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Detection of Virulence Factors Produced by Local Isolates of Aeromonas hydrophila

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Abstract

Aeromonas hydrophila was isolated from different local sources includes fresh fish, water, and lake of fish farm in Baghdad governorate, and identified according to their morphological and cultural characteristics and biochemical tests. Virulence factors produced by A. hydrophila isolates were detected to select the more virulent isolate. Results showed that these isolates have the hemolytic activity, slime production and with high ability in protease production. Antibiotic susceptibility of the more virulent isolate was examined. Results showed that A. hydrophila H4 was resistant to many antibiotics, while it was sensitive to others. Plasmid profile A. hydrophila H4 was studied by extraction of plasmid DNA and electrophoresis on agarose gel. Results showed that this isolate was harboring two small plasmids which may be responsible for the production of virulence factors and/or the antibiotics resistance. After curing of these two plasmids by using SDS (6%), it was found that cured colonies of A. hydrophila H4 was lost its ability to resist many antibiotics, while it was still having the ability to produce different virulence factors, which means that genes encoding these virulence factors are chromosomally located.

Keywords: Aeromonas hydrophila, Virulence factors, DNA plasmid, curing,

1. Introduction

Aeromonas hydrophila is a gram-negative, facultative anaerobic rod approximately 0.8-1.0 × 1.0-3.5 µm in size that is motile via single polar flagella that morphologically resembles members of the family Enterobacteriaceae (Altarriba et al. 2003). A. hydrophila has been identified as causative agent of human diseases such as septicemia, meningitis, wound infections as a result of exposure to contaminated marine environment and diarrhea (Odeyemi et al. 2012). A. hydrophila is capable of expressing a number of virulence factors such as haemolysin, aerolysin, cytotoxin, enterotoxin, cytotonic enterotoxin, endotoxin lipopolysaccharide, slime production, outer membrane proteins and enzymes such as proteases, lipases, DNases, elastase and gelatinase (Seethalakshmi et al. 2010). These virulence factors are used as survival means, self defense mechanism and establishment of pathogenicity. In a research in 1995, some researchers stated that virulence factors are determinant of bacterial pathogenicity (Janda and Duffey 1988). A. hydrophila is the most studied due to its presence in food (Radu et al. 2003) water (Asmat and Gires 2002), estuary (Odeyemi et al. 2012), antibiotic resistance and it ability to cause infections in human and animals (Evangelista et al. 2010). A. hydrophila has been identified as causative agent of human diseases such as septicemia, meningitis, wound infections as a result of exposure to contaminated marine environment and diarrhea (Evangelista et al. 2010).

A. hydrophila is one of the causative agents for diarrheal infections in children and immunocompromised patients. These are ubiquitous water borne organisms and have gained importance as human pathogens causing gastrointestinal and extraintestinal infections (Subashkumar et al. 2006). This study was aimed to isolate A. hydrophila and detecting virulence factors produced by these isolates.

2. Materials and Methods

2.1 Isolation and Identification of Aeromonas hydrophila

For the isolation of *A. hydrophilia*, a total of 120 samples of fresh fish samples (80 sample), and water samples (20 sample) from pool of living fish were collected from lake of fish farm in Baghdad governorate, while no isolates from 20 samples of chicken and meat during the same period. Aliquots of 1ml of each water sample were diluted with 9 ml of alkaline peptone water (pH 8.9) and incubated at 35ºC for 4-6 h., then 1 ml aliquots of each sample was added to test tubes containing 9 ml of distilled water and mixed vigorously. Each sample was serially diluted, then 0.1 ml aliquots from the appropriate dilution were taken and spread on blood and MacConky agar medium as differential medium, and on TCBS agar medium as a selective medium for

Aeromanas ssp. and incubated at 35ºC for 24h. Morphological and culture characteristics and biochemical tests were achieved for the suspected colonies according to (Atlas et al. 1995).

2.2 Detection of virulence factors

2.2.1 Hemolytic activity

Hemolytic activity for the bacterial isolates were determined by using blood agar assay method Pattern of haemolysis around the colonies on blood agar plates containing 5% (v/v) human blood was recorded after 24 hr. incubation at 35 °C (seethalakshmi et al. 2010).

