

## PCR Detection of Putative Hemolysin and Aerolysin Genes in An *Aeromonas Hydrophila* Isolates from Diarrhea in Babylon Province

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### Abstract

*Aeromonas* spp are considered as opportunistic infection and enterotoxigenic pathogen and can cause severe diarrhea. A total of one hundred and forty two stool samples were collected from patients with diarrhea at Babylon hospital for maternity and pediatrics of a period between December 2012 to June 2013. The 6(4.22%) strains of *Aeromonas hydrophila* were isolated from 142 samples of diarrhea stools. The *Aeromonas hydrophila* identified based on colony and microscopic morphology, biochemical tests and confirmed by Api 20E. Then investigation was done on hemolysin, All the isolates of *Aeromonas hydrophila* have the  $\beta$ -hemolytic activity on the blood agar. The polymerase chain reactions carried out to detect the presence of hemolysin and aerolysin genes in 6 *Aeromonas hydrophila* isolates. A 100% (6 isolates) of *Aeromonas hydrophila* isolates were contained hemolysin genes (*ahh 1a*) and 50% (3 isolates) were contain aerolysin gene (*aerA*). The band appearance in amplified gene bacteria shows molecular weight of hemolysin (130 p) and the molecular weight of aerolysin (309 bp).

**Key word:** *Aeromonas*, aerolysin, hemolysin, PCR

### 1. Introduction

*Aeromonas* spp are Gram negative, short rod shape, facultative anaerobes resistance to O/129 vibriostatic & non spore forming (Alperi *et al.*, 2010). They are ubiquitous microorganisms found in both aquatic and environmental habitats such as estuary sediment, sea water, sea grass, sea food, water used water, food and drinking water (Abbott *et al.*, 2003; Matyer *et al.*, 2007; Martinez Murcia *et al.*, 2000). *Aeromonas* spp. Are considered as opportunistic infections and enterogenic pathogen, *Aeromonas* can cause severe diarrhea, dysentery and bacteremia (Trower *et al.*, 2000; Blair *et al.*, 1999). Virulence of *Aeromonas* spp. is multifactorial and incompletely understood. Factors contributing to virulence include toxins, proteases, hemolysins, lipases, adhesins, agglutinins, and various hydrolytic enzymes (Janda and Abbott 1996). Virulence factors are present in two forms, cell-associated structures, and extracellular products. The main route of transmission for *Aeromonas* gastroenteritis is considered to be fecal oral. Studies on the etiology of travelers' diarrhea revealed *Aeromonas* to have a prevalence of about 3% in diarrheic patients returning from Asia and Africa (Shah *et al.*, 2009). Among other clinically relevant aeromonads like *Aeromonas caviae*, *A. trota*, and *A. veronii* biovar *sobria*, the most frequently isolated pathogen *A. hydrophila* is mainly associated with diarrheal illness accompanied by abdominal pain and nausea (Adamki *et al.*, 2006). Virulence factors of *aeromonas* spp. including toxin, protease S, hemolysin, lipase, adhesin, agglutinins and various hydrolytic enzymes (Janda and Abbott, 1996).

*A. hydrophila* is mainly associated with diarrheal illness accompanied by abdominal pain and nausea (Adamki *et al.*, 2006).

*A. hydrophila* is the most commonly involved in human infection such as septicemia and gastroenteritis (Chopra and Houston, 1999). The pathogenicity of *A. hydrophila* infection by producing virulence factors such as cytotoxin, proteases, S-layer and aerolysin (Rahaman *et al.*, 1997). Some researcher states the virulence factors are determinant of bacterial pathogenicity (Vadivelu *et al.*, 1995). The virulence of *A. hydrophila* is closely related to  $\beta$ -hemolysin produced. Screening of hemolysin genes the most effective way to detect and characterize *aeromonas* virulence factors (Yousre *et al.*, 2007).

Two hemolytic toxins have been described in *A. hydrophila*: a hemolysin (*hyl A*) (Hirono & Aoki, 1991) and aerolysin (*aer A*) (Howard *et al.*, 1987). Aerolysin produced by some *Aeromonas* sp. and possesses both hemolytic and enterotoxigenic activity (hemolytic enterotoxin) (Xu *et al.*, 1994).

