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Study of Some Virulence Factors of Aeromonas Hydrophila Isolated from Clinical Samples (Iraq)

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Abstract- The present study included the detection of Aeromonas hydrophila in clinical samples were taken from patients suffering from various infection at Al-Sadder Medical City and Al-Zahraa Educational Hospital during the period from October 2011 to March 2012.The samples were collected from stool, sputum ,urine, blood , cerebral spinal fluid (C.S.F), wound and burns samples. A.hydrophila isolates which were diagnosed by four methods as (Culture method, biochemical tests, Api20E system and Polymerase chain reaction (PCR)). Api20E kit was the important method for diagnosis, which has led to isolate and diagnosis of (28) isolate of A.hydrophila, but the PCR method of gene (16S rRNA) was the best methods for diagnosis, which has led to isolate and diagnosis as (25) clinical isolates were includes (23)isolate from diarrhea samples and (1)isolate for each wound and sputum samples, with no isolated from cerebral spinal fluid ,blood and urine samples. The virulence factors of bacteria were detected and showed that all isolates were produced of heamolysin, protease, lipase, phospholipase, gelatinase, sidrophore, and capsule formation. Also, A.hydrophila has the ability to adhere to uroepithelial cell of human.

Keywords: *Aeromonas hydrophila , 16S rRNA gene Capsule, Sidrophore, heamolysin, phospholipase and Lipase*

I. 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 animal's 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 multifactorial [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, lactamase, enterotoxin and sidrophore that seem to play an important role in pathogenesis[2].

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 clinical cases and identification by API20E system and the PCR technique, with used specific primer (16S r RNA) diagnostic gene.

2- Detection the virulence factors of A.hydrophila isolates such as heamolysin, lipase, protease, gelatinase hydrolyze, phospholipase, sidrophore, capsule formation and adherence to uroepithelial cells of human among clinical isolates.

II. MATERIALS AND METHODS

A. Samples collection

159 clinical samples were involved 110 diarrheas, 11 sputum, 6 cerebral spinal fluid (C.S.F), 7 wounds, 6 burns, 9 Blood, and 10 Urine from patients who attended to AL-Sadder Medical City and AL-Zahraa Educational Hospital in Najaf governorate (IRAQ) during the period from October 2011 to the March 2012.They were swabbed onto thiosulfate citrate bilesalts sucrose (TCBS) agar and MacConkey agar (MC) the plates were incubated overnight.

B. Identification of A. hydrophila

1) Microscopic Properties:

Gram's stain was used to examine the isolated bacteria for studying the microscopic properties as initial identification of A.hydrophila [6].

2) Cultural Characteristics:

Morphological colonies characteristics were recorded on the specific media for primary identification of A. hydrophila

3) Biochemical Tests :

Biochemical tests of *A.hydrophila* 1-2 colony were tested for oxidase, catalase, Simmone Citrate, Kligler Iron agar and Indole tests and these entire tests positive [7].

4) API 20 E System

Strains identification of A.hydrophila using API 20E (Biomerieux, France).

5) Molecular Identification

16S rRNA gene for confirmation the identification of A.hydrophila [8]. All the cultures were grown on brain heart infusion agar at 37°C for 18-24 h. The genomic DNA of each strain was obtained through the wizard genomic DNA purification kit (Promega kit). 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 [9]. The PCR assay was performed to detect the (16S rRNA) gene for confirmation the identification of A. hydrophila, shown in [Table-1]. This primer synthesized by Alpha DNA company, Canada.

Concentration of DNA was determined spectrophotometrically (Orient research, USA) 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 [10]. 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.

C. Detection virulence factors of A.hydrophila

1) Heamolysin Detection

The strains were tested for β-hemolytic activity on base agar (Himedia, India) supplemented with 7% sheep erythrocytes. A loopful of an overnight growth from nutrient agar was cultured on blood agar by streaking method, incubated at 37°C for 24 hr. The presence of a clear colorless zone surrounding the colonies indicated hemolytic activity [11].

2) Lipase Detection

Lipase activity was determined by Tween 20 agar. A loopful of an overnight growth from nutrient agar were cultured on Tween 20 agar by streaking method, incubated at 37°C for 24h. Turbid zone around colonies with change to blue color after added the regent (CuSO4.5H2O Solution), indicates a positive result [11].

3) Protease Detection.

