The Role of Plasmids In Pathogenicity of Locally isolated *Plesiomonas shigelloides*

دور البلازميدات في أمراضية بكتريا *Plesiomonas shigelloides* المعزولة محلياً

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Abstract

One hundred and twenty diarrhea samples were collected from different hospitals in Baghdad, four isolates 3.3% (P1, P2, P3, P4) were obtained and identified as *Plesiomonas shigelloides* from these samples. Four isolates were also obtained and identified as the same bacteria from the environmental samples, two isolates 10% from twenty water samples (termed W1, W2) and two isolates 10% from twenty fish samples (termed F1, F2). Investigation of antibiotic susceptibility was done for all isolates. Some of the virulence factors were determined in this bacterium, like its ability to produce some enzymes. Ability of bacterial isolates to adhesion on epithelial cells was studied as one of important virulence factors; it was found that all isolates have the ability to adhesion on living and non-living surfaces. The plasmid profile for all isolates was studied, and the results showed that the plasmid profile was similar for all isolates. All isolates contained two small plasmid DNA bands approximately in the same sizes. The role of plasmids in pathogenicity of *P. shigelloides* P1 were studied, throughout cure this isolate using SDS. These results indicated that the trait of β-lactamas, haemolysin and proteinase production in addition to resistance trait for tetracycline and streptomycin were plasmids born. While the lipase and lecithinase production trait were chromosomal born in *Plesiomonas shigelloides* P1.

المستخلص

جمعت 120 حالة إسهال من مستشفيات مختلفة في بغداد ، حيث أظهرت أربع عزلات 3.3% عائديتها لبكتريا *Plesiomonas shigelloides*(رمزت P1، P2، P3، P4) . كما شخصت أربع عزلات تعود للبكتريا نفسها من عينات بيئية ، عزلتين 10 % من 20 عينة ماء (رمزت W1،W2) وعزلتين 10 % من 20 عينة من الأسماك (رمزت F1،F2) . اختبرت حساسية العزلات لعدد من المضادات الحيوية و حددت بعض عوامل الضراوة في هذه البكتريا منها قابليتها على إنتاج بعض الأنزيمات و كذلك درست قابلية عزلات هذه البكتريا على الالتصاق على سطوح الخلايا الطلائية كأحد عوامل الضراوة المهمة ، ووجد إن جميع العزلات لها قابلية الالتصاق على الأسطح الحية والغير حية . درس النمط البلازميدي لبكتريا *P.shigelloides* ، و تبين احتواء العزلات على النمط البلازميدي نفسه ، إذ احتوت جميع العزلات على حزمتين بلازميديتين صغيرتين تمتلك نفس الحجم الجزيئي تقريباً . درس دور البلازميدات في امراضية بكتريا *P.shigelloides* P1 من خلال تحييدها بطريقة استخدام مادة SDS أشارت النتائج إن صفة إنتاج البيتا لاكتاميز و الهيمولايسين و البروتييز ، فضلاً عن صفة المقاومة لمضادات التتراسايكلين و الستربتومايسين كانت محمولة على البلازميدات أما صفة إنتاج أنزيمي اللايبيز و اللسثين فهي كروموسومية في العزلة *P.shigelloides* P1 .

Introduction

Plesiomonas shigelloides, previously was belonged to the family *Enterobacteriaceae*, but because of morphological and biochemical differences, now belonged to the family *Vibrionaceae*, which include beside *Plesiomonas*, *Vibrio* and *Aeromonas* [1].

Plesiomonas shigelloides is aquatic microorganism, Gram negative, motile with lophotrichous flagella, non-spore-forming, facultative anaerobic, and pleomorphic microorganism. It can be isolated from human and numerous animals such as fish, birds and insects. Also from several aquatic environments (e.g. rivers, fresh water, lakes, sludge).

It have been implicated as a cause of gastroenteritis infection in adults and children [2,3], also cause food poisoning and acute diarrhea beside that it cause extraintestinal infection, which include meningitis, osteomyelitis, septic arthritis, cholecystitis, peritonitis and other non diarrheal diseases.Various virulence factors have been determined in this bacterium like enterotoxin, and haemolysin also produce other enzymes which is important as virulence factors like elastase, lecithinase and proteinase [4]. It have also a higher adhesion ability to living and non living tissues, and have cytotoxin which is important for promoting colonization or invasion of epithelial cells. Other virulence factors are tetradotoxin which cause food poisoning, lipopolysaccharide (LPS) or what is called endotoxin also play an important role to the pathogenicity of this bacterium [5]. Most strains of *Plesiomonas shigelloides* are resistant to beta-lactam group of antibiotics, because bacteria may be produce beta-lactamase enzyme. Some strains are susceptible to aminoglycosides group especially to gentamicin, tobramycin [6]. It was found that this bacteria contain different molecular weight plasmids, some with small molecular weight ranging from (2-8 MDa), which are may be associated with antibiotic resistant and bacteriocin production, and the other with large molecular weight (140 MDa) which may be has been associated with adhesion of the bacteria [7].