The present study was therefore carried out to document the presence of pathogenic *A. hydrophila* in diarrheal stool samples at Babylon province /Iraq. Two hemolytic toxins, hemolysin and aerolysin have been described in *A. hydrophila*. No report is available from Babylon province, in this study a search was made for the presence of hemolysin and aerolysin genes in the genome of *A. hydrophila* isolated from diarrhea specimens.

## 2. Materials and methods.

### 2.1 Collection of samples

one hundred and forty two (142) Stool samples were collected from patients attending to Babylon hospital for maternity and pediatrics during the period from Dec 2012 to April 2013. Samples were collected in screw capped bottles and transported to the laboratory in ice box with ice packs. Information was also obtained from patients regarding age groups. All samples were analyzed within 8 h. for collection.

### 2.2 Isolation of *Aeromonas hydrophila*

One gram of each sample was briefly emulsified in 3ml of sterile 0.85%(w/v) saline and vortexed for 30 sec. Organ debris was allowed to settle down for 5 min. the samples were put it in alkaline peptone water (oxid pH 9) and sub cultured after incubation at 37C for 6 h. onto to macconkey agar and aeromonas agar at 37C for 24 h. (Nzeaka *et al.*,2002; Jatau and Yalubu, 2004). All the isolates were grown on trypticase soy agar (TSA) at 37° for 18 h. the strains first identified as *Aeromonas* spp. According to colony morphology on Macconkey and *Aeromonas* agar and by microscopic Morphology(gram stain)and by chemical tests (Oxidase, Catalase, Motility, H<sub>2</sub>S production, Citrate utilization, Indole, Methyl red & Vogas Proskure,)and by String test, Lysine decarboxylase, Arginine dehydrogenase, Ornithine decarboxylation for differentiated from *Vibrio cholera* and the diagnostic of these strains confirmed by Api 20E (Biomérieux, france)

### 2.3 Hemolytic activity

The strains were tested for β-hemolytic activity on a the blood base agar (oxid)supplement with 5% sheep erythrocytes. five micro liters of each suspension was streaked onto plates and incubated at 22 C and 37C for 24 h. the presence of clear colorless zone surrounding the colonies indicated β-hemolytic activity (Gerhardt *et al.*, 1981).

### 2.4 Oligonucleotide primers and PCR conditions

The polymerase chain reaction (PCR) was used to detect the presence of hemolysin and aerolysin in all *Aeromonas* isolates. the primer used for hemolysine gene and aerolysin gene (table 1)The *ahh1a* primer set was designed to amplify a 130-bp fragment of *A. hydrophila* extracellular hemolysin gene *ahh1* (Wang *et al.*, 2003). The AH-*aerA* primer set amplified a 309-bp fragment of the *A. hydrophila* aerolysin gene *aerA* (Wang *et al.*, 2003).

TABLE( 1)Primer pairs used for PCR amplification

Primer pair	Sequence (5to 3)	Target gene	Size of PCR amplicon (bp)	Reference or GenBank accession no.
AHH1F AHH1R	GCCGAGCGCCCAGAAGGTGAGTT GAGCGGCTGGATGCGGTTGT	<i>ahh1a</i>	130	Wang <i>et al.</i> ,2003
AH- <i>aerA</i> F	CAAGAACAAGTTCAAGTGGCCA ACGAAGGTGTGGTTCCAGT	<i>aerA</i>	309	Wang <i>et al.</i> ,2003

PCR was carried out on cyler using the following cycle :preheating at 95 C for 5 min followed by 30 cycles at 95C for 2 min ,55 C° for 1min and 72 C for 1 min followed by 7min final extension at 72 C. PCR products were examined by electrophoresis in 1.5 %agrose gel in TBE buffer. the gel stained with EtBr and saw under U.V. light (Yogananth *et al.*,2009)

### 2.5 DNA extraction

DNA extraction from gram negative bacteria was performed according to the genomic DNA purification kit supplemented by the manufacturing company(Gene aid) and it was stored in 2-8 C.

## 3. Results

### 3.1 isolation of *Aeromonas hydrophila*.

Out of the one hundred and forty two (142) diarrheic stool samples analyzed .6( 4.22%) were found to be positive for *Aeromonas hydrophila*. The prevalence per age groups as shown in figure (1) showed the age groups ,<6years having height rate of 2.11%(3 isolates) from total samples analyzed .age groups 7-12years having 1.40%(2 isolates) and >13 years having 0.70%(1 isolate).