Protease hydrolysis 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. The presence of a transparent zone around the colonies indicated protease activity [12].

4) Phospholipase (Lecithinase) Detection

The specific media for detection phospholipase was inoculated with a single colony of overnight culture from nutrient agar, incubated for (24-72) hr at 37°C. Changing the color of precipitation zone around the colonies from white to brown color considered a positive result [13].

5) Gelatinase Detection

Gelatinase was assayed by the conventional technique. The tubes of medium containing (1.2g of gelatin for 100 ml of Nutrient broth) were inoculated with a single colony of overnight culture from nutrient agar, they were incubated at 37°C for 24 h, tests for gelatin liquefaction were are made after 24-48 h, incubation by refrigerating the tubes and comparing with an uninoculated control tube. If the gelatin was liquefied as the result of gelatinase activity, the medium no longer solidifies when refrigerated [11].

6) Sidrophore Detection

Sidrophore detection was performed on M9 media which is prepared as suggested by [14]. The media was inoculated with single colonies of overnight culture by streaking method incubated for 24hr at 37°C. The appearance of the growth of A.hydrophila on M9 media indicated a positive result.

7) Capsule Production Detection

Capsulation was be demonstrated through India ink preparations. India ink preparation counterstained with crystal violet showing unstained clear halo indicative of a capsule surrounding individual bacilli or Capsule Production was performed as described by [15].

8) Adherence to Human Uroepithelial Cells

The human epithelial cell was maintained in nutrient broth medium used for maintained the cell without antibiotics. Log-phase cultures of Aeromonas spp. were diluted in phosphate buffer solution supplemented with Dulbecco's Mineral Salt Solution (BDH, England) 0.5% (PBSS) to a concentration of (2-3) x lo6 cfu/ml as determined by serial dilution and plating on to BA. Semiconfluent monolayers human epithelial cell of grown for 20 h on glass coverslips in 24- well trays (Costar Tissue Culture Cluster, Cambridge, MA, USA) were washed with 2 ml of PBSS. Bacterial cultures were added in 1-ml volumes to the wells, and three wells were used for each test. After incubation for 90 min at 37°C in an atmosphere of C02 5% in air, nonadherent bacteria were removed from the infected monolayers by washing four times in situ with 2 ml of PBSS. The cells were fixed for 5 min; the over slips were then mounted on glass microscope slides and stained by Geimsa stain method. Adhesion was assessed by bright-field microscopy at a magnification of 1000 x under oil immersion. Used some strains control for this assay.The human epithelial cell adherence assay was performed as described by Lomberg et al., (1986). All strains were tested in duplicate. The following strains were included as positive controls: Which stains by Geimsa stain [16].

III. RESULTS

A. Isolation and Identification of A.hydrophila

A total of (159) samples were collected from patients suffering from different infections. 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.

Microscopical examination has revealed that 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.

B. Identification of A.hydrophila by API 20 E System:

This system is a standardized colorimetric identification system for enteric bacteria and it is considered as an acceptable method for identification of the more commonly-occurring members of the family Aeromonadeace. This system is characterized by fast detection of bacteria without the need for several culture media as well as reduce cultural contamination, API 20E system is used to confirm identification of A.hydrophila included in this study. The results demonstrate that (28) clinical 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% = 97.8).

C. Molecular Identification of A.hydrophila by PCR Technique

Polymerase chain reaction 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 isolates of A.hydrophila, producers carrying 16S r RNA gene that is characteristic of Aeromonas hydrophila including:(25)clinical isolates, as shown in [figure-3].

D. Detection of some Virulence Factors for A.hydrophila

The ability of A.hydrophila (25) isolates to produce some virulence factors. All isolates of A.hydrophila were positive for heamolysin production (100%), type beta (β-heamolysin). Also these isolates were capsulated 25(100%) for clinical isolates [figure-4]. The protease and lipase production were detected of A.hydrophila as shown in [figure -5, 7]. All isolates had the ability to produce protease by hydrolyze the protein(100%). Also the ability to hydrolyze fats by lipase enzyme 25/25(100%) clinical isolates. In addition, the bacteria were able to produce phospholipase, gelatinase enzyme, siderophores. The Phospholipase production was 22/25(88%) for clinical isolates. Likewise clinical isolates of A.hydrophila were siderophores and gelatinase production (100%. All isolates of A.hydrophila isolates have no ability to produced urease enzyme. On the other hand, the ability of A.hydrophila isolates to adhere to epithelial cells of human was shown in [figure-6]. It has given positive results to adhesion by the rang of adhesion (40-76 cell/ml) for 22(88%) of the clinical isolates.

