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Correlation between virulence factor and biofilm formation in *Proteus* spp.

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Abstract

Urinary Tract Infection is an infection that caused by the members of the genus *Proteus* that depends mainly on the availability of virulence factors ;Various virulence factors including biofilm, swarming migration , polysaccharide ,heamolysin,protease, DNase, urease production weredetermined for 45*Proteus* isolates that obtained from clinical specimens of Urinry Tract Infection patient . The distribution of virulence factors was showed variation among the testedisolates and strain specific in most cases. All *Proteus* isolates showed 45 (100%)biofilm , polysaccharide andSwarming capabilities with different extents. High ureaseproduction was demonstrated in most isolates 40 (88.8%);In addition, they were abling to produce protease,DNase and heamolysin, in 30(66.6%) , 28 (62.2%) and26(57.7%) respectively.

The result of susceptibility test for *Proteus spp*. against seven antibiotics commonly used to treat UTI infection caused by *Proteus spp*. ; *P.mirabilis* were 90% sensitive for Meropenem , 80% to Ciprofloxacin , 60% for both Cephalothin , Nitrofurantion , 50% , 40% and 30% for Amikicin, gentamicin and ceftriaxone respectively ,but*P.vulgaris* isolates were sensitive to 80% for Meropenem ,70% Ciprofloxacin and Nitrofurantion and sensitive to 60%,50% ,40 % and 30 % for Cephalothin, gentamicinAmikacinand ceftriaxone respectively.(This studyaimed to detect and evaluate the presence of some virulence factors in *Proteus* species isolates causing Urinry Tract Infection .

Keywords:-Biofilm, Swarming, DNase.

العلاقة بين عوامل الضراوه وتكوين الغشاء الحياتي في جنس *Proteus spp.*

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الخلاصة

التهاب المسالك البولية هو التهاب يصيب جزء من المسالك البولية والناجمة عن الاصابه بجنس*Proteus spp.*حيث يعتمد بشكل رئيسي على توافرعواملالفوعة . حددت عوامل امراضية مختلفة والمتضمنة الغشاء الحياتي والانتشار السطحيوانتاج السكريات المتعدد وقابليه انتاج انزيم اليوريز والدينيز والبروتيز والهيمولايسين ل 45 عزلة تم الحصول علية من مرضى التهاب المسالك البولية. لقد اظهرت النتائج تباينا في توزيع عوامل الفوعه بين العزلات المختبرة وهي خاصيه متعلقة بالسلاله في معظم الاحيان. اظهرت معظم العزلات45 (100%) قابليات مختلفه على انتاج الغشاء الحياتي والسكريات المتعدده والانتشار السطحي، كانت العزلات قادره على انتاج انزيم اليوريزبنسبة عالية (88.8) 40 عزلة وبالاضافه الى قدرتها على انتاج انزيم البروتيزوالدنايز والهيمولايسين بنسب (66.6%)30 ، (62.2%) 28و (57.7%)26.

اظهرت نتائج اختبار الحساسيه لعزلات *Proteus .spp* ضد سبع مضادات حياتيه شائعه الاستعمال لمعالجه الاصابه بالتهاب المجاري البوليه الناتجه من *Proteus .spp* حيثاظهرت العزلات العائده ل *Proteus mirabilis* بانها حساسه للMeropenem وCiprofloxacinبنسبه 90%و80% والCephalothin وNitrofurantion بنسبه 60% وللAmikicin بنسبه 50%لل gentamicin وceftriaxone بنسبه 40% و30% على التوالي اما بالنسبه لعزلات *Proteus vulgaris فانها كانت حساسه بنسبه 80%* Meropenem و70 % لكل من Ciprofloxacin وNitrofurantion وولل Amikacinو Cephalothin وgentamicin وceftriaxone بنسبه 60%،50%،40% و30% على التوالي .(الهدف من الدراسة هوكشف وتقييم وجود عوامل الفوعة في عزلات محلية تابعة الى جنس*Proteus spp.* تسبب الاصابه في المسالك البوليه).

Introduction

*Proteus spp.*is a common causative agent of human urinary tract infection (U.T.I.) and a major cause of nosocomial infection [1].These bacteria also cause wound infection, pneumonia and septicaemia [2]. Several pathogenic factors -associated with *Proteus spp.* including swarming (3), urease [4], haemolysin [5]., protease [6] , DNases [7] and polysaccharide [8].