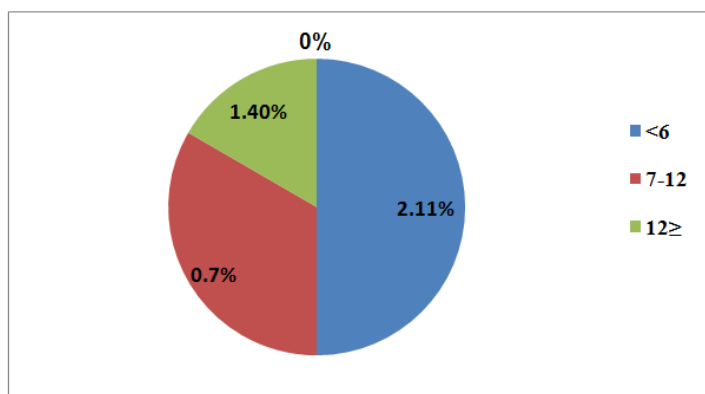


Figure (1) prevalence of the *Aeromonas hydrophila* in diarrhea samples according to age groups and percentage from total samples

### 3.2 Identification of *A. hydrophila*

The colonies of bacteria grown in culture media appeared 1-3 mm in diameter. *A. hydrophila* showed a yellow shine color on TCBS agar and non lactose fermenters on Macconkey agar . and it was smooth ,convex ,rounded ,β-hemolytic colonies and pale white to grey color on blood agar . this bacteria appeared gram negative, rod shaped, singly ,in pairs ,or even as short chains at the microscopic examination The biochemical tests used to confirmed the initial diagnosis of *A. hydrophila* (table 2) . *A. hydrophila* presented appositve result to each of the oxidase , catalase , indole , methyl red , simmon citrate,motility test,vogas prokauer and gelatin liquefaction this results

Table 2:- biochemical tests of *A. hydrophila* isolates

No.	Type of test	Result
1-	<b>Oxidase</b>	+
2-	<b>catalase</b>	+
3-	<b>Indole test</b>	+
4-	<b>MRtest</b>	+
5-	<b>Citrate test</b>	+
6-	<b>Gelatin liquefaction</b>	+
7-	<b>Motility test</b>	+
8-	<b>VP test</b>	+

To differentiated *A. hydrophila* from *V. cholera* by string test that the all *A. hydrophila* was gave negative results to it) and the *A. hydrophila* was gave positive results to arginine dehydrogenase and lysine decarboxylation and negative result to ornithine decarboxylation in compare to *V. cholera* was positive result to string test ,and ornithine and lysine decarboxylation and negative result to arginine dehydrogenase (table3)

Table (3):-differentiated between *A. hydrophila* and *V. cholera* by string test and amino acid utilization

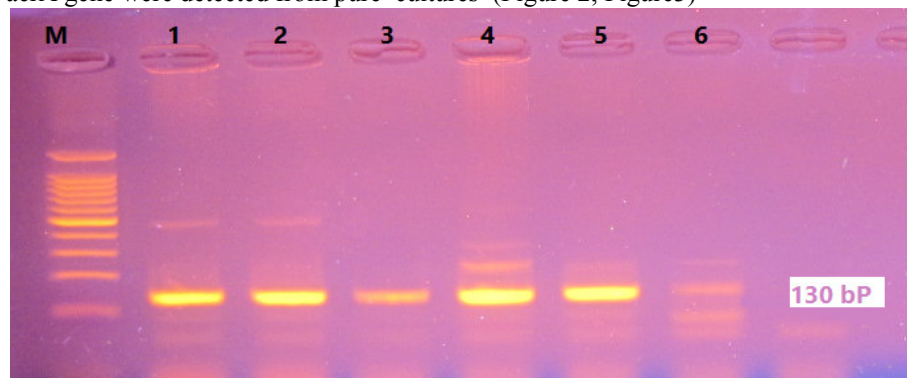
Test type	<i>V. cholera</i>	<i>A. hydrophila</i>
String test	+	-
Lysine decarboxylation	+	+
Argnine dehydrogenase	-	+
Ornithine decarboxylation	+	-

In this study standard of biochemical tests by API 20E were used to confirm identification of *A. hydrophila*, According to the result of API 20E test, the isolates were identical to the reference of Bergey's Manual of Determinative Bacteriology. Characterization (based on their morphological and biochemical reactions using the API 20E test showed that these isolates were phenotypically identified as *A. hydrophila*

