Isolation and Characterization of *A. hydrophila* from the Al-Jadryia River in Baghdad (Iraq)

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Abstract In this paper, we described detection of *Aeromonas hydrophila* in water of AL-Jadryia river (Baghdad city, Iraq), during the period from July, 2012 to April, 2013. The samples collected from water of Jaderia river and transfer to Bacteriology laboratory for diagnosis. *A. hydrophila* isolates which were diagnosed by three methods (Culture method, biochemical tests, Api20E system), in Culture method was isolated (71) isolate on blood agar and TCBS media and (44) isolates by biochemical tests while Api20E kit was the important method for diagnosis, which has led to isolate and diagnosis of (36) isolate of *A. hydrophila*. Also which used the PCR method of gene Tetracycline gene (*tetA-E*) was the best methods for diagnosis, which has led to isolate and diagnosis as (27) isolates of *A. hydrophila* have *tetA-E* sequence gene of all samples. The virulence factors of bacteria were detected and showed that all isolates produced of heamolysin, protease, lipase enzymes and also detection of tetracycline resistant gene of these samples.

Keywords: *Aeromonas hydrophila* of water, tetracycline gene (*tetA-E*), lipase, heamolysin, protease

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1. Introduction

The genus *Aeromonas* is a member of the family Aeromonadaceae that are primarily aquatic organisms found in water [1]. *Aeromonas* infections are one of the most common bacterial diseases diagnosed in marine and cultured freshwater fish. *Aeromonas hydrophila* is found in diverse habitats, including soil, widely in fresh and salt water also frequently found in chlorinated and non-chlorinated drinking water, and is pathogenic to warm and cold-blooded animals [2]. Aquatic environment along with water and seafood is thus important potential source for the transmission of *A. hydrophila* resulting in human infections. *Aeromonas* spp. have been involved in wound infections, sepsis, outbreaks of water, and food-borne gastroenteritis [3]. Direct contact with contaminated water and soil is the most frequent cause of gastrointestinal and wound infections in humans [4].

There has been an increasing incidence of antimicrobial resistance among *Aeromonas* sp. isolated from aquaculture environments, five classes of genetically distinguishable tetracycline resistance determinants designated A through E, have been described among aerobic enteric gram-negative bacteria and Several studies have shown *tetE* to be the predominant determinant among the different classes of tetracycline-resistant genes (4,15).

The pathogenicity of *A. hydrophila* infection have been implicated in the cause of numerous human infections, such as gastroenteritis, cellulitis, meningitis, bacteremia, soft-tissue infections, peritonitis, and broncho pulmonary infections [5]. Various putative virulence factors (aerolysin/hemolysin, proteases, lipases, DNases, enterotoxins) that may play an important role in the development of diseases, either in humans or in fish, have been described in several species of the genus [6]. Some authors reported that the production of cytotoxins and hemolysin is related to *A. hydrophila* and *A. veronii* bv, and hemolytic molecules seem to be related to enterotoxigenicity [7]. Virulence in *Aeromonas hydrophila* is multifactorial which these enzymes that play significant role in pathogenesis. *A. hydrophila* has been an increasing incidence of antimicrobial resistance among *Aeromonas* sp. isolated from aquaculture environments [8]. Broad-spectrum antibiotics, such as tetracycline, are prescribed clinically for the treatment of such infections. Several studies have shown *tetE* to be the predominant determinant among the different classes of tetracycline resistant genes of *A. hydrophila* [6,9].

Molecular studies on *Aeromonas* species have received a little attention in Iraq and this study was considering the first molecular study in Iraq. The present study is carried out to achieve the following objectives:

1- Isolation of *A. hydrophila* isolates among the environmental samples and identification by API20E system and used the PCR technique, with specific primer (*tetA-E*) sequence gene.

2- Detection the virulence factors of *A.hydrophila* isolates such as heamolysin, lipase, protease and Phospholipase.

2. Methods

A total of samples collection (440) samples of water were obtained from Al- Jadryia bridge water in Baghdad who attended to Bacteriology laboratory in Sciences faculty during the period from August,2012 to the April,2013. They were swabbed onto thiosulfate citrate bilesalts sucrose (TCBS) agar and MacConkey agar (MC) the plates were incubated overnight.

2.1. Identification of *A.hydrophila*

Morphological colonies characteristics were recorded on the media that are used (MacConkey agar, blood agar and TCBS agar) for primary identification of *A.hydrophila* and microscopic properties by Gram's stain was used to examine the isolated bacteria for studying the microscopic properties such as gram reaction, shape and motility [10]. While Biochemical tests used Oxidase test, Catalase test (Hydrogen Peroxide 3%), Simmon's Citrate test, Indole Production test, Motility test were all these tests and urease test result according to [11]. Also, API20E system was carried out according to the procedure of (Biomerix company, France).

