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Detection of Virulence Factors of Local Isolates of *Proteus mirabilis*

التحري عن عوامل ضراوة بكتريا *Proteus mirabilis* المعزولة محليا

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Abstract

A total of eighty five samples were collected from different clinical and food samples includes twenty samples of urine, forty five samples from wounds, and twenty samples of chicken and meat. From the overall samples a total of ١٤٦ isolates were obtained, and among the monly fifteen isolates were belong to *P. mirabilis* according to the results of identification by studying their morphological and cultural characteristics and biochemical tests. Results of identification were confirmed by using Api٢٠E system. Pathogenicity of local isolates of *P. mirabilis* was examined by detecting several virulence factors includes invasion and adhesion ability and protease production. Results showed that the adhesion of these isolates to uroepithelial was ranged between ٣٠-٥٥ bacterial cell/uroepithelial cell, and number of bacterial cells invade uroepithelial cells was ranged between ١-٢٠ bacterial cell/uroepithelial cell. Furthermore all of the isolates of *P.mirabilis* were able to produce protease as another virulence factor forpathogenicity. the diameter of zone of hydrolysis on skim milk agar was ranged between ٥-١٤ mm for different isolates. Specific activity of protease produced in culture filtrates of these isolates was ranged between ١٤,٥ and ٠,١٢١ U/mg protein. The most virulent isolate was *P. mirabilis* P١٥ isolated from urine of urinary tract infection because of its high ability to produce these virulence factors.

Keywords**:** *Proteus mirabilis*, virulence, adhesion, invasion, protease.

Introduction

Proteus species, members of the family Enterobacteriaceae are motile Gram negative enteric bacteria. They are important pathogens of the urinary tract and are the primary infectious agent in patients with indwelling urinary catheters. Individuals suffering from urinary tract infections caused by *Proteus mirabilis* often develop bacteriuria, cystitis, kidney and bladder stones, catheter obstruction due to stone encrustation, acute pyelonephritis, and fever (١). Several potential virulence factors of *Proteus* had been studied in relation to its virulence and pathogenicity, including hydrolysis of urea by urease, cell invasiveness, cytotoxicity induced by hemolysins, and adherence to the uroepithelium mediated by fimbriae. There were many proposed mechanisms and influencing factors for the invasive properties of *P.mirabilis*. Microbial invasion could be facilitated by virulence factors, microbial adherence, and resistance to antimicrobials. Virulence factors assisted pathogens in invasion and resistance of host defenses. Bacterial proteins with enzymatic activity (e.g. protease, elastase, and collagenase) facilitated local tissue spread. Microbial adherence to surfaces helps microorganisms establish a base to penetrate tissues. The adhesive properties in the Enterobacteriaceae were generally mediated by different types of pili(٢).

Haemolysin, which is cytotoxic for cultured urinary tract epithelial cells, had been shown to be correlated with the ability of bacteria to invade cells. The abilityof *P.mirabilis* to express virulence factors, including urease and haemolysin, and to invade humanurothelial cells, is coordinately regulated with swarming differentiation (٣). According to these findings, this study was aimed to isolate *P.mirabilis* from different clinical and food samples and studying there virulence factors affects their pathogenicity.

Methods

Collection of Samples

Clinical samples (urine specimens and swabs samples from wounds) were collected from patients in Al-Yarmok hospital and food samples (chicken and meat samples) collected from local markets in Baghdad city.

Isolation of *P. mirabilis*

For the isolation of *P. mirabilis* from urine samples, loop full of undiluted urine was taken and spread on blood agar and MaConkey agar plates, while swabs taken from wounds were streaked on blood agar medium and McConkey agar medium, then plates were incubated at ٣٧°C for ١٨ hrs. On the other hand, ١ g of each meat sample was transferred to containers of ٩٩ ml of nutrient broth and incubated at ٣٧°C for overnight under aerobic conditions. Then ١ ml of broth of each sample was transferred to test tubes containing ١٠ ml of Tetrathionate broth and ١ ml of the same sample to ١٠ ml of Rappaport-Vassiliadis RV broth and incubated at ٣٥°C for overnight. After incubation aloop full of sample was taken and spread on Xylose Lysine deoxycholate XLD agar, blood agar and MaConkey agar plates and incubated at ٣٧°C for overnight under aerobic conditions. After incubation, suspected colonies were selected and subjected to identification according to their morphological and cultural characteristics and biochemical tests (٤).