2.2.2 Slime production

Bacterial isolates *of A. hydrophila* were plated on Brain heart infusion agar medium containing 0.8 g/L Congo red, and incubated at 35 °C for 48 hr. Slime production was examined by detection of Congo red uptake according to (seethalakshmi et al. 2010).

2.2.3 Production of protease

Specific activity of protease in crude filtrates of bacterial cultures was determined according to (Manachini et al. 1989).

2.3 Determination of protein concentration by Bradford method

Protein concentration in crude filtrates of bacterial cultures was determined according to **(**Bradford 1976).

2.4 Antibiotic susceptibility of bacterial isolates

Disc diffusion method was used to determine the antibiotic susceptibility of bacterial isolates according to (Atlas et al*.,* 1995).

2.5 Extraction of plasmid DNA

Plasmid DNA extraction was achieved by salting out procedure described by (Kieser 1995).

2.6 Curing of plasmid DNA

In order to determine the relationship between plasmid content and virulence factors of *A.hydrophila,* curing experiment was performed on the selected isolate using SDS as a curing agent (Trevors 1986). After treatment of bacterial isolates with SDS, survivors were analyzes for the presence or absence of antibiotics resistance as a result of eliminating the plasmid.

3. Results and Discussion

3.1 Isolation of Aeromonas spp

In order to isolate Aeromonas hydrophila, one hundred and twenty samples were collected from different locations in Baghdad governorate during the period from 10-2011 to 1-2012. From all of these samples, ninety one isolates were obtained, these isolates were maintained and grown on blood and MacConky agar medium as differential medium, and on TCBS agar medium as a selective medium for Aeromonas spp. Among the total isolates, thirty five were suspected to be belonging to Aeromonas spp. and were subjected to identification according to their morphological and cultural characteristics and biochemical tests. Results mentioned in table (1) showed that twelve of these isolates were positive for catalase, oxidase and indole tests, while all the twelve isolates were negative for urease test, and they gave a variable results for citrate utilization in which seven of them were negative and the other five isolates were positive for this test, this may be attributed to the different ecotypes of Aeromonas spp in different environments as mentioned by (Mange et al.1998).

To confirm the identification of the twelve isolates of A.hydrophila, these isolates were re-identified by using Api 20E system. Results showed that these isolates gave the same results of identification given by the standard isolate of A. hydrophila (ATCC35654).

3.2 Detection of the virulence factors for A. hydrophila isolates

3.2.1 Haemolytic activity

Ability of local isolates of A. hydrophila in haemolysin production as a major virulence factor was studied. Haemolytic activity was examined by measuring the diameter of hydrolysis zone around each bacterial isolate on

blood agar medium containing 5% human blood after 24 hours of incubation at 35°C. Results indicated in table (2) showed that the local isolates were able to produce haemolysin to hydrolyze zone the blood constituent of the medium and forming a halo of hydrolysis around each colony ranged between 2mm to 7mm.

3.2.2 Slime Production

Slime production in term of Congo red uptake has been shown to be a marker for virulence in several enteropathogenic and nonenteropathogenic bacteria (Maurelli 1984). Results indicated in table (3) showed that local isolates of A.hydrophila were able took up congo red and produce viscous slimes around their colonies with variable degrees. This means that these isolates differ in pathogeneses, and this may be associated with the presence of virulence plasmid. (Barry and George 1987) found that motile Aeromonas strains of diverse clinical origin universally took up this dye, this finding suggests that all isolates were potential enteric pathogens.

3.2.3 Protease production

Ability of local isolates of A. hydrophila in protease production was examined. Results indicated in table (4) showed that all of the isolates were protease producers with variable degrees. Specific activity of protease in culture filtrates was ranged between 2.11 and 30.01 U/mg protein, among them, A. hydrophila H4 was the most efficient in protease production because the specific activity of protease in crude filtrate of this isolate was 31.01 U/mg protein.

According to the results of the detection of virulence factors for the bacterial isolates of A. hydrophila, the isolate H4 was selected for further studies because it was the most virulent isolated among the other local isolates of A. hydrophila.