Biofilms are aggregates ofmicroorganisms, which are formed due tothe attachment of cells to each otherand/or to a host surface in an aqueous environment [9].Bacterial adhesion to surfaces is one ofthe initial steps that lead to biofilmformation [10].Biofilms are a matter of concern tomany medical industries, since bacteria can colonize medical devices, altering their properties. Furthermore, it may represent an important source of contamination, releasing pathogenic bacteria. Biofilms are more resistant to antimicrobial agents, impairing the control of this form of microbial organization, when compared to free cells [10]. The crystalline biofilms formed by *Proteus mirabilis* can seriously complicate the care of patients undergoing bladder catheterization. They are capable of generating ammonia from urea by urease and elevating the pH of the urine and biofilm. The generation of alkaline causes calcium and magnesium phosphates to precipitate from urine and accumulate in the catheter biofilm, blocking the flow of urine from the bladder. Aggregates of crystals and bacteria form in the urine and these also become incorporated into the developing crystalline biofilm [11].Deu to biofilm important in bacterial pathogenecity for human ,thebehavior of antibiotic susceptibility was detected against highest biofilm production among *Proteus spp.*

Materials and methods

Bacterial isolate: *Proteus spp.* Isolates were isolated from a patientwith U.T.I. attended to different Hospitals in baghdad,as, Medical City, Hospital Ibn al-Nafis and Al-Kindi hospital and identified by morphological characteristic and biochemical tests beside to re-conformed by API 20 E system.

Swarming motility assays:Overnight culture (5ul) was inoculated centrally onto the surface of the dried swarming plates containing nutrient broth ,agar 1.5% and incubated overnight at 37C◦[12].

Blood agar plate was used to detection of hemolysin production, skim milk agar was used to detection protease production and Urea agar was used to detection of urease production [13]. Congo red agar wasused to detection polysaccharide production [14].DNase agar was used to detection DNase production [15].

Quantitative Assays of BiofilmFormation

Twenty micoliter of *P. mirabilis* overnightculture was used to inoculate microtiterwells containing 180 µl of brain heartinfusion broth supported with 2% glucose andcovered sealed withparafilmthen incubated at 37°C fordeferent periods. Cultures were removed and the wells were rinsed with (PBS phosphate buffer solution) pH 7.2. to determine the optical density at 630nm (OD630), and drying at roomtemperature for 15 min, then 200 µl of crystalviolet (1%) was added to the wells for 20 min. The stained biofilms were rinsedthree times with PBS pH 7.2, and allowed todry at room temperature for 15 min, thenextracted twice with 200 µl of 95% ethanol. The OD 630 was estimated usingautomatic microtiter plates reader [16]

Antimicrobial susceptibility test

This test was performed by modified Kirby-Bauer method[17] Susceptibility test were determined for allisolates to 7 different of (Meropenem , Ciprofloxacin , Cephalothin's , Nitrofurantion ,Amikicin, gentamicin and ceftriaxone) antibiotics by disc diffusion method recommended by National Committee for Clinical Laboratory Standards (NCCLs) (2011)[18].

Results and disscustion

Out of 185 clinical specimens urine collected from patients attended to different hospitals in Baghdad ,45 isolatesthat represent 24.3% were successfully diagnosed as *Proteus spp.*, 30(67%)isolatesbelong to*P.mirabilis* and 15 (33***%)*** isolates belong to *P.vulgaris* . The investigation for7 virulence factors showed that not all theisolates had the same number of the factors.All isolates appeared (100%) positive for biofilm , swarming and slime layer production,m Fortyisolates (88.8%) were urease producer , 30(66.6%) of the isolates showed the abilityto produce protease .DNase production was appearedin 28 (62.2%) of isolates, also the *Proteus spp.* were showedlysis of human RBCs by haemolysin production with26(57.7%) isolates, (table-1).

The virulence score of each of 45 isolates were also estimated. Nine isolate (20%) only have all factors, 21 (46%) isolates produced six factors ,while 12(26.6%) isolates produced five factors , twoisolates (4.4%) produced four factors and only one isolate (2.2%) produce three factors ,(table -2)

