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MOLECULAR STUDY OF *Aeromonas hydrophila* ISOLATED FROM STOOL SAMPLES IN NAJAF (IRAQ)

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Abstract- The present study included the detection of *A. hydrophila* in some clinical cases in the governorate of AL-Najaf, during the period from October 2011 to March 2012.The samples was collected from stool samples for *A. hydrophila* isolates. The PCR method of gene (16S rRNA) was the best methods for diagnosis, which has led to isolate and diagnosis of (23) isolate of *A. hydrophila* from stool samples. The PCR technique was used for detection of some genes in *A. hydrophila* like heamolysin *(hyl)* and aerolysin (*aerA*) genes which were responsible for pathogenicity of bacteria. The (*hyl)*and *(aerA)* genes presented in (80%, 72%) respectively for the clinical isolates.

Keywords- Aerolysin and heamolysin genes, *16SrRNA, Aeromonas hydrophila,* PCR

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Introduction

Aeromonas hydrophila is one species of the genus *Aeromonas* that received increasing attention as opportunistic pathogens because of its association with human diseases and aquatic and terrestrial animals infections [1]. *A. hydrophila* is associated with both diarrheal and extraintestinal infection in human disease especially dangerous for children and persons with impaired immune system. The symptoms of the pathological features associated with infection caused by *A. hydrophila* refer to local enema, tissue necrosis, sepsis and mortality [2], resulting in the occurrence of disease entities such as gastroenteritis, wound infections, septicaemia, meningitis, peritonitis, endocarditis, osteomyelitis, etc [3]. This bacterium is linked to two types of gastroenteritis, the first type is a disease similar to cholera which causes rice- watery diarrhea and the other type of disease is dysenteric gastroenteritis that causes loose stools filled with blood and mucus. Dysenteric gastroenteritis is the most severe out of the two types [4].

The pathogenicity of *A. hydrophila* infection is complex and multifactoral [5], and it's attributed to a multiple virulence factors, including cell structural : lipopolysacchariede (LPS), outer membrane proteins (OMPs), pili and flagella, type III secretion system(T3SS) acts as adhesion structures and extracellular factors such as exotoxin, aerolysins, heamolysins, β-lactamase, enterotoxin and sidrophore that seem to play an important role in pathogenesis [2].

The PCR methods, have been developed for routine identification of the species of *Aeromonas*' most frequently involved in human disease [6] and to genetically detect putative virulence genes [7]. Molecular studies on *Aeromonas* species have received a little attention in Iraq. The present study is carried out to achieve the following objectives

1. Isolation of *A. hydrophila* from diarrheal samples and identification by PCR technique, which used specific primer (16S rRNA) diagnostic gene.
2. Identification aerolysin and heamolysin genes in clinical isolates.

Material and Methods

Samples Collection- A total of 110 of diarrheal samples were obtained from patients who attended to Hospitals in AL Najaf governorate during the period from October 2011 to the March 2012.

Identification of *A. hydrophila*

- **Microscopic Properties-** Microscopic properties: Gram's stain was used to examine the isolated bacteria for studying the microscopic properties [8].
- **Cultural Characteristics-** Morphological colonies characteristics were recorded on the specific media for primary identification of *A. hydrophila*
- **Biochemical Tests-** Oxidase, catalase, Simmone Citrate, and Indole tests all these tests positive [9].

API 20 E System

API20E system was carried out according to the procedure of Biomerix company, France.

Molecular Identification

16S rRNA gene for confirmation the identification of *A. hydrophila.*

PCR Assay:- The wizard genomic DNA purification kit is designed for isolation of DNA from G- bacteria. Gel electrophoresis was used for detection of DNA by UV transilluminator [10]. The PCR assay was performed to detect the (*16S rRNA*) gene for confirmation the identification of *A. hydrophila* and to detect the virulence factors genes, shown in [Table-1]. These primers synthesized by Alpha DNA company, Canada [11,12].

Table 1- Sequence and Concentration of Forward and Reverse Primers.

Primer type	Primer sequence	Concentration In picomoles	Product size
16Sr RNA - F	5-CCAGCAGCCGCGGTAATACG-3	86900	300 bp
16Sr RNA - R	5-TACCAGGGTATCTAATCC-3	128177	
aerA-F	5'-AGCGGCAGAGCCCGTCTATCCA-3'	92012	416 bp
aerA-R	5'-AGTTGGTGGCGGTGTCGTAGCG -3'	75793	
hyl -F	5'-GGCCCGTGGCCCGAAGATGCAGG-3'	73167	597 bp
hyl -R	5- CAGTCCCACCCACTTC-3'	94775	

Concentration of DNA was determined spectrophotometrically by measuring its optical density at 260 nm (Extinction coefficient of dsDNA is 50 µg/ml at 260 nm) the purity of DNA solution is indicated by ratio of OD260/OD280 which is in the range of 1.8± 0.2 for pure DNA [13]. PCR program that apply in the thermocyler illustrate in [Table-2]. The PCR products and the ladder marker are resolved by electrophoresis on 1.2% agarose gel [11,12].