IV. DISCUSSION

A. Isolation and Identification of A.hydrophila

The present study is conducted to isolation and identification of A.hydrophila bacteria from clinical samples, (25) isolate of A.hydrophila has been isolated from clinical infections. Clinical samples were involve diarrhea, wound, burns, sputum, cerebral spinal fluid (C.S.F), blood and urine samples A.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 humans [17]. The taxonomy of the genus Aeromonas has been confusing because of lack of matching between phenotypic and genotypic characteristics of species and multiple methods that are required for accurate classification [18]. 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 [19]. In addition, those colonies appear as pale like shaped on the MacConkey agar that indicated A.hydrophila is unable to ferment lactose sugar. A.hydrophila when grow on the blood agar produces smooth, convex, rounded and β-hemolytic colonies and pale white to grey color. These results are agree with [20,21,22]. According to the results, A.hydrophila consists of straight, coccobacillary to bacillary gramnegative 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 may be formed on solid media by some species [17,23,24]. The biochemical tests of A. hydrophila was the ability to grow (Alk/Acid) on kligler iron agar and positive for simmone citrate, indole, catalase tests, and oxidase test that is different from enteric family bacteria, but does not urease and H2S produced. The biochemical tests were accordance with the standard characteristics [6,7,11]. 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 [1], and these results agree with [25,26,27,28].

B. Molecular Identification of A.hydrophila by PCR Technique

Polymerase chain reaction technique has been used to amplify 16SrRNA gene from genomic DNA of all A.hydrophila isolates. Hybridization groups (HGs) are recognized as having no reliable phenotypic characteristics, resulting in confusion among microbiologists and physicians. Some of the newly recognized Aeromonas are described from a handful of strains, making it difficult to describe the species phenotypically. The current taxonomy of the genus Aeromonas is based upon DNA-DNA hybridization and 16S ribosomal DNA related studies. The genera of the family Aeromonadaceae now include Aeromonas[17], A.hydrophila [29]. The first attempts to identify Aeromonas to genotype

rely upon differences in 16S ribosomal DNA sequences are doing [30], and several investigators developed probes for detection of various Aeromonas spp. [31]. Results of the present study demonstrated 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 (25) isolate were diagnostic as belong to A.hydrophila for clinical isolates, depending on the diagnostic gene 16S rRNA ,that selected specific primer to this gene [9]. The ribosomal mainly 16S r RNA gene has proven to be a stable and specific molecular marker for the identification of Aeromonas hydrophila bacteria [32,33]. The results agree with [9,34,35] which refer that 16rRNA gene was specific marker in all strain of A.hydrophila.

C. Virulence Factors of A.hydrophila

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[2], 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 [36,37]. Besides, diarrheal illness, Aeromonas spp. cause wound infections, septicemia, meningitis, ophthalmitis and endocarditis. Factors contributing to virulence include toxins, proteases, heamolysins, lipases, adhesins, agglutinins and various hydrolytic enzymes [38]. 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 [39]. Polar flagellins function as adhesions, while lateral flagellum's serve as colonization factors [40]. The present study identifies some virulence factors associated with pathogenicity of A.hydrophila, where it is observed that all isolates for A.hydrophila produce haemolysis. All isolates had ability to produce β-heamolysin (100%) which cause complete hydrolysis of RBCs on blood agar, this result similar to [27,41]. As shown to be cytolytic for the erythrocytes and mammalian cells in culture. All isolates are able to lysis of erythrocytes. As researchers indicate that clinical isolates had high pathogenicity were responsible for diseases occurs in human because they secrete different toxins [42]. Some reports are indicated that β-heamolysin 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. Heamolysin is made in the logarithmic phase of cell growth, and consider virulence factor important for A.hydrophila bacteria. Where observed that all clinical isolates of A.hydrophila under study were produced of capsule[43]. Our results reveal that A.hydrophila isolates were able to hydrolyze the protein by protease enzyme(100%) when tested on skim milk agar, and these results were agree with [25] 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 [44]. A.hydrophila produce gelatinase enzyme (100%). This enzyme act on hydrolysis of gelatin, which leads to the loss of gels attributes and conversion to liquid form even at low temperatures [45]. According to the results a clear variation has been obtained in the viability of the isolates for A.hydrophila to produce lipase and phospholipase enzymes and all isolates were lipase producer (100%), when grow on 1%Tween agar. Lipase is able to catalyze both the hydrolysis and synthesis of ester bonds of triacylglyceride, [46].The clinical isolates produce (88%) phospholipase. Phospholipase produced by bacteria is involved in different pathogenic process associated with intestinal damage [47].