Identification of *P. mirabilis*

Morphological and cultural characteristics (size, shape, edge, color, and margin) of the bacterial coloniesand biochemical tests were examined as described by Atlas*et al*. (٥). *P. mirabilis* isolates were further identified according tobiochemical reaction by using Api ٢٠E system (٦).

Detection of Virulence Factors

Adherence and Invasion assay

Adherence and Invasion assayof local isolates of *P. mirabilis* was achieved according to Iwahi*et al*. (٧)

Production of Protease

Semi Quantitative Screening for detection of protease production by local isolates of *P. mirabilis* was achieved according to Sneath*et al*. (٨) by measuring the diameter of hydrolysis zones around colony of each bacterial isolate. While quantitative screening was achieved according to Manachini*et al*. (٩) by measuring the release of trichloroacetic acid TCA soluble peptides from ١٪ (w/v) casein solution. Enzyme activity and specific activity was calculated as follows:

$$\text{Enzyme activity (U/ml)} = \frac{\text{absorbance at ٢٨٠ nm}}{\text{k(٠.٠١)}\times\text{time(٣٠min.)}\times\text{enzyme vol.(٠.٢ ml)}}$$

$$\text{Specific activity (U/mg)} = \frac{\text{activity (U / ml)}}{\text{protein concentration (mg / ml)}}$$

Protein concentration

Protein concentration in culture filtrates was determined according to the method described by Bradford, (١٠)

Results and Discussion

Isolation of *P. mirabilis*

In order to isolate *P. mirabilis*, different samples (clinical and food samples) were collected from different locations in Baghdad city during the period from ١١-٢٠١٢ to ٢-٢٠١٣. Results indicated in Table (١) show that ٦٥ of clinical samples (includes urine samples from patients with urinary tract infection and swabs from wounds) and ٢٠ of food samples (chicken and meet) were obtained.

Table (١)
Bacterial isolates obtained from different sources.

Source of sample	*No. of samples*	*No. of isolates*	*No. of Proteus spp. isolates*
Urine	٢٠	٥	١
wounds	٤٥	٩٥	١٢
Chicken and meat	٢٠	٤٦	١٣
Total	٨٥	١٤٦	٢٦

From all of these samples, a total of ١٤٦ different isolates were obtained, these isolates were cultured on blood and MacConky agar medium as a differential medium to differentiate *Proteusspp*. from clinical samples, and on blood, MacConky agar and XLD agar medium for chicken and meat samples. Among the total isolates, ٢٦ of them were suspected to be belonging to *Proteus* spp. according to their morphological and cultural characteristics as they were able to grow on MacConky agar medium for ٢٤ hrs. at ٣٥^{o}C, and appeared as pale colonies with swarming motility on blood agar medium. Under light compound microscope they were appeared Gram negative, rods, and non-spore formers.

Identification of *P. mirabilis*

Several biochemical tests were achieved to identify the isolates of *Proteus* spp. Results mentioned in Table (٢) show that ١٥ of these isolates were positive for catalase, urease, phenylalanine - deaminase, motile and Kligleriron agar, and gave negative results for utilizing Simoncitrate, oxidase and indole tests. These results were agreed with those obtained by Stankawska*et al*. (١١).

Table (٢)
Biochemical tests for identification of Proteus mirabilis isolates.

Isolate / *Symbol Test*		*P١*	*P٢*	*P٣*	*P٤*	*P٥*	*P٦*	*P٧*	*P٨*	*P٩*	*P١٠*	*P١١*	*P١٢*	*P١٣*	*P١٤*	*P١٥*
Oxidase		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenylalanine - deaminase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Simon citrate		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kligler iron agar	**Slat/ Butt**	**K/ A**	**K/ A**	**K/ A**	**K/ A**	**K/ A**	**K/ A**	**K/ A**	**K/ A**	**K/ A**	**K/ A**	**K/ A**	**K/ A**	**K/ A**	**K/ A**	**K/ A**
	H٢S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	CO٢	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(-): Negative results, (+): positive results, K/A: alkaline/acid

These ١٥ isolates were subjected to identification by using Api ٢٠E system. Results of Api ٢٠E system confirmed the above results.