3.3 Antibiotic susceptibility of A. hydrophila H4

The susceptibility of A.hydrophila H4 against different antibiotics was studied. Results indicated in table (5) showed that these bacteria were resistant to six antibiotics, while it was sensitive to the other eleven antibiotics. Resistant to these antibiotics may be encoded by chromosomal and/or plasmid genes. This resistance could be attributed to degradation of these antibiotics by β- lactamase enzyme or to lack of penicillin binding protein or the microorganisms change outer membran permeability to the drug (Scott et al. 1999; Avesion et al. 2000). Aeromonas spp. can possess a conjugative plasmid that confers multiple antibiotic resistances (Chang and Bolton 1987).

3.4 Plasmid profile of A. hydrophila H4

Plasmid profile of the locally isolated A. hydrophila H4 was studied by extraction genomic DNA according to salting-out procedure described by (kieser 1995). Results mentioned in figure (1) showed that these bacteria have two small plasmids after electrophoresis on agarose gel. The molecular sizes of A.hydrophila plasmids ranged from approximately 2 to 110 megadaltons (Chang and Botton, 1987).

3.5 Curing of plasmid DNA

Plasmid curing for the locally isolated A. hydrophila H4 was achieved by using SDS to determine whether the genes responsible for virulence factors and antibiotic resistance are chromosomally located or encoded by plasmid. Results indicated in table (6) showed that the highest concentration of SDS that allows the growth of A.hydrophila H4 was 8%,

After treatment with SDS, a total of 100 colonies were selected and tested on a selective medium containing the appropriate antibiotic to which the wild type was resist (Amoxicillin, Cefotaxime, Cephradine) in order to detect the cured colonies which lost their ability to conferring the resistance phenotypes to those antibiotics.

Results showed that 18 from the total selected colonies were unable to grow in the presence of Cefotaxime, Cephradine and became sensitive to these antibiotics. One of these colonies was selected randomly and examined for the presence of its own plasmid by extraction of genomic DNA and electrophoresis on agarose gel.

Result mentioned in figure 2 showed that this cured colony of *A. hydrophila* was lost their own plasmid, and this referred that the plasmid is responsible for resistance Cefotaxime, Cephradine in *A. hydrophila* as shown in table (7). In other word, gene(s) responsible for resistance of these antibiotics are located on plasmid in this bacterium.

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Table 1 Morphological characteristics and biochemical tests for identification of A. hydrophila isolates

Test		Isolate symbol												
		ATCC 35654	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
shape		Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
motility		+	+	+	+	+	+	+	+	+	+	+	+	+
Gram stain		-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase		+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase		+	+	+	+	+	+	+	+	+	+	+	+	+
Indole		+	+	+	+	+	+	+	+	+	+	+	+	+
urease		-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization		-	-	-	-	+	+	-	-	+	+	+	-	-
Glucose		+	+	+	+	+	+	+	+	+	+	+	+	+
manitol		+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose		+	+	+	+	+	+	+	+	+	+	+	+	+
lactose		-	-	-	-	-	-	-	-	-	-	-	-	-
Triple Sugar Iron	Slant/ Buttm	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
	H2S	-	-	-	-	-	-	-	-	-	-	-	-	-
	CO2	-	-	-	-	-	-	-	-	-	-	-	-	-
Hemolysis		β	β	β	β	β	β	β	β	β	β	β	β	β

(-): negative result, (+): Positive result, β: beta (Complete haemolysis).

Table 2 Diameter of hydrolysis zones around each of A.hydrophila isolates on blood agar medium after incubation for 24h. at 35°C

Isolate Symbol	Diameter (mm)
H1	4
H2	5
H3	2
H4	7
H5	3
H6	5
H7	3
H8	4
H9	5
H10	4
H11	2
H12	3
ATCC 35654	4

Table 3 Slime Production by A. hydrophila isolates after 48houres at 37°C

Isolate Symbol	Slime Production
H1	++
H2	++
H3	+++
H4	++
H5	++
H6	+
H7	+
H8	+++
H9	+++
H10	+
H11	+++
H12	+++
ATCC 35654	++

+ = Low viscous growth, ++ = Moderate viscous growth, +++ = High viscous growth

Table 4 Specific activity of protease produced by local isolates of A.hydrophila after 24hrs of incubation at 35°C in production medium (pH 8.0) in shaker incubator at 150 rpm.