The current study demonstrates that all A.hydrophila are able to produce to sidrophore (100%). Siderophores are secreted molecules with a high affinity for iron that scavenge iron from the bacterial cells' environment for growth. Production of siderophores has been reported in different species of Aeromonas and might be related to virulence properties. However, the role of siderophores, whether enterobactins or the recently characterized and named amonobactins, in A.hydrophila infections [48]. It 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 Aeromonas. Earlier studies have reported that the ability to produce siderophores correlated with higher virulence in Aeromonas hydrophila [49]. Results in the present study concluded that A.hydrophila has the ability to adhesion to epithelial cells of human, which is the important virulence factors and because they contain fimberia and S-layer that protects it from the growth effects as well as being the first step in the process pathogenicity. The result shows that clinical isolates of A.hydrophila are able to adhere on uroepithelial cells (88%) and these results indicated to found signification difference between isolates in statistical analysis at level $P \leq 0.05$ (ANOVA). When A.hydrophila isolates were attached on uroepithelial cell for human the range was (38 to 58 bacteria/ cell). Bacterial adherence to the epithelial cells surface is considered as one of the important factors in the infection. Adherence is an interaction between adhesion molecules on the bacterial cell surface and complementary receptor molecules on the host cell surface. Microorganisms adhere to epithelial cells in a highly selective manner and thus cannot be removed by unspecific defense mechanisms of the urinary, respiratory, gastrointestinal, and genital tracts[50]. There are three patterns of adhesion (diffuse, localized, and aggregative). In the diffuse pattern, adherent bacteria are randomly and individually dispersed over the cell surface. The localized and aggregative patterns are characterized by the formation of adherent micro-colonies corresponding to small or large clusters of bacteria, respectively [51]. The results agree with the findings of [25,47,49].

V. CONCLUSIONS

The following conclusions are extracted from the present study: 1-Isolation of A.hydrophila from the clinical cases in Najaf (Iraq) and identification by API20E system and the PCR technique, with used specific primer (16S r RNA) diagnostic gene. 2- Detection the virulence factors of A.hydrophila such as heamolysin, lipase, protease, gelatinase hydrolyze, phospholipase, sidrophore, capsule formation and adherence to uroepithelial cells of human of clinical isolates.

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TABLE I. SEQUENCE AND CONCENTRATION OF FORWARD AND REVERSE PRIMERS

Primer type	Primer sequence	Concentration In picomoles	Product size
16Sr RNA - F 16Sr RNA - R	5-CCAGCAGCCGCGGTAATACG-3 5-TACCAGGGTATCTAATCC-3	86900 128177	300 bp

TABLE II. PCR PROGRAM THAT APPLY IN THE THERMOCYLER

Gene	Initial denaturation	Cycles			Final elongation
		Denaturation	Annealing	Elongation	
16S r RNA	94°C for 3 min	30 cycles			72°C for 10 min
		94°C for 30 sec	52°C for 30 sec	72°C for 30 sec	