Detection of the virulence Factors Produced by *P. mirabilis* Isolates

Virulence factors produced by local isolates of *P. mirabilis* were investigated. These virulence factors include adhesion and invasion and protease production in different growth media.

Adherence and Invasion

Ability of *Proteus mirabilis* local isolates to adhere to uroepithelial cells is considered as an important virulence factor in pathogenesis of urinary tract infections (١٢). In this study, the adherence of bacterial cells of*P. mirabilis* isolates to uroepithelial cells was detected under oil-immersion lens of light compound microscope as shown in Fig.(١). This figure showed bacterial cells infectsuroepithelial cell that appears as rods. On the other hand, results indicated in Table (٣) show that the highest number of adhering bacteria to UEP was ranged from ٣٠-٥٥ bacterial cell / uroepithelial cell. These results were in agreement with Perez-Serrano *et al.*(١٣) who found that the adherence of *P.mirabilis* to UEPCs ranged between ٣٠-٥٥ bacterial cell/ uroepithelial cell. In other study on the adhesion of *P. mirabilis* to uroepithelial cell Al-kabby, (١٤) found that the adhesion range of *P. mirabilis* to uroepithelial cells was ٣١,١.

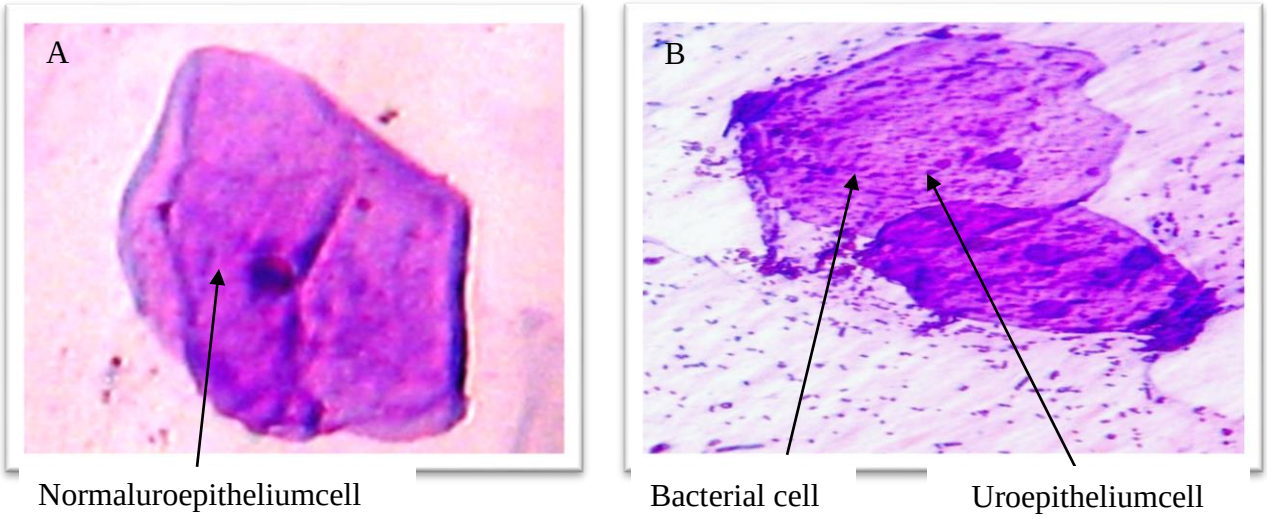

Fig. (١) Adherence of Proteus mirabiliscellsto uroepithelium (١٠٠٠X).
(A) Normal uroepithelium cell.
(B) Uroepithelium cell after adherence of Proteus mirabilis.

Table (٣)
Adhesion ability of P. mirabilis touroepithelial cells.

Isolate	*Adhesion (bacterial cell/uroepithelial cell)*
***P. mirabilis* P١**	٣٠
***P. mirabilis* P٢**	٣٣
***P. mirabilis* P٣**	٣٣
***P. mirabilis* P٤**	٣٥
***P. mirabilis* P٥**	٣٠
***P. mirabilis* P٦**	٣٥
***P. mirabilis* P٧**	٣٢
***P. mirabilis* P٨**	٣٨
***P. mirabilis* P٩**	٤٢
***P. mirabilis* P١٠**	٣٢
***P. mirabilis* P١١**	٤٠
***P. mirabilis* P١٢**	٤٥

P. mirabilis P١٣	٣٥
P. mirabilis P١٤	٣٨
P. mirabilis P١٥	٥٥

The difference in adhesion capabilities to uroepithelial cells may be related to the virulence variation between bacterial strains. Gram-negative uropathogensproduce an assortment of adhesins including those attached to the tip of hair-like projections, known as fimbriae or pili, as well as adhesins anchored directly within bacterial cell membranes, known as nonfimbrial adhesions (١٥).