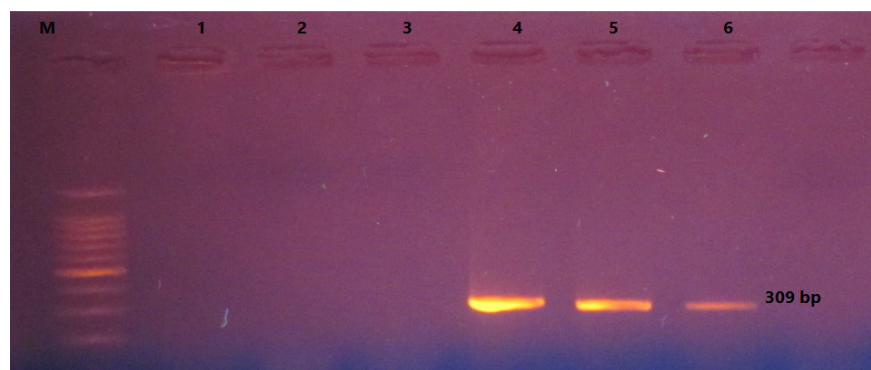
### 3.3 Hemolysin activity

The results of this study revealed that the all *A. hydrophila* positive to the β-hemolysin on blood agar .Nucleic

acid amplification methods targeting virulence genes of hemolysin and aerolysin *A. hydrophila* isolates. The specific PCR products corresponding to the 130 bp fragment of the *ahh1* gene and the 309 bp fragment of the *aerA* gene were detected from pure cultures (Figure 2, Figure3)



**Figure (2 ) Electrophoresis of PCR amplification products on 2% agarose gel, Lane 1 to Lane 6 *ahh1* gene of *Aeromonas hydrophila* Lane M marker DNA standard (100-1000) bp**



**Figure (3) Electrophoresis of PCR production on 2% agarose gel, Lane 1 to Lane 6 *aerA* gene for *Aeromonas hydrophila* Lane M marker DNA standard (100-1000) bp**

#### 4. Discussion

Among bacterial etiologies of diarrhea *A. hydrophila* is recognized increasingly as a clinically significant enteric pathogen. However, there are limited data on prevalence and associated severity of diarrheal disease caused by *A. hydrophila* in many regions (Mansour *et al.*, 2012).

Out of the one hundred and forty two (142) diarrheic stool samples analyzed, 6 (4.22%) were found to be positive for *Aeromonas hydrophila*. Our results agree with the findings of 4.7% incidence of *A. hydrophila* in Chennai, India (Vila *et al.*, 2003), and higher than the finding of 1.28% (Vasaikar *et al.*, 2002) and 1.4% of *A. hydrophila* from Mumbai, India (Deodhar *et al.*, 1991) and 3.12% in Nigeria (Rogo *et al.*, 2009). Alavandi and Anandhan (2003) reported *Aeromonas* associated diarrhea in 13% samples in Chennai, while Kuijper *et al.* (1987) and Ogunsanya *et al.* (1994) reported 3.7% in Netherlands and 1.4% in Lagos, Nigeria respectively. However, higher prevalence of 17.7 and 28.1% were recorded during 2000 and 2001 in Kolkata, India (Sinha *et al.*, 2004). The recovery of *A. hydrophila* from children <6 years represented the highest percent (2.11%) in this study compared with other age groups. It is believed that gastroenteritis caused by *A. hydrophila* occurred more commonly in children with acute diarrhoea and adults with traveller's diarrhea (2%). Self-limiting watery diarrhea but could be more severe in children (Kuijper *et al.*, 1987). Recovery rates among children with diarrhea vary geographically: 0.62 to 4% in Malaysia (Lee and Puthuchery 2001; Lee and Puthuchery 2002), 0.75% in Nigeria (Kehinde *et al.*, 2001), 2% in Sweden (Svenungsson *et al.*, 2000), 2.3% in Taiwan (Juan *et al.*, 2000), 4.8% in Switzerland (Essers *et al.*, 2000), and 6.8% in Greece (Maltezou *et al.*, 2001). The isolation rates for human fecal specimens vary widely, as geographical areas, patient populations, food habits, level of sanitation, and culture methods influence the recovery rates (Dumontet *et al.*, 2003).