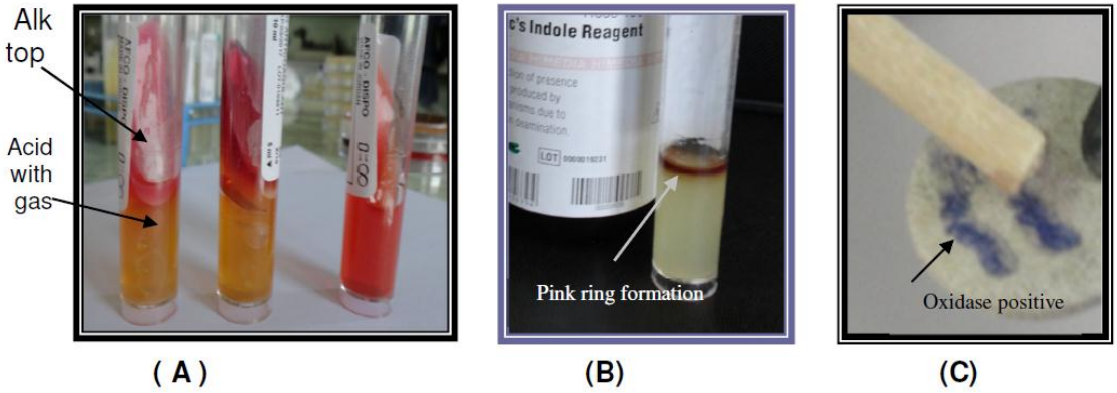

Figure 1. The Biochemical Tests of A.hydrophila (A) – Kligler iron agar (B)- Indole test (C) – Oxidase test

Figure 2. The Identification Results of API20E System for one A.hydrophila isolates

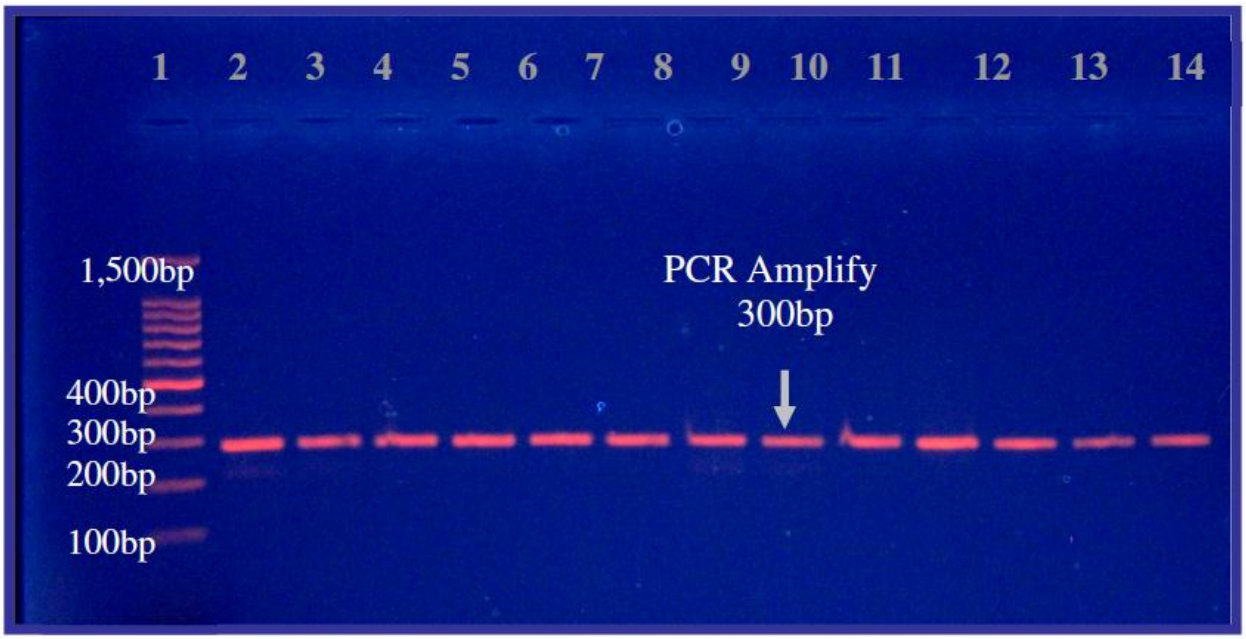

Figure 3. Agarose Gel Electrophoresis (1.2%) of PCR Amplified of 16S r RNA Gene (300)bp of A.hydrophila Isolates for (55) min at (100) volt. Lane (1), DNA marker (100bp ladder). Lane (2 ,3,4,5,6,7,8,9,10,11,12,13,14) Amplify of 16Sr RNA gene in clinical isolates of A.hydrophila.