A gentamicin resistances assay was used as a measure to determine the host invasion mediated by the local isolates of *P. mirabilis*. Results indicated in Table (٤) show the number of bacterial cells not susceptible to gentamicin and gain entry to the uroepithelial cell was ranged between ١ and ٢٠ bacterial cell/ uroepithelial cell. These results revealed that different isolates of *P. mirabilis* exhibits high invasion ability to epithelial cell and survive intracellular. These results were in agreement with those mentioned by Jacobsen *et al.* (١٦) who found that the invasion, cytotoxicity and biofilmformation of *P. mirabilis* and *Escherichia coli*have been observed *in vitro* and *in vivo* to be internalized by bladder and renal epithelial cells. Adhesin-mediated invasion of uroepithelial cells is dependent upon the presence of the decay accelerating factor receptor on host cells and may contribute to persistence within the upper urinary tract.

Table (٤)

Ability of P. mirabilislocalisolate to invade uroepithelial cells.

Isolate	*Invasion (bacterial cell/ uroepithelialcell)*
P. mirabilis P١	—
P. mirabilis P٢	١
P. mirabilis P٣	١٤
P. mirabilis P٤	٣
P. mirabilis P٥	—
P. mirabilis P٦	٥
P. mirabilis P٧	—
P. mirabilis P٨	٤
P. mirabilis P٩	١٦
P. mirabilis P١٠	١١
P. mirabilis P١١	١٢
P. mirabilis P١٢	١٦
P. mirabilis P١٣	٨
P. mirabilis P١٤	—
P. mirabilis P١٥	٢٠

Ability of *P. mirabilis* isolates for protease production

Two methods for screening the ability of the localisolates of *P. mirabilis* for protease production as another factor for virulence and pathogenicity:

a. Semi- quantitative screening

Semi-quantitative screenings for protease production by the local isolates of *P. mirabilis* was achieved by detection the formation of zone of hydrolysis around each colony when grown on skim milk agar medium. Results mentioned in Fig. (٣) and Table (٥) show that these isolates were able to hydrolyze skim milk agar medium around each colony and forming halo of hydrolysis with variable degrees.

Results mentioned in Table (٥) also show that the diameter of zone of hydrolysis was ranged between ٥-١٤ mm for different isolates, among them the isolates *P. mirabilis* P١٥ (isolated from urine) was the most efficient in protease production because it gives the highest diameter of hydrolysis (١٤ mm) on skim milk agar. It was well known that protease is a proteolytic enzyme responsible for hydrolyzing proteins by attacking peptide bounds in the basic structure of proteins, hence protease produced by bacterial isolates attack casein (the constituent of skim milk protein) in the culture medium and forming a halo of hydrolysis around each colony (١٧).

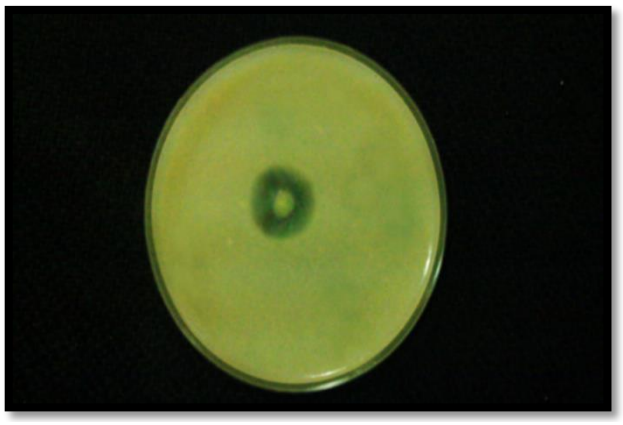

Fig.(٣) Protolytic activity of protease produced by P. mirabilis on skim milk agar after incubation at ٣٥℃ for ٢٤h.

Table (٥)
Diameter of clear zones around colonies of P. mirabilis local isolates grown on skim milk agar medium after incubation for ٢٤ hrs at ٣٥℃.