According to those mentioned above and because of limited studies about the genetics of this *Plesiomonas shigelloides*, the study aimed to determine the virulence factors and attempt to determine the plasmid content and its role in pathogenecity of this bacterium.

Material & Methods

Collection of samples: One hundred and twenty stool samples were collected from patients with watery diarrhea and from patient with gastrointestinal infection of AL-Yarmooke Hospital and AL-Kadhymia Hospital, and twenty samples were collected from deep water river. Other twenty swabs sample were taken from hands and tools of fish sailor in a local market.

Isolation of *Plesiomonas shigelloides*: Samples were cultured on MacConky's agar plate (Oxoid) and tryptic soy broth (Oxoid). Cultured plates were incubated overnight at 37ºC. After incubation suspected *Plesiomonas* colonies were again selected and streaked on deoxycholate citrate agar (Oxoid) and Hekton enteric agar (Oxoid). Suspected *Plesiomonas* colonies were selected and stored in pure form for further identification.

Identification of bacterial isolates

Bacteria isolates were identified according to its morphological, physiological and biochemical characteristic as recommended by [8].

Detecting of virulence factors

Number of virulence factors (amylase, DNase, Gelatinase, lipase, lesithenase, proteinase, Haemolysine) were investigated for all bacterial isolates as recommended by [8, 9].

β – lactamase production assay

Plesiomonas shigelloides isolates were screened for β-lactamase production using Rapid Iodometric Methods [10].

Adhesion test

Adhesion test described by [11] done by making a single streak of bacteria on Adhesion medium and incubated at 37 ºC for 24 hrs. After incubation the observation of dark colonies indicates a positive result.

DNA extraction

DNA extraction was done by salting out method which described by [12].

Agarose gel electrophoresis

Samples of DNA were loaded in agarose gels 0.7% in Tris- borate EDTA buffer (TBE 1X). Samples of DNA were mixed with 1 /10 volume of the loading buffer and added to the wells in the gel. Generally, gels were run for (2-3) hrs at 5 v/cm and the gel buffer added up to the level of horizontal gel surface as in [13].

Curing of plasmid DNA

In order to determine the correlation between plasmid content and virulence factors of *Plesiomonas shigelloides* curing experiment was performed on *P. shigelloides* (P1) isolate by using SDS as a curing agent depending on [14].

Result and Discution

Isolation

One hundred and twenty diarrhea samples were collected from patients. Four 3.3% isolates (P1, P2, P3, P4) of *Plesiomonas shigelloides* were isolated from diarrhea samples, while from environmental samples two 10% isolates (W1,W2) were isolated from twenty water samples and two 10% isolates (F1, F2) were isolated from twenty fish samples, during the same period.

The environmental isolates have the highest share of isolates compared with clinical isolates, and that explained by presence of *Plesiomonas shigelloides* as a normal flora in water and in the gut of aquatic animals especially fishes. The results were close to those reported by [15,16] who found that percentage of clinical isolates were 2.9% and 3.2% respectively.

Identification

Bacterial isolates showed morphological, through direct microscopic examination**,** bacterial isolates appeared as non spore forming gram negative pleomorphic bacteria. When examine the movement of this bacteria by using hanging drop method it was found that all bacterial isolates were motile. Bacterial isolates reveals different colonial appearance depending on the selective or differential media used. Colonies of all isolates appeared in medium size, mucoid, low convex on MacConkey agar,

colonies of some isolates appeared pale as a non lactose fermenter in 24 hr, but some isolates (P2, P3, P4) ferment lactose in 24 hr.They were also appeared in pale color (non lactose fermenter) on deoxycholate citrate agar with irregular edge and shape while on hekton enteric agar appeared as a flat colonies with bright green color, and appeared as a flat with red color in xylose – lysine – deoxycholate agar (XLD). Physiological, and biochemical characteristics Table (1) Were in agreement with that mentioned by [8, 17].