Table 1-virulence factors of *Proteus spp.*

Proteus spp.	Slime layer	Biofilm	Swarming	Urease	Protease	Dnase	Heamolysin
pm1	++++	0.693*	90.00	++	++	++	++
pm2	+++	0.235	77.00	++	++	++	++
Pm3	++++	0.573*	78.00	++	++	++	++
Pm4	+++	0.424	89.00	++	++	_	++
pm5	++++	0.573*	84.00	++	_	++	++
Pm6	++++	0.830*	79.00	++	_	_	_
Pm7	+++	0.437	69.00	++	++	++	_
pm8	++++	1.13*	86.00	++	++	++	_
Pm9	++++	0.351	76.00	++	_	++	++
pm10	++++	0.656*	73.00	++	++	_	_
Pm11	+++	0.214	85.00	++	_	_	++
pm12	++++	0.703*	69.00	++	++	++	++
Pm13	+++	0.513	74.00	++	++	_	++
1m14	++++	0.610*	71.00	++	++	++	++
Pm15	+++	0.325	90.00	++	_	_	++
Pm16	+++	0.505	87.00	++	++	++	_
Pm17	++++	0.397	78.00	++	_	++	++
Pm18	++++	0.459	69.00	_	++	++	_
Pm19	+++	0.432	82.00	++	++	_	++
Pm20	++++	0.450	77.00	++	++	++	_
Pm21	+++	0.513	67.00	++	++	_	++
Pm22	+++	0.291	88.00	++	_	_	_
Pm23	+++	0.501	77.00	++	++	_	++
pm24	++++	0.863*	90.00	++	++	++	_
pm25	++++	0.653*	83.00	++	++	++	_
Pm26	+++	0.531	66.00	++	++	_	_
Pm27	++++	0.472	86.00	++	_	++	++

Pm28	+++	0.459	68.00	++	__	++	++
Pm29	++++	0.354	72.00	++	++	++	__
Pm30	+++	0.355	85.00	++	++	__	++
pv1	++++	0.523*	71.00	++	++	++	++
Pv2	+++	0.327	87.00	__	__	__	__
pv3	++++	0.770*	89.00	++	__	++	__
pv4	++++	0.553*	68.00	++	++	__	__
Pv5	++++	0.348	77.00	++	++	++	++
pv6	++++	0.526*	90.00	++	__	__	++
pv7	++++	0.830*	77.00	++	__	__	++
pv8	++++	0.786*	75.00	++	__	++	__
pv9	++++	0.916*	81.00	++	++	++	__
Pv10	++++	0.448	89.00	++	++	++	++
Pv11	++++	0.8438*	84.00	++	++	++	++
Pv12	+++	0.510	80.00	__	__	++	++
Pv13	++++	0.432	90.00	++	++	__	__
Pv14	++++	0.550*	73.00	++	++	++	__
Pv15	++++	0.352	78.00	__	++	++	+

*(best biofilm producing strain) ++++(very good slime layer producing strain) +++(good slime layer producing strain) ++(positive producing strain) – (neativenon producing strain)

Table 2- Virulence factors score of *Proteus* isolates.

No.of the virulenceFactors	Number ofisolates	%	Cumulative percentage
3	1	2.2	2.2
4	2	4.4	6.6
5	12	26.6	33.2
6	21	46.6	79.8
7	9	20.2	100
Total	45	100	

Pathogenic bacteria have developed numerous means ofadapting to their host environment [19].Quantification of the main *P. mirabilis* virulence factors would increaseour understanding of their role in the infectionand colonization of the human urinary tract [20]. As the present study aimed to investigate the isolation rate of *Proteus* species from UTI patients and to explore their relevant virulence factors. Results found that 24.3% of the urine culture were positive for *Proteus* species. Actually, these results were almost dissimilar to that reported by another worker such as [21, 22). Our result disagree with result of [23], who found

that 100% *Proteus spp.*wereurease producer , 91 % biofilm producer and 48.3% protease producer ,and our results were lower than result reported by other workers [24,25] found that 64% of *P. mirabilis* isolated from UTIs protease enzyme producer, and in another study he reported that 94% of *P. mitabilis,* 71% of *P.vulgaris* [26] In a final conclusion, *Proteus spp.* appeared as one of the main causative agent of urinary tract infection .Most of these isolates had multiple virulence factors that increase their infectivity and worsen the clinical picture of the disease and may interfere with the efficiency of antimicrobial therapy.

Our results showed *P.mirabilis* isolates were sensitive to meropenem with90% ,80% forciprofloxacin , 60% for both nitrofurantionandcephalothin ,sensitive to 50% ,40% ,30%. foramikacin , gentamicin and ceftriaxone respectively .While,*P .vulgaris* isolates were sensitive to meropenem with80% ,70% for both ciprofloxacin,nitrofurantion and sensitive with60%,50%,40 % and 30% for cephalothin ,gentamicin, amikacinand ceftriaxone respectively(fig 1 and 2)Our result showed variation in antibiogram with other auther report such as [27] ,theyfound that *P.mirabilis*were resistance to amikacin , to ciprofloxacin and gentamicin . Also ,[28] and [29] they reported that *P.mirabilis* were Resistance to gentamicin and sensitive for ciprofloxacin, While [30] results were shown 100% *P. ,vulgaris* resistant tocephalothin and 93.3% fornitrofurantion and 96.6%of isolate were sensitive for ciprofloxacin and 80% for gentamicin and 65.7% for amikacin ;Howerer , [31], they are demonstrated that *P.vulgaris*were 100% sensitive to ciprofloxacin, and 85% were sensitive to gentamycin . The antibiotic susceptibility tests demonstrated that *Proteus spp.* have a wide range of