2.2. Detection Virulence Factors of *A.hydrophila*

Heamolysin was detection according to [12] tested for β -hemolytic activity on base agar (Himedia, India) supplemented with 7% sheep erythrocytes. A loopful of an overnight growth from nutrient agar were cultured on blood agar by streaking method, incubated at 37°C for 24 hr. Protease was detected by [13] was tested on 2% agar-agar (Himedia, India) containing 10% (w/v) skimmed

milk (Himedia, India). A loopful of an overnight growth from nutrient agar were cultured on agar by streaking method, incubated at 37°C for 24 h. While Lipase detection was performed on Tween 20 agar [12].

2.3. Molecular Identification Tetracycline Resistant Gene of *A.hydrophila*.

PCR Assay:

The wizard genomic DNA purification kit is designed for isolation of DNA from G- bacteria by a universal extraction kit of (Promega company). Gel electrophoresis was used for detection of DNA by UV transilluminator [14]. Concentration of DNA was determined spectrophotometrically by measuring its optical density at 260 nm (Extinction coefficient of dsDNA is 50 μ g/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 [16]. PCR program that apply in the thermocycler. The PCR products and the ladder marker are resolved by electrophoresis on 1.2% agarose gel. [15].

3. Results

3.1. Isolation and Identification of *A.hydrophila*

A total of (440) samples were collected from water of AL-Jadryia river (Baghdad city, Iraq), and was observed (71) isolate on media. The colonies of *A. hydrophila* are yellow shin color on TCBS agar, 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 [Figure 1].

Figure 1. A- *A.hydrophila* produces smooth, convex, rounded colonies: A- β -hemolytic colonies and white to grey color on Blood agar. B- Pale colonies on MacConkey agar

Microscopical examination has revealed that (44) isolate of *A.hydrophila* a gram negative bacillus, straight shape, singly or pairs and rarely as short chains, and not spore forming. The results of biochemical tests were adopted as a complementary characteristic of the initial diagnosis of *A.hydrophila*, where the results indicated that isolates belong to *A.hydrophila*, all isolates are positive result for oxidase test. [Figure 1] reveals that *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, Indole, simmone citrate tests.

API 20E system is used to confirm identification of *A.hydrophila* included in this study. The results demonstrate that (36) isolate were positive in identification by API20E, as shown in [Figure 2]. Using the analytical profile index of this system the identification percentage is (id% = 93.8).

Figure 2. API 20E system for identification of *A. hydrophila* after 24 hrs. incubation

The ability of *A. hydrophila* (36) isolates to produce some virulence factors. All *A. hydrophila* showed positive result for hemolysin production (100%), type beta (β - hemolysin), when cultured on blood agar medium. The isolates also showed the ability to produce protease to hydrolyze the protein (100%), when inoculated on skim

milk agar for 24 hours at 37°C. Also the ability to hydrolyze fats by lipase enzyme when inoculated on 1% Tween agar for (3-5) days at 37°C. While all isolates of *A. hydrophila* have no ability to produce urease enzyme as shown in [Figure 3].

Figure 3. Some Virulence Factors of *A. hydrophila* (A)-Lipase production (B)- Protease production

While, molecular identification by PCR technique has been used to amplify gene of the (*tet-E*) 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 (*tet-E*) detection clarify that isolates of *A. hydrophila*, producers carrying (*tet-E*) gene by 26/36 (72.22). Specific primers of *aerA* gene with genomic DNA of *A. hydrophila* isolates were used in this study as shown in [Figure 4]. The PCR assay was performed to detect the (*tetE*) gene of *A. hydrophila*, tetA-E – Forward Primer (F) 5'- ATGAACCGCACTGTGATGATG-3' and tetA-E

– Reverse Primer (R) 5'- ACCGACCATTACGCCATCC-3' with size (744 bp), these primers synthesized by Alpha DNA company, Canada [15]. The thermocycler for this primer by following : Initial denaturation 94°C for 3 min and (94°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec for 30 cycles) and 72°C for 10 min final elongation. The amount (concentrations) of the PCR components were included final volume 25 μ l in tube PCR by DNA template (5 μ l), Forward primers (2.5 μ l) Reverse primers (2.5 μ l), Gotaq master mix (12.5 μ l) and distilled water (2.5 μ l).

Figure 4. Agarose Gel Electrophoresis (1.2%) of PCR Amplify of (*tetE*) gene of *A. hydrophila* isolates for 55 min at 100 Volt

Lane (M) Marker (1kb DNA ladder).

Lane (1,2, 3, 4, 5, 6) Positive isolates of *A. hydrophila* to

Lane (7) Negative isolates of to (*tetE*) gene

4. Discussion

The present study is conducted to isolation and identification (36) isolate of *A. hydrophila* bacteria from

water samples of AL-Jadryia river (Baghdad city, Iraq). *Aeromonas. hydrophila* causes a wide range of human illnesses possible routes of transmission include contaminated water, food and exposure of wounds to environments that contain the pathogen. In general, the

genus *Aeromonas* are facultative anaerobic, oxidase positive, Gram negative bacteria whose natural habitat is in the aquatic environment. Some species are pathogenic for animals and human.