Table (1) : Biochemical and physiological characteristics of *Plesiomonas shigelloides* isolates

Test		Isolates							
		P1	P2	P3	P4	W1	W2	F1	F2
Oxidase		+	+	+	+	+	+	+	+
Catalase		+	+	+	+	+	+	+	+
Indole		+	+	+	+	+	+	+	+
Citrate utilization		+	+	+	+	-	-	-	-
Voges proskaur		-	-	-	-	-	-	-	-
Methyl red		+	+	+	+	+	-	-	-
Triple sugar iron	Slant/But t	K/A	A/A	A/A	A/A	K/A	K/A	K/A	K/A
	H_2S	-	-	-	-	-	-	-	-
	CO_2	-	-	-	-	-	-	-	-
Growth in 8 °C		+	+	+	+	+	+	+	+
Growth in 45 °C		+	+	+	+	+	+	+	+
Growth in 55 °C		-	-	-	-	+	+	-	-
Growth in 60°C		-	-	-	-	+	+	-	-
Asculine hydrolysis		-	-	-	-	-	-	-	-
Carbohydrate fermentation									
Glucose		+	+	+	+	+	+	+	+
Lactose		+	+	+	+	+	+	+	+
Galactose		+	+	+	+	+	+	+	+
Inositol		+	+	+	+	+	+	+	+
Trehalose		+	+	+	+	+	+	+	+
Salicin		-	-	-	-	-	-	-	-
Mannitol		-	-	-	-	-	-	-	-
Mannose		-	-	-	-	-	-	-	-

(K) : Alkalin , (A) : Acid ; P1,P2,P3,P4 : Clinical isolates ; W1,W2: Water isolates; F1,F2: Fish isolates

Detection of virulence factors of *Plesiomonas shigelloides* isolates

Number of virulence factors were investigated for all *P. shigelloides* isolates

Table (2): Virulence factors for *Plesiomonas shigelloides* isolates

Enzymes	Isolates							
	P1	P2	P3	P4	W1	W2	F1	F2
Amylase	-	-	-	-	-	-	-	-
DNase	-	-	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	-	-
Lipase	+	+	+	+	+	-	-	+
Lecithinase	+	+	+	+	+	+	+	+
Proteinase	+	+	+	+	+	+	+	+
β– lactamase	+	+	+	-	+	+	-	+
Haemolysine	β	β	β	β	β	β	β	β

β: beta (Complete haemolysis), (-): Absence, (+): Present

Results

Table (2) were in agreement or closed to those reported by other study (18) who referred that some strains of *P. shigelloides* cannot produce haemolysin and others produce beta or alpha haemolysine which can utilize the iron found in hemoglobin. DNase production test is important for distinguish *P. shigelloides* (producer) from *Aeromonas sp.* (non producer) [19]. Most strains of *P. shigelloides* were produced β-lactamase enzymes, while the other strains don't produce this enzyme [20].

Adhesion test

By using blood agar containing Gongo red, it was found that *P. shigelloides* have the ability to adhesion by forming dark pigment around the colonies, that refers to the bacterium ability to adhere on Gongo red dye.Adhesion on human epithelial cells were also studied, it was found that *P. shigelloides* have high ability to adhere on epithelial cells, and this may be because *P. shigelloides* have slime layer which make it adhere on the different surfaces [21].

Antibiotic susceptibility

The standard disk diffusion method was used to determine the sensitivity of *P. shigelloides* isolates to several antibiotics.

Table (3): Antibacterial resistance of *Plesiomonas shigelloides* isolates

Antibacterial	Code	P1	P2	P3	P4	W1	W2	F1	F2
Amikacin	AK	S	S	S	S	S	S	S	S
Ampicillin	AMP	R	R	R	R	R	R	R	R
Carbenicillin	PY	R	R	R	R	R	R	R	R
Cefotaxime	CTX	S	S	S	S	S	S	S	S
Chloramphenicol	C	R	S	S	R	S	S	S	S
Ciprofloxacin	CF	S	S	S	S	S	S	S	S
Cloxacillin	CLX	R	R	R	R	R	R	R	R
Erythromycin	E	R	S	S	R	S	R	S	S
Gentamicin	GM	S	S	S	S	S	S	S	S
Nalidixic acid	NA	S	S	S	S	S	S	S	S
Norfloxacin	Nor	S	S	S	S	S	S	S	S
Penicillin	P	R	R	R	R	R	R	R	R
Pipracillin	PC	R	R	R	R	R	R	S	R
Rifampicine	Rif	S	S	S	S	S	S	S	S
Streptomycin	S	R	R	R	R	R	R	R	R
Tetracyclin	TE	R	R	R	S	R	S	S	R
Tobramycine	TM	S	S	S	S	S	S	S	S
Trimethoprim	TR	S	S	S	S	S	S	S	S
Ticarcillin	TC	R	R	R	S	R	R	R	R
Vancomycin	VA	R	R	R	S	S	R	R	R
O_{129}	O_{129}	S	S	S	S	S	S	S	S

R: Resistance, S: Sensitive

Results

Table (3) was close to those reported by other studies, which found that most strains of these bacteria were multiresistant [22].