Isolate	*Diameter (mm)*
***P. mirabilis* P١**	٥
***P. mirabilis* P٢**	٦
***P. mirabilis* P٣**	٥
***P. mirabilis* P٤**	٦
***P. mirabilis* P٥**	٧
***P. mirabilis* P٦**	٦
***P. mirabilis* P٧**	٥
***P. mirabilis* P٨**	٥
***P. mirabilis* P٩**	٨
***P. mirabilis* P١٠**	٧
***P. mirabilis* P١١**	٧
***P. mirabilis* P١٢**	٨
***P. mirabilis* P١٣**	٥
***P. mirabilis* P١٤**	٧
***P. mirabilis* P١٥**	١٤

b. Quantitative screening for protease production by local isolates of *P. mirabilis* Local isolates of *P. mirabilis* were screened quantitatively to examine their ability in protease production. This was achieved by Manachini *et al*. (٨). Results indicated in Table (٦) showed that all the isolates were protease producers with variable degrees. Specific activity of protease in culture filtrates was ranged between ٠,١٢١ and ١٤,٥ U/mg protein. Among them, *P. mirabilis* P١٥ was the most efficient in protease production because the specific activity of protease in crude filtrate of this isolate was ١٤,٥ U/mg protein. The differences in the ability of the isolates to produce protease are due to genetic variations of the genes responsible for the production of protease (١٨).

Table (٦)
Specific activity of protease produced byP. mirabilis isolates.

Isolate	*Specific activity (U/mg)*
***P. mirabilis* P١**	٠,١٢١
***P. mirabilis* P٢**	٢,١١
***P. mirabilis* P٣**	٥,٠٣
***P. mirabilis* P٤**	١,٣٢
***P. mirabilis* P٥**	٣,٠٧
***P. mirabilis* P٦**	٣,٨٦
***P. mirabilis* P٧**	٢,٠٤
***P. mirabilis* P٨**	١,٩٦
***P. mirabilis* P٩**	٦,٠٤
***P. mirabilis* P١٠**	١٢,٠٣
***P. mirabilis* P١١**	٧,٨٩
***P. mirabilis* P١٢**	٤,٥٥
***P. mirabilis* P١٣**	٥,٠٣
***P. mirabilis* P١٤**	٣,٢٠
***P. mirabilis* P١٥**	١٤,٥٠

Conclusions

From these results, it could be concluded that *P. mirabilis* was associated with urinary tract infection and were contaminants of meat and chicken, On the other hand, these local isolates of *P. mirabilis* are able to produce different virulence factors such as invasion and adhesion production and protease production with variable degrees.

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الخلاصة

جمعت ٨٥ عينة سريرية وغذائية من مناطق مختلفة من محافظة بغداد شملت ٢٠ عينة إدرار و ٤٥ عينة جروح و ٢٠ عينة من اللحوم الحمراء والبيضاء (الدجاج). ومن المجموع الكليلتلك العينات فقد تم الحصول على ١٤٦ عزلة بكتيرية، شخصت ١٥ عزلة منها على انها *P. Mirabilis* وفقا لخصائصها المظهرية والمزرعية و صفاتها الكيموحيوية. وقد تم تأكيد تشخيصها باستخدام العدة التشخيصية Api-.

بين ١ و ٢٠ خلية بكتيرية/ خلية طلائية، فضلا عن قابلتها على إنتاج إنزيم البروتييز بفعالية نوعية تراوحت بين ٤,٥_٠,١٢١ وحدة/ ملغم بروتين. وقد تميزت *P. mirabilis* P١٥) منبينتلك العزلات البكتيرية العزلة المعزولة من عينة إدرار بضراوتها العالية لقدرتها العالية على إنتاج عوامل الضراوة.

٢٠E اختبرت القابلية الامرضية لعزلات بكتريا *P. mirabilis* بالتحري عن بعضع وامل الضراوة كقابليتها على الالتصاق بالخلايا الطلائية واختراقها وقابليتها على إنتاج إنزيم البروتييز. وقد أظهرت النتائج قابلية العزلات على الالتصاق بالخلايا الطلائية بمدى يتراوح بين ٣٠ و ٥٥ خلية بكتيرية/ خلية طلائية، وقابليتها على الاختراق بمدى يتراوح