Identification of *A. hydrophila* depends on the colonial morphology, biochemical tests, Api20E system and molecular identification for (*tet-E*) gene. The colonies of *A. hydrophila* are yellow shiny /green color on TCBS agar due to sucrose ferment, with diameter ranged from (2-3)mm. In addition, those colonies appear as pale like shaped on the MacConkey agar that indicated *A. hydrophila* is unable to ferment lactose sugar. But it is grow on the blood agar with produces smooth, convex, rounded and α -hemolytic colonies and pale white to grey color, [Figure 1], these typical characteristics being described by referential studies [17]. These results are agree with [18,19,20]. API20E system is characterizes by fast detection of bacteria without the need for many of culture media as well as reduce cultural contamination, and it is used to confirm identification of *A. hydrophila*, [21]. The isolation rate of *Aeromonas hydrophila* in many developing countries. In environmental isolates, *Aeromonas hydrophila* has been isolated in rates as high as 82% in Senegal [22] and in 77% of food samples in Kenya [23]. In the Venda region of South Africa, *Aeromonas species* are isolated from clinical and environmental samples [24], while the studies in Iraq refer that the isolation rate of *A. hydrophila* by 6.6% from stool cases in Baghdad [25] who study of Ecological and physiological on *A. hydrophila* and role enterotoxin in pathogenicity, and 8.9% in Baghdad [26] which studied, some characteristics and immunological effects of *A. hydrophila* enterotoxin. Whereas [27] refer that the isolation rate reach to 9% from stool samples and 20% from Tigris water, when studied the crude growth extract effect *A. hydrophila* on normal and Cancer cell lines.

A. hydrophila bacteria have produced a variety of biologically active extracellular products similar to the virulence factors of enteropathogenic bacteria and these virulence factors associated with health effects in humans [28] and causes many diseases as significant human pathogens causing a variety of extra-intestinal infections. Extra-intestinal and gastrointestinal infections were known to occur in previously healthy hosts as well as immunocompromised or otherwise susceptible populations [29]. Virulence factors were present in two forms, cell-associated structures, and extracellular products. The cell-associated structures involved pili, flagella, outer membrane proteins, lipopolysaccharide, and capsules. The major extracellular products include cytotoxic, cytolytic, hemolytic, and enterotoxic proteins. *Aeromonas* produce an array of filamentous structures, including short rigid, and long wavy pili, as well as polar and lateral flagella. Polar flagella and lateral flagella are described by [30,31]. Polar flagellins function as adhesions, while lateral flagellum's serve as colonization factors [32].

The present study identifies some virulence factors associated with pathogenicity of *A. hydrophila*, where it is observed that all environmental isolates had ability to produce β -hemolysin (100%) which cause complete hydrolysis of RBCs on blood agar, this result similar to [33,34]. As shown to be cytolytic for the erythrocytes and mammalian cells in culture. However, the hemolytic activity of *A. hydrophila* has been used as an indicator of

enterotoxigenicity and may be responsible for outbreaks of diarrhea [35]. As researchers indicate that isolates had high pathogenicity and also environmental are responsible for diseases occurs in human because they secrete different toxins [36]. Some reports were indicated that β -hemolysin which is produced from *A. hydrophila* has a close relationship to the production of toxins in the cell producing enzyme, and toxin called Cytotoxic factors. Hemolysin is made in the logarithmic phase of cell growth [37]. and it is virulence factor important for *A. hydrophila* bacteria. On other hand, in this study was reveal that *A. hydrophila* were able to hydrolyze the protein by protease enzyme (100%) when tested on skim milk agar, and these results were agree with [38,39] who indicates that *A. hydrophila* is producing protease enzyme. Protease enzyme secreted outside of the cell through a process of growth as they accumulate significantly in the phase stability of the bacteria, and it is one of virulence factors important for *A. hydrophila* bacteria [40].

A variety of antibiotics have been used to treat infection caused by *A. hydrophila* and have proved useful in many cases, but multiple antibiotic resistances are common among *A. hydrophila* [28]. Many strains of *A. hydrophila* are known to harbour mobile elements that encode antibiotic resistance and can be transferred among themselves or to other bacterial species' to establish multiple antibiotic resistances [41]. The widespread use of antibiotics has been identified as a major factor responsible for the increased incidence of antibiotics resistance [1]. Molecular identification of (*tetE*) gene by PCR 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 (*tetE*) gene of *A. hydrophila* [42]. The present study showed that most of isolates of *A. hydrophila* produces carrying (*tet-E*) gene by 26/36 (72.22). Specific primers of *aerA* gene with genomic DNA of *A. hydrophila* isolates were used in this study as shown in [Figure 4]. The results of this study agree with several studies [37,43]. This results leading to higher level of resistance to is recorded for these antibiotic may be caused by initial mutation located in a single site on the *A. hydrophila* chromosome [43].

5. Conclusions

The following conclusions are extracted from the present study:

- 1- The frequency of *A. hydrophila* isolates in water of AL-Jadryia river (Baghdad city) was higher in environmental isolates.
- 2- The molecular study provides definite identification of antibiotics gene such as tetracycline gene.
- 3- Most environmental isolates produced many virulence factors involve hemolysin, lipase and protease.

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