Table (3) showed that the clinical isolates were multiresistant to antibiotics more than the environmental isolates, and this result is in agreement with [23] .The multidrug resistance of this bacteria to several antibiotics could be due to the permeability of the outer membrane, which might prevent the entry into the cell of most of these antibiotics or due to certain mutations that occur as a result of overuse and misuse of antibiotics, in addition to mutation, plasmids that carrying resistance gene play an important role in spreading the multidrug resistance between bacteria [22].

Plasmid isolation

Salting out method (12) was used successfully to determine the plasmid profile of *P. shigelloides* isolates.

Figure (1) indicated that all the isolates containing a small plasmid DNA bands and all these plasmid bands approximately in the same size compared with each other, and with pBR322 plasmid (which is not shown in this figure). The bacterial isolates, tested in this study may be containing another plasmids does not detected (may be because it's large size).

As shown in Figure (1) the plasmid bands were common in all isolates of clinical and environmental sources. The plasmid was similar for all isolates. It can be concluded that they are belong to related strains, since the related strains often contain the same number of plasmids with the same molecular weight and similar phenotypes [24].

There are limited studies dealing with plasmid isolation from *P. shigelloides*, which reported that this bacteria contain small plasmids ranging from (2-8 MDa) responsible for antibiotics resistance and bacteriocin, and large plasmids (140 MDa) coded for a protein that is responsible for adhesion to the epithelial cells [7].

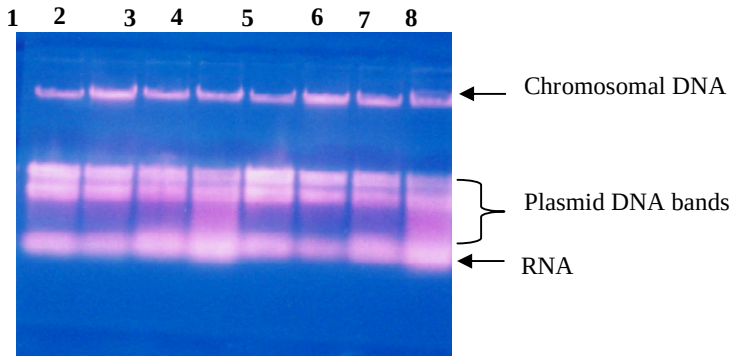

Fig (1): Gel electrophoresis of isolated plasmid from the bacterial isolates migrated on agarose gel (0.7%) in TBE buffer at 5V/cm.)

1: *P. shigelloides* (P1). 5: *P. shigelloides* (W1).
2: *P. shigelloides* (P2). 6: *P. shigelloides* (W2).
3: *P. shigelloides* (P3). 7: *P. shigelloides* (F1).
4: *P. shigelloides* (P4). 8: *P. shigelloides* (F2).

The relationship between Plasmid content of *Plesiomonas shigelloides* and virulence factors

Plasmid curing of *P. shigelloides* (P1**)** was performed by using SDS (1-10)% to determined whether the genes responsible for virulence factors are located on the plasmid or not. The highest concentration of SDS that allows the growth of *P. shigelloides* (P1) was 6%. From this treatment, appropriate dilutions were made and spread on brain heart infusion agar. Then 100 colonies were selected and tested on a selective media containing a specific antibiotic (ampicillin, streptomycin) in order to determine the cured colonies, which cannot grown on this antibiotics and then they obtained cured colonies were tested on another specific antibiotic (penicillin and tetracycline). A cured colonies was obtained and their ability to loss some virulencs factor was tested.

Table (4): Curing strains of *P. shigelloides* (P1) that lost resistance to antibiotics and some virulence factors

	P1	P1c1	P1c2	P1c3	P1c4	P1c5	P1c6	P1c7	P1c8	P1c9
Lipase	+	+	+	+	+	+	+	+	+	+
Lecithinase	+	+	+	+	+	+	+	+	+	+
Haemolysin	β	-	-	+	-	-	+	-	-	-
Casein	+	-	-	-	-	-	-	-	-	-
β-Lactamase	+	-	-	+	-	-	+	-	-	-
Ampicillin	R	S	S	R	S	S	R	S	S	S
Penicillin	R	S	S	R	S	S	R	S	S	S
Tetracyclin	R	S	R	S	S	R	R	S	S	S
Streptomysin	R	S	S	S	R	R	S	S	S	S

β: beta (Complete haemolysis), R : Resistance , S : Sensitive, (-) :Absence, (+):Existance

Table (4) indicated that streptomycin and tetracycline resistance genes are carried on different plasmids (two plasmids), while ampicillin and penicillin resistance genes are carried on one plasmid, because no cured strain was obtained that lost one of them only.

