in target host cells (15). It has been proposed that hemolytic activity helps *P. mirabilis* spreading into the kidneys during infection (18).This is probably mediated through the increased ability of hemolytic *P. mirabilis* cells to invade host tissue (49). Hemolysin is not as critical for infection as urease, however, similar to urease and IgA protease, it is overexpressed in swarmer cells (57).

For biofilm formation (89.5%) of *P. mirabilis* isolates demonstrated the ability to biofilm formation. The results of this study were in agreement with the results of (5) and (3) who found that (91.7% and 88.88%) respectively, of *P. mirabilis* isolates showed ability of biofilm formation, but (64) were observed the biofilm formation in all *P. mirabilis* isolates, while other isolates of *P. mirabilis* showed a moderate ability (65.1%) of biofilm formation (8). There are many environmental factors which may affect the on slime layer production such as: O_2, temperature and others

which may be give various results (28). One of the most important pathogens in this regard is *P. mirabilis* (31).

In this study, all *P. mirabilis* isolates (100%) exhibited swarming motility on agar plates. These results are in agreement with the results obtained by (7, 1, 3 and 6) who found that all *P. mirabilis* isolates (100%) exhibit swarming activity.

P. mirabilis is a flagellar peritrichious bacterium. This microorganism has the ability of swarming motility. This phenomenon occurs on 1.5% of agar surface and describes flagellum-dependent movement across the surface, resulting in a characteristic bull's eyes pattern (27, 10). The contribution of swarming motility to virulence of *P. mirabilis* remains a topic of great debate in the field (33). Interestingly, it appears that the expression of at least some virulence genes seems to be higher in swarmer cells than vegetative cells (24). However, *in vivo*, it appears that swarmer cells are in the minority (33). It has been well documented that *P. mirabilis* can swarm across the surface of urinary catheters (67, 35).

Finaly, Siderophore production by *P. mirabilis* was (10.52%). This result was in agreement with (3) who showed ability to production siderophore by *P. mirabilis* was (11.11%).

Members of the family *Enterobacteriaceae*, to which *Proteus* belongs, typically produce siderophores of the enterobactin, aerobactin, and/or yersiniabactin type (68). However, *P. mirabilis* produce two types of siderophores; protobactin and yersiniabactin (30). The genome sequencing of *P. mirabilis* HI4320 revealed the presence of a large number of proteins which may play a role in iron acquisition (58).

Conclusion

The study documented that all *P. mirabilis* isolated from different clinical specimens have the ability *to* possess more than one virulence

factors (phenotypic and genotypic) such as Urease, extracellular protease, adherence factors, swarming activity, hemolysin and haemagglutination.

التحري المظهري والجزيئي لعوامل ضراوة المتقلبات *Proteus mirabilis* المعزولة من مصادر سريرية مختلفة

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الخلاصة

هدفت الدراسة إلى التحري المظهري والجزيئي لبعض عوامل الضراوة في بكتريا المتقلبات *Proteus mirabilis* المعزولة من الإنسان. خلال الفترة من حزيران إلى أب 2014 عزلت ١٩عزلة من *P.mirabilis* من مستشفيات محافظة الديوانية. شخصت العزلات اعتمادا على الفحوصات البايوكيمياويه التقليدية وأكدت باستخدام تقنية الفايتك Vitek -2. درست بعض عوامل الضراوة لهذه البكتريا منها إنتاج الإنزيم الحال لليوريا, والإنزيم الحال لكريات الدم الحمراء, والإنزيم الحال للبروتين, وتكوين الغشاء الحيوي Biofilm , إنتاج حاملات الحديد siderophores, فعالية الحركة الزاحفة swarming و عوامل الالتصاق للعزلات أعلاه المعزولة من حالات التهابية مختلفة هي ٧ عزلات (36.9%) من خروج أطفال مصابين بالإسهال, ٥ عزلات (26.3%) من الأذن الوسطى, ٥ عزلات (26.3%) من الجروح/ قيح وعزلتان (10.5%) من الحروق. أظهرت النتائج إن جميع العزلات (100%) كانت تملك جينات الإنزيم الحال لليوريا *UreC* , الإنزيم الحال للبروتين*ZapA* , و الفلاجلين *flaA* وعوامل الالتصاق *mrp*. كما وأظهرت النتائج إن جميع العزلات (100%) كانت منتجة للإنزيم الحال لليوريا, والإنزيم الحال لكريات الدم الحمراء, والإنزيم الحال للبروتين والتلازن الدموي(CFA), بالإضافة إلى قدرتها على النمو الزاحف. فيما أعطت (89.5% , 10.52%) على التوالي من العزلات نتيجة موجبة لاختباري تكوين الغشاء الحيوي وإنتاج حاملات الحديد.

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