Table 2- PCR program that apply in the thermocyler.

Gene	Initial denaturation	Cycles Denaturation	Annealing	Elongation	Final elongation
16S r RNA	94ºC for 3 min	94ºC for 30 sec.	30 cycles 52ºC for 30 sec.	72ºC for 30 sec.	72ºC for 10 min
aerA *hyl*	95ºC for 5 min	95ºC for 30 min	30 cycle 55ºC for 1 min	72ºC for 1 min	72ºC for 7 min

Results

Isolation and Identification of *A. hydrophila*

A total 110 samples were collected from patients suffering from diarrheal infection. The colonies of *A. hydrophila* are yellow shin color on TCBS agar [Fig-1] with diameter ranged from 2-3 mm. In addition to those colonies appeared as pale like shaped on the MacConkey agar indicated that *A. hydrophila* is unable to ferment lactose sugar. On blood agar *A. hydrophila* produces smooth, convex, rounded and β-hemolytic colonies and pale white to grey color, as show in [Fig-2].

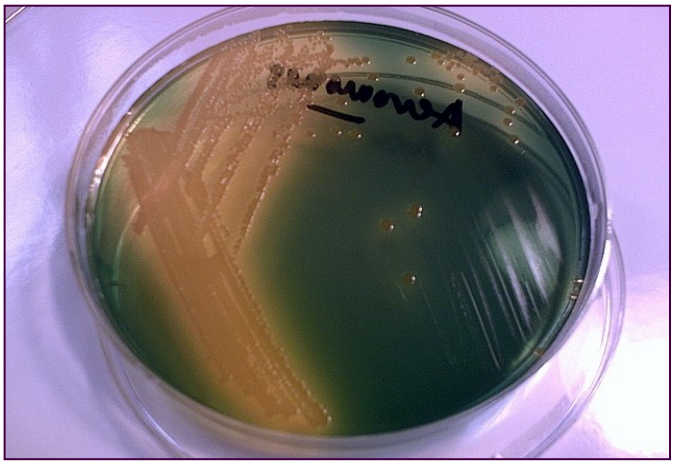

Fig. 1- *A. hydrophila* on Culture Media such as TCBS agar

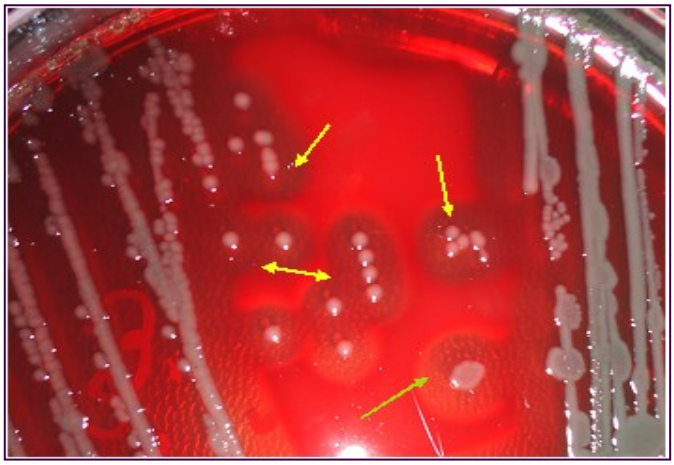

Fig. 2- *A. hydrophila* on Culture Media such as Blood agar

The results of biochemical tests indicated that 44 isolates belong to *A. hydrophila,* all isolates are positive result for oxidase test. *A. hydrophila* isolates are characterized by their ability to ferment glucose with gas formed on kligler iron agar (Alk/Acid), it produces (Alkaline) red color top and bottom (acidic) yellow color with gas formed but not H2S; it gives a positive result to, catalase, vogues-Proskauer, Indole, simmone citrate tests. *A. hydrophila* isolates gives negative result to string test, which is differentional test between *A. hydrophila* and *V. cholera*.

API 20E system is used to confirm identification of *A. hydrophila* included in this study. The results demonstrate that 26 clinical isolates are positive in identification by API 20E, Using the analytical profile index of this system the identification percentage is (id% = 97.8), and the rest strains give negative result.

Molecular Identification of *A. hydrophila* by PCR Technique

Detection of *16Sr RNA* Gene of *A. hydrophila.*

PCR technique has been used to amplify genes of *16Sr RNA* gene from genomic DNA of all *A. hydrophila* isolates. DNA is extracted from all isolates. The results of isolates diagnosis using the PCR technique for 16SrRNA detection clarify that 23 isolates of *A. hydrophila,* producers carrying *16S r RNA* gene that is characteristic of *A. hydrophila,* as shown in [Fig-3].