Isolate	Specific activity (U/mg protein)
H1	0.121
H2	12.03
H3	3.86
H4	31.01
H5	0.06
H6	5.03
H7	7.89
H8	3.07
H9	1.69
H10	4.55
H11	6.04
H12	5.78
ATCC 35654	12.01

Table 5 Antibiotic susceptibility of A.hydrophila H4

Antibiotic	Abbreviate	Susceptibility
Amikacin	AK	S
Amoxicillin	AX	R
Ampcillin	AM	R
Chloramphenicol	C	S
Cephradine	CE	R
Cephalothin	KF	R
Cefotaxime	CTX	R
Ciprofloxacin	CIP	S
Clindamycin	DA	S
Doxycycline	DO	S
Imipenem	IPM	S
Gentamicin	GM	S
Penicillin	P	R
Rifampin	RA	S
Tetracycline	TE	S
Trimethoprim	TMP	S
Vancomycin	VA	S

R: Resistance; S: Sensitive

Table 6 Effect of SDS on the growth of A. hydrophila H4

SDS Con.(%)	Bacterial growth
1	+++
2	+++
3	+++
4	++
5	++
6	+
7	+
8	±
9	_
10	_

(+++): very good growth, (++): Good growth, (+): Moderate growth,
(±): Slightly growth, (-): No growth

Table 7 Curing strains of A. hydrophila that lost resistance to antibiotics after treatment with SDS

Antibiotic and virulence factors	A. hydrophila H4	
	Wild-type	Cured
Haemolysin	β	β
protease	+	+
Amoxicillin	R	R
Cefotaxime	R	S
Cephradine	R	S

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

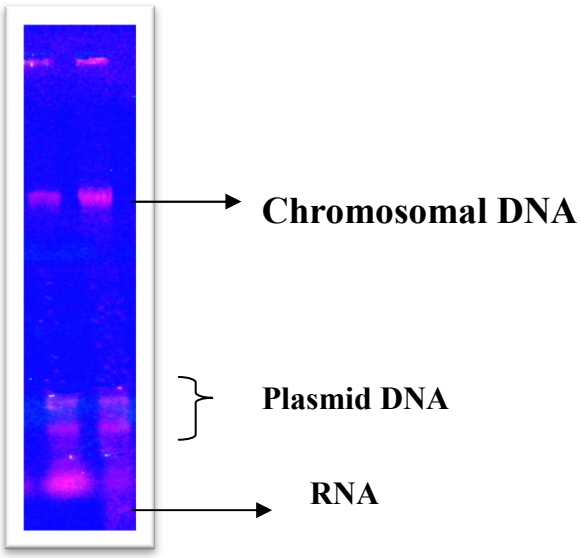

Figure 1. Gel electrophoresis of plasmid DNA extracted from A. hydrophila H4 on agarose gel (0.8%) in TBE buffer at 5V/cm

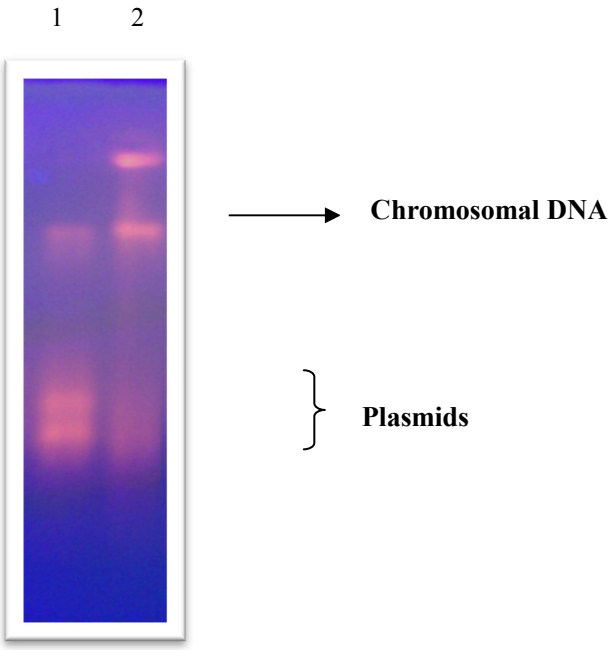

Figure 2 Plasmid profiles of wild type and cured A. hydrophila on agarose gel (0.8%) at (5 v/cm) for 2 hrs.

Lane (1): A. hydrophila H4 (wild-type).

Lane (2): A. hydrophila H4 (cured).