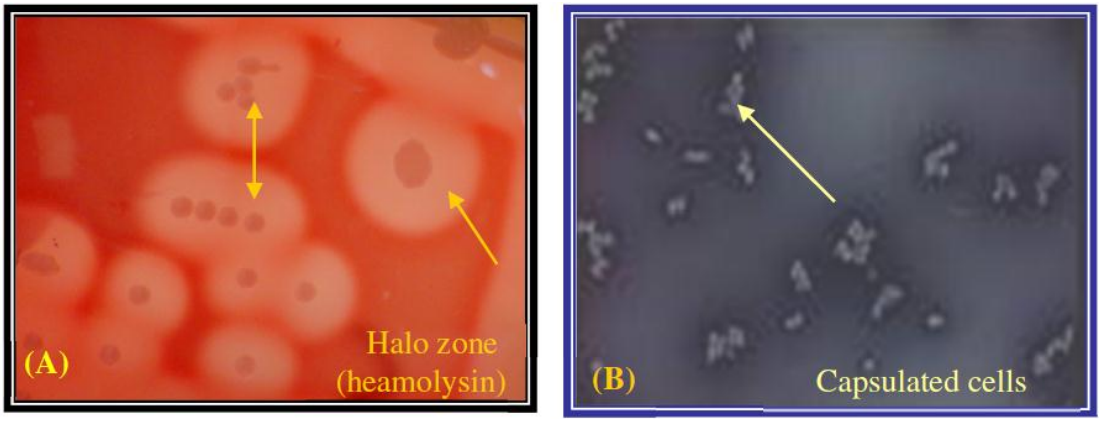

Figure 4. Some Virulence Factors of A.hydrophila (A) – Heamolysin lysis (B)- Capsules formed

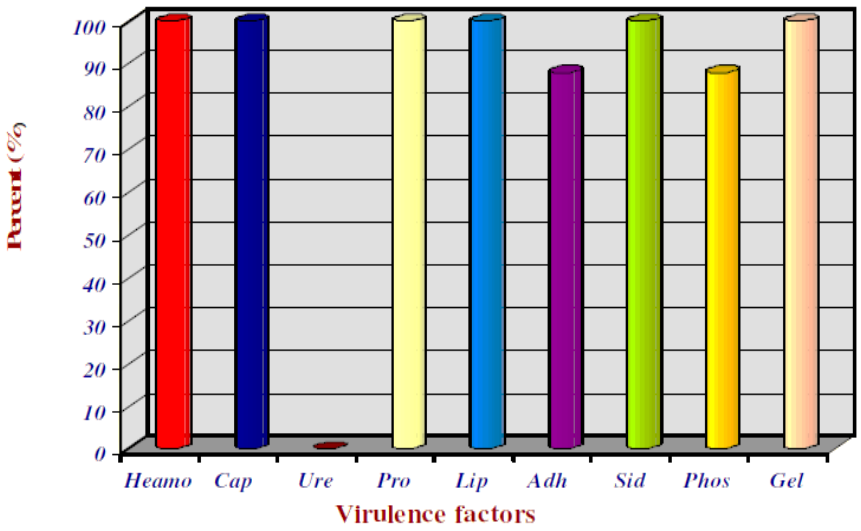

Figure 5. Production for Some Virulence Factors from the Clinical isolates of A.hydrophila
(*Heamolysin(Heamo),Capsule(Cap),Urease(Ure),Protease(Pro),Lipase(Lip),Adherence(Adh),Sidrophore(Sid),Phospholipase(Phso),Gelatinase(Gel))

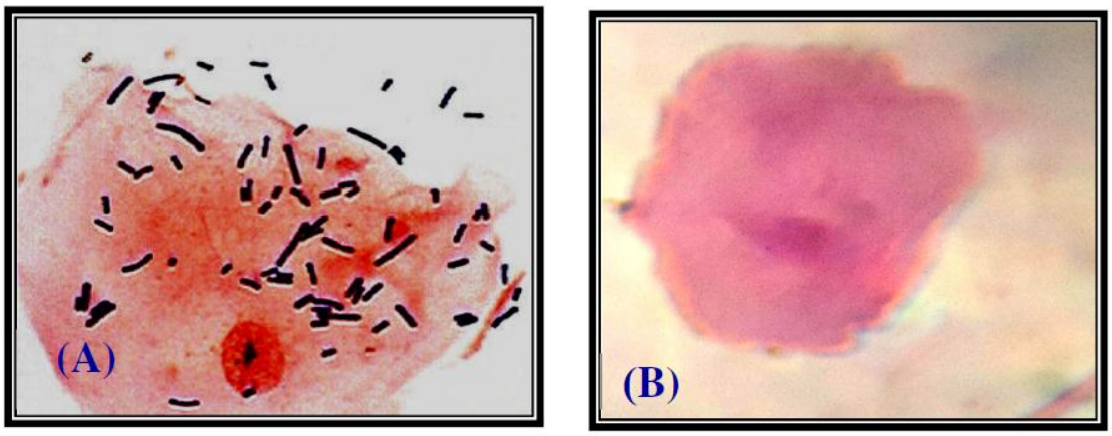

Figure 6. Adhesion of A.hydrophila to Epithelial Cells: (A)– A.hydrophila attached on Epithelial cell. (B)- Normal Epithelial cell