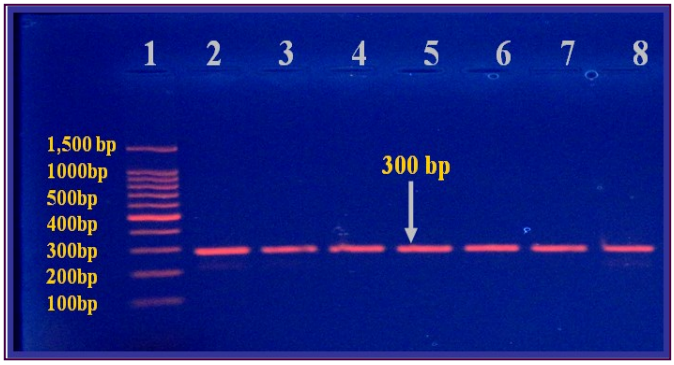

Fig. 3- Agarose Gel Electrophoresis 1.2% of PCR Amplified of *16S rRNA* Gene 300 bp of *A. hydrophila* Isolates for 55 min at 100 volt.
Lane 1 DNA marker (100bp ladder).
Lane 2,3,4,5,6,7,8 Amplify of 16Sr RNA gene in clinical isolates.

Detection of Heamolysin Gene (*hyl*)

PCR technique has been used to amplify heamolysin gene (*hyl*) from genomic DNA of all *A. hydrophila* isolates. Isolates with specif-

ic primer, which responsible for heamolysin toxin, as shown in [Fig-4]. Most of clinical isolates contain (80%) of (*hyl*) gene of *A. hydrophila*.

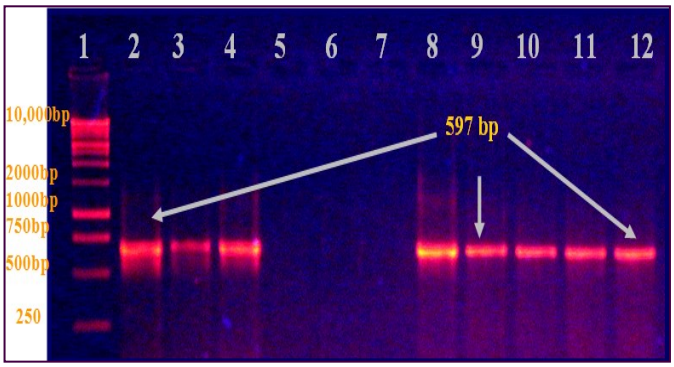

Fig. 4- Agarose Gel Electrophoresis 1.2% of PCR Amplified of (*hyl*) gene 597 bp of *A. hydrophila* isolates for 55 min at 100 Volt.

Lane 1 Marker (1kb DNA ladder)
Lane 2,3,4,8,9,10,11 positive result for clinical isolates of A. hydrophila while 5,6,7 were negative result.

Detection of Aerolysin Gene (*aerA*)

Detection of Aerolysin gene (*aerA*) by PCR technique is presented in [Fig-5]. Clinical isolates of *A. hydrophila* possess 72% of this gene. Specific primers of *aerA* gene with genomic DNA of *A. hydrophila* isolates were used in this study

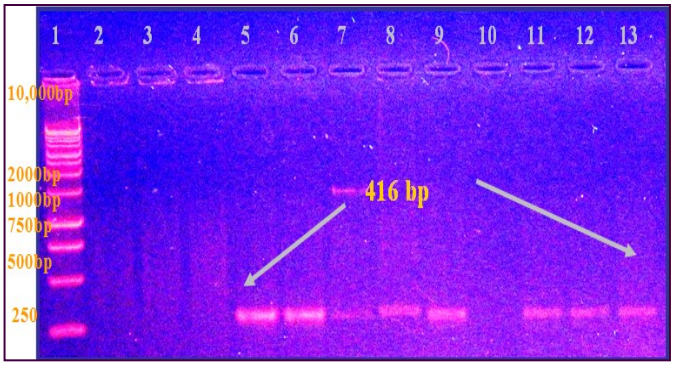

Fig. 5- Agarose Gel Electrophoresis 1.2% of PCR Amplify of (*aerA*) gene of *A. hydrophila* isolates for 55 min at 100 Volt.

Lane 1 Marker (1kb DNA ladder)
Lane 5,6,7,8,9,11,12,13 were positive result isolates of A. hydrophila while 2,3,4 and 10 were negative result.