The colonies of bacteria grown in culture media appeared 1-3 mm in diameter and this result agreed with Brenner *et al.* (2005). *A. hydrophila* showed a yellow shine color on TCBS agar and non-lactose fermenters on MacConkey agar. It was smooth, convex, rounded,  $\beta$ -hemolytic colonies and pale white to grey color on blood agar. This is in agreement with Janada & Abbott (2010) and Rogo *et al.*, (2009). This bacteria appeared Gram negative, rod-shaped, singly, in pairs, or even as short chains at the microscopic examination (Brenner *et al.*, 2005).

The biochemical tests used to confirmed the initial diagnosis of *A. hydrophila* . *A. hydrophila* presented appositve result to each of the oxidase , catalase , indole , methyl red , simmon citrate,motility test,vogas prokauer and gelatin liquefaction this results are almost finding in other researchers report (Erdem *et al.* ,2011;Kivanc *et al.*,2011).

To differentiated *A. hydrophila* from *V. cholera* by string test that the all *A. hydrophila* was gave negative results to it (Martin –Carnahan and Joseph ,2005) and the *A. hydrophila* was gave positive results to argnine dehydrogenase and lysine decarboxylation and negative result to ornithine decarboxylation in compare to *V. cholera* was positive result to string test ,and ornithine and lysine decarboxylation and negative result to argnine dehydrogenase (Parija,2009).

In this study standard of biochemical tests by API 20E were used to confirm identification of *A. hydrophila* .indeed Adel *et al.* ,(2011)and Orozova *et al.*,(2010)noticed that all suspected colonies were subsequently confirmed to be *A. hydrophila* using API 20E system and analytical profile index give very good identification .

$\beta$ -hemolysin s as an important bacterial virulence factors which promoting channel formation leading to cell death . the results of this study revealed that the all *A. hydrophila* positive to the  $\beta$ -hemolysin on blood agar this results agreements with Janada and Abott(2010) and EPA (2006). the -hemolytic activity of *A. hydrophila* has been used as an indicator of enterotoxicity and may be responsible for outbreaks of diarrhea (Rahim *et al.* , 1984) Nucleic acid amplification methods targeting virulence genes are used for detection of pathogenic bacteria and to differentiate pathogenic from non-pathogenic strains (Chacon *et al.* , 2003; Sen and Rodgers 2004). Two hemolytic toxins have been found , hemolysin and aerolysin. When the genotypes of known virulent strains as defined in Wong *et al.* (1996) were compared, it was apparent that all the *A. hydrophila* isolates with the a *hh1a* and *aerA* genotype were virulent in the suckling mouse model. These isolates also demonstrated  $\leq$ - hemolytic and cytotoxic activities. Due to the fact that the *aerA* and hemolysin genes were found in the vast majority of the diarrhoeal isolates from this species (Michelle *et al.* , 1999),this results agreement with Howard *et al.* , (1987)The two haemolytic toxins, haemolysin and aerolysin have been described in *A. hydrophila*. When the PCR was performed to detect aerolysin gene (*aerA*), we found that *aerA* were associated with *A. hydrophila*( 52.6% ) harbored *aerA*(Yours *et al.* ,2007). The major hemolysin produced by aeromonads is called aerolysin, though it is know by several other names (cytotoxic enterotoxin, Asao toxin, and cholera toxin cross-reactive cytolytic enterotoxin). Aerolysin is produced by some strains of *A. hydrophila*, Wang *et al.* (2003) developed a multiplex PCR method for detection of hemolysin and aerolysin genes in *A. hydrophila* and *A. sobria* The range of virulence of aeromonads is thought to result from the variety of genotypes present in the environment. Both phenotypic and genotypic heterogeneity are common among aeromonads, Xia *et al.* (2004) cloned the  $\beta$ -hemolysin gene from *A. hydrophila* isolated from freshwater fish in China. The cloned  $\beta$ -hemolysin sequences were used in a PCR assay to survey environmental isolates to detect potential pathogenic *A. hydrophila* strains.( Alperi *et al.* ,2010).

## 5. Conclusions

*Aeromonas hydrophila* recognized one of the most important factors that cause diarrhea disease especially in children under 6 years old and the *Aeromonas hydrophila* have virulence factors such as hemolysin and aerolysin, that confirm pathogenicity this bacteria.

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