resistance to several antibiotics. This could be a result of the extra outer cytoplasmic membrane which contains a lipid bilayer, lipoproteins, polysaccharides and lipopolysaccharides. And the abuse and misuse of antibiotics could be part of the contributing factors of resistance to antibiotics (31).The ciprofloxacin was a bacterial antibiotic that interferes with nucleic acid synthesis by inhibiting the gyrase enzyme, this antibiotic has several binding sites on the enzyme . This decreases the likelihood of resistance (32; 33), therefore, it appeared to be drug of choice for urinary tract infection. The routine use of antibiotics in both medical and veterinary medicine has resulted in wide spread antibiotic resistance and development of antibiotic resistance genes especially within the gram negative organisms (33) .

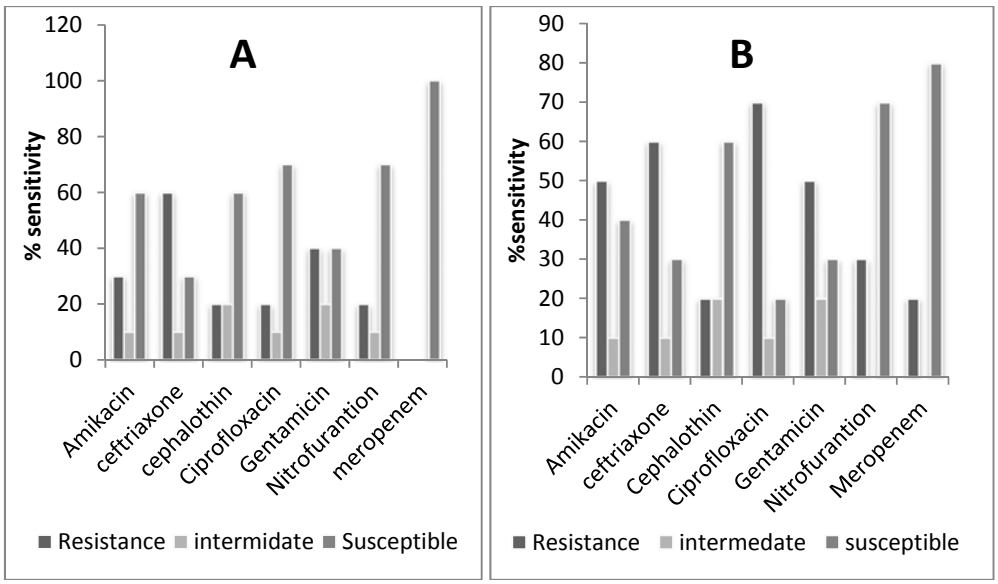

Figure 1- Antibiotics susceptibility pattern for *Proteus spp.*,A :*P. mirabilis* ,B:*P.vulgaris*

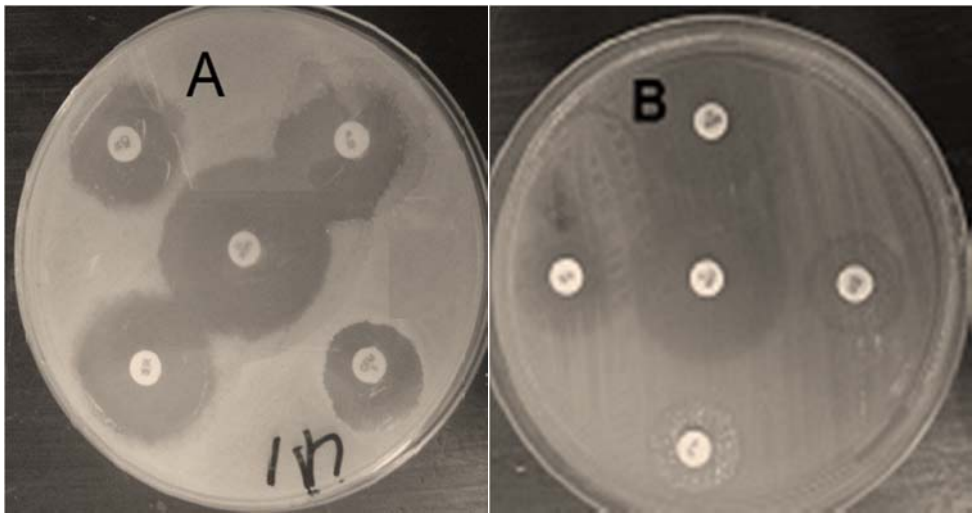

Figure 2-Antibiotic susceptibility test of *Proteus spp,* A :*P. mirabilis* B:*P.vulgaris*

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