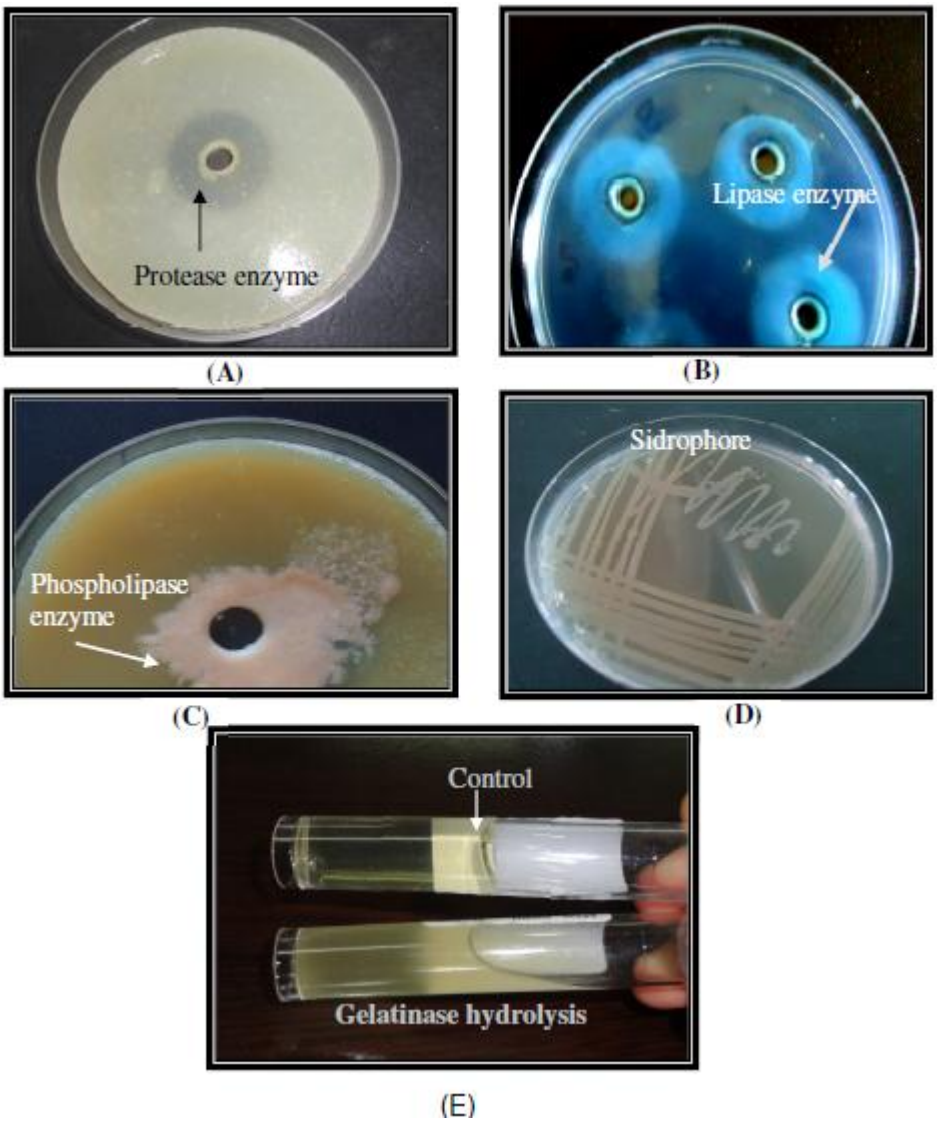

Figure 7. Some Virulence Factors of A.hydrophila (A)-Protease production (B)-Lipase production (C)-Phospholipase production (D)-Siderophores production (E)-Gelatinase hydrolysis