Discussion

Isolation and Bacteriological Diagnosis

Identification of *A. hydrophila* depends on the colonial morphology, biochemical tests, Api20E system and molecular identification. The colonies of *A. hydrophila* are yellow shin /green color on TCBS agar due to sucrose ferment, with diameter ranged from 2-3 mm,these typical characteristics being described by referential studies [14]. In addition, those colonies appear as pale like shaped that indicated *A. hydrophila* is unable to ferment lactose sugar on the the Mac-Conkey agar. *A. hydrophila* produces smooth, convex, rounded and β -hemolytic colonies when grow on the blood agar. These results are agree with [15,16]. *A. hydrophilla* consists of straight, coccobacillary to bacillary gram-negative bacteria with rounded ends, it occurs singly, in pairs, and rarely as short chains. Motile strains produce a single polar flagellum, though peritrichous or lateral, flagella [17]. The biochemical tests of *A. hydrophila* involved its ability to ferment glucose produces acid and gas, it ferments many sugars such as galactose, and, sucrose, but non-fermented arabinose and lactose,. It was able to grow on kligler iron agar and positive for catalase, Vogus-Proskaur reaction, and oxidase test but does not urease and H2S produced API20E system is used to confirm identification of *A. hydrophila* [1]. The findings demonstrate that 59 clinical isolates are positive results in API20E, these results agree with [18,19].

Table 3- Virulence factors genes of A. hydrophila in present study.

	Virulence factors genes		
No. of isolates	(*hyl*) gene	Gene (*aerA*)	Both genes
A.H 1 S	+	-	-
A.H 2 S	+	-	-
A.H 3 S	+	-	-
A.H 4 S	-	+	-
A.H 5 S	-	+	-
A.H 6 S	-	+	-
A.H 7 S	+	+	+
A.H 8 S	+	+	+
A.H 9 S	+	-	-
A.H 10S	+	+	+
A.H 11S	+	+	+
A.H 12 S	+	+	+
A.H13 S	-	-	+
A.H14 S	+	+	+
A.H15 S	+	+	+
A.H16 S	+	+	+
A.H17 S	+	+	+
A.H18 S	+	+	+
A.H19 S	+	+	+
A.H20 S	-	+	-
A.H21 S	+	+	+
A.H22 S	+	+	+
A.H23 S	+	+	+

A.H.: A. hydrophila isolate, (hyl):heamolysin gene, (aerA): aerolysin gene, S : Stool sample

Molecular Identification of *A. hydrophila* by PCR Technique

PCR technique has been used to amplify *16SrRNA* gene from genomic DNA of all *A. hydrophila.* Results of the present study demonstrate significant differences between the methods used and PCR in the diagnosis for *A. hydrophila* isolates. Where the method of PCR was more sensitive compared to other methods, whereas it shown that 23 isolate were diagnostic as belong to *A. hydrophila* isolates, depending on the diagnostic gene *16S rRNA*, that selected specific primer to this gene according to [12]. Most isolates of *A. hydrophila* isolate gave a positive result to detected for (*16SrRNA*) gene [7,20]. who noted that the ribosomal mainly 16S r RNA gene has proven to be a stable and specific molecular marker for the identification of *A. hydrophila* bacteria. The results agree with [12,21] which refer that 16rRNA gene was specific marker in all strain of *A. hydrophila*.

Detection of Heamolysin gene (*hyl*) and Aerolysin gene (*aerA*)

PCR is used to amplify a precise fragment of DNA from a complex mixture of starting material usually template genomic DNA. A number of reports are available for PCR amplification of conserved aerolysin gene and heamolysin gene of *A. hydrophila* [22]. Other virulence genes of *A. hydrophila* have also been detected by PCR [7].

The present study is conducted by PCR assays to detect the presence of specific virulence traits or the genes encoding these traits. The results of the present study show that most of *A. hydrophila*

isolates from clinical samples gave a positive results to detection for (*hyl A*) gene by using the PCR technique, whereas these results show that clinical isolates of *A. hydrophila* are 80% to (*hyl A*) gene while Aerolysin gene (*aerA*) were 72% by using PCR technique as shown [Fig-4], [Fig-5], and through statistical analysis was not observed any signification difference at level $p \leq 0.05$ (ANOVA) in all results between clinical isolates in the present study.

Whereas, (*hyl*) gene is observed as responsible for disease occurrence especially diarrhea, which is confirmed by some studies that some bacteria cause diarrhea by production of enterotoxins or by invasion of the gastrointestinal epithelium [2].

Aerolysin is a hemolytic toxin protein secreted by *Aeromonas hydrophila*. It is known that pathogenic isolates of *A. hydrophila* secrete aerolysin toxin that causes the lysis of the RBCs [23]. The results of this study agree with several studies [11,22,24].

Conclusions

The following conclusions are extracted from the present study :1- The molecular techniques is necessary for detection of pathogenic bacteria of clinical sources. 2- The molecular study provides definite identification of virulence factors such as aerolysin (*aerA*) and heamolysin (*hyl*).

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