In order to determine whether the cured strains have lost other virulence factors in addition to those investigated previously, all these strains were tested for their virulence factors.

Table (4) indicated that all cured strains 100% lost their ability to hydrolyze casein, which means that this trait in *P. shigelloides* (P1) is plasmid born. Seven strains lost their ability to produce β-lactamase enzyme and these strains also lost the resistance to ampicillin and penicillin, and this indicate that resistance to ampicillin and penicillin in (P1) isolate is due to β- lactamase enzyme, which encoded by a plasmid. These strains, that lost β-lactamase production, also lost haemolysin production ability, the genes responsible for β- lactamase production also kept β- haemolysin ability; this result indicated that haemolysin genes and β- lactamas gene are carried on single plasmid, because no cured strain was obtained that lost one of them only.

Results indicated also that none of the cured strains lost their ability to produce lipase and lecithinace, which means that gene responsible for these traits in *P. shigelloides* (P1) are located on chromosome.

The previous results indicated that *P. shigelloides* (P1) contains four plasmids, and these results are closed with results of [7] who found that *P. shigelloides* contain beside the large plasmid two plasmids responsible for antibiotic resistance and other plasmid responsible for bacteriocin; other study found that tetracycline resistance gene was carried on a plasmid [23].

Proteinase coded by a plasmid may have a relationship with adhesion protein which is very important for adhesion properties in *P. shigelloides*, so when all cured strains tested for adhesion properties, it was found that their adhesion properties were decreased, in which the bacterial cell was not lost its adherent properties completely. Adhesion of bacteria to the epithelial cell is very important for colonization of bacteria, this adhesion mainly is adherent protein or sometimes by pili. Any reduction in one of these factors may affect the adhesion of bacteria [25].

In order to prove the results of curing experiment, plasmid DNA was isolated from one of the cured strains, (strain P1c1), which lost different virulence factors Table (4)

and compared with plasmid content of the original isolate. Result figure(1) showed that the cured strain (P1c1) lost their plasmid DNA bands. This result supported the results of curing experiments which indicated that genes responsible for antibiotics resistance (tetracycline and streptomycin), β- lactamase, haemolysin and casein hydrolysis in *P. shigelloides* (P1) are located on plasmid.

According to the results of plasmid curing experiment, which refers that *P. shigelloides* (P1) contains at least four plasmids, but Figures (1, 2) showed only two plasmid bands and they have been lost from the cured strain (P1c1) which means that the other plasmids were not appeared and this may be attributed to its large sizes.

According to the results of plasmid curing experiment, which refers that *P. shigelloides* (P1) contains at least four plasmids, The first use of SDS as a curing agent with this bacterium in this study showed that SDS is a powerful agent in eliminating different plasmids, hence, the curing percentage for *P. shigelloides* was 9%, this result was agreement with [26] who found that SDS was used successfully as a curing agent.

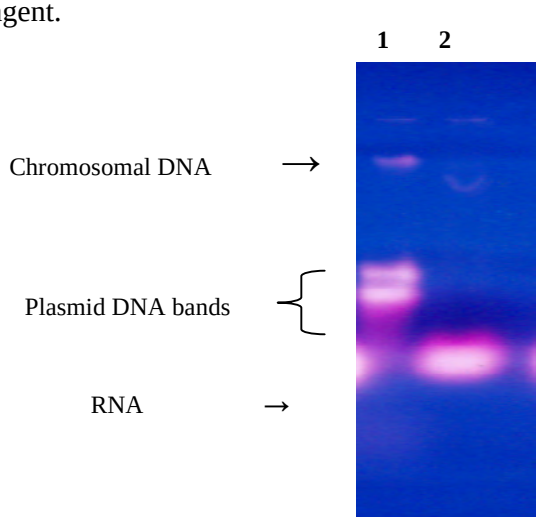

Figure (2): Gel electrophoresis of plasmids content of *P. shigelloides* before and after treatment with SDS on agarose gel (0.7%) in TBE buffer at 5V/cm.
1: Plasmid content of *P. shigelloides* (P1).
2: Plasmid content of cured strain (P1c1).

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