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Assessment of the bacterial load of the fish ponds and identification of A. Hydrophilla; haemolytic and proteolytic activity

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Abstract

The aim of study to assess the bacterial load of the three fish ponds (Tilapia, cat and carp) and bacteria were counted by the previous methods. Total viable count of bacteria flora we focus on *A. hydrophila* isolated from Qassim area in Saudi Arabia. A total of 150 strains were obtained from three types of fish ponds (50 Tilapia fish, 50 Cat fish and 50 Carp fish). Sample from Tilapia, cat and carp were cultivated on NA (Nutrient Agar), TSA and modified Rimler–shott (mRS) agar showed 1.2×10^6 cfu/ml, 8.7×10^5 cfu/ml, 6.5×10^5 cfu/ml; 1.2×10^6 cfu/ml, 2.7×10^7 cfu/ml, 1.5×10^4 cfu/ml; 5.5×10^5 cfu/ml, 2.5×10^6 cfu/ml, 6.7×10^4 cfu/ml respectively. A total of 80 colonies showed a yellow color in mRS in which further biochemical and morphological study showed that 35% of isolates were confirmed as *A. hydrophila* from the all presumptive aeromonads. In the present study the 68 % isolates of the *A. hydrophila* produced β-haemolysis while 43 % isolates of *A. hydrophila* exhibited significant protease production. In the present study the chi-square test showed that there was no significant difference between the hemolytic and protease activity. Also there was no statistical difference found in the presence of genes. Molecular techniques revealed that isolates were confirmed as *A. hydrophila* with 98% similarity to the GenBank BLAST. it was

resulted that the 57% isolates of *A. hydrophila* possess haemolysin gene (*hlyA*), 130 bp. While 35% isolates were positive for aerolysin gene (*aer*) showing expected band at 309 bp. There was a large bacterial flora found in fish ponds although the fish farms have good and optimal conditions in Qassim area of Saudia. The bacterial specie *A. hydophila* also identified from this flora. *A. hydophila* virulence showed haemolysis activity and proteases activity the genes of hemolysin and aerolysin confirmed its virulence.

INTRODUCTION

Fish and fishery products are of great importance worldwide due to their nutritional value, clear health benefits and wholesome properties and the seafood are nutritive, they are highly susceptible to contamination [1] *A. hydrophila* has been shown to be widely distributed [2]. Aeromonas species are mainly organisms of aquatic environments and are present in fresh water, estuarine and coastal water bodies, not excluding chlorinated water [3]. While According to [4]. Aeromonas species are isolated from rivers, lakes, streams, canals, sediments, marine as well as chlorinated water especially during hot months and it can be isolated from many sources, such as food, drinking water, sewage, environmental water [5]. Moreover it can isolated from food including fish, vegetables and the intestine of apparently healthy humans with diarrhea [6]. *A hydrophila* are universal and opportunistic bacterium that constitutes the normal microflora of fish and can induce diseases in fish under stressful conditions and the damages induced to both wild and farmed freshwater fish population is extensive, leading to mortality and severe loss of income [7, 8]. *A. 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 [9] also it is oxidase and catalase positive [10]. Although Aeromonas was initially positioned in the family Vibrionaceae, successive phylogenetic analyses point out that the genus Aeromonas is not closely related to vibrios resulting in the relocation of Aeromonas from the family Vibrionaceae to a new family, the Aeromonadaceae [11]. *A. hydrophila* is the causative agent of MAS (Motile Aeromonas Septicemia). Both wild and

farmed fishes have been reported to be affected by this disease [12]. There are many reports said *A. hydrophila* may play role of the disease for fishes as a causative agent of outbreaks such as hemorrhagic septicemia, ulcer syndrome, and enteritis [13]. Fish species affected by MAS include tilapia [14], catfish [15] and common carp [16]. On other hand *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 [17].Currently, the genus comprises of 17 DNA hybridization groups (HGs) or genomospecies and 14 phenospecies [18]. Aeromonas are divided into three species that are phenotypically and genetically differed namely *A. hydrophila*, Aeromonas caviae and Aeromonas sobria. Aeromonas species produce a variety of virulence factors, including cytotoxic and cytotonic enterotoxins, aerolysins, haemolysins, proteases, haemagglutinins and lipases [19]. The hemolysin genes it is most effective way to detecting and characterizing aeromonas virulence factors [20]. Two hemolytic toxine have been described the *A. hydrophila* a hemolysin (hyl A) (Hirono,I,et al 1991) and aerolysin (aer A) [21]. The 16S rRNA is very useful in classifying Aeromonas spp. up to genus level [22]. Carp fishes and catfishes the importance of the agriculture of these species increased particularly in concrete tanks, and it can be produced in many confined water bodies in Saudi Arabia. In contrast, the tilapia fishes is fast growing and it is able to growth on brackish water so this support to impartment of this fishes. Therefore, the present study was designed with a view to achieve the following objectives. The evaluation of bacterial flora in (tilapia, carp, and cat) fish pond and isolation and identification of *A. hydrophila*. To determine hemolysis and aerolysin genes in *A. hydrophila*, which is still not studied in Saudi Arabia.

Materials and Methods

2.1: Study area:

This study was carried out to evaluate the bacterial flora in three concrete fish ponds containing tilapia fishes (*Oreochromis niloticus*), catfishes (*Clarias gariepinus*) and carp fishes (*Cyprinus carpio*) in Qassim area of Saudia Arabia. The total area for each pond was $6\times6\times1.5$ m^2.

2.2 Sampling:

Five hundred ml water was collected from three types of fish pond with Carp fish, Catfish and Tilapia fish. The sample were transported by ice box (4 °C) to keep temperature to lab and then were analyzed within 4 hours of after collection. Aliquots of 1 ml of each bottle were diluted with 9 ml of distilled water and then mixed vigorously. A volume 0.1 ml of serially diluted were spread into following enrichment media TSA, Nutrient, selective media RSm. Finally, all pleated were incubated at 31 °C for 24 hours.

2.3: Aerobic plate count (APC)

For bacteria count were incubated at 31°C for 24 hours. The colony forming units (cfu) were counted by Quebec dark field colony counter (Leica, Buffalo. New York, USA) a guide plate ruled in square centimeter was used. The colonies 30-300 were used to final calculated bacteria population result.

2.4: Phenotypic and biochemical characterization:

A total of 150 colonies show 70 green and 80 yellow colors on RSm and TCBS (Thiosulfate Citrate Bile salts Sucrose) media and the yellow color were the presumptive aeromonads. These samples were picked and selected sample were restricted on fresh media for purification. The colonies examined under microscope for identification of the shape. The species identification of *A. hydrophila* was performed by API 20E test system (bioMerieux). Moreover, these colonies were examined by the following tests gram stain, oxidase test,

catalase test, H_2S production and Esculin hydrolysis. Also, physiological characters were recorded through observing the growth of each isolate temperature 4 °C, 32 °C and 40 °C. Isolates were incubated in different concentration of NaCl as 0%, and 6%. Finlay, Vibriostat sensitivity 0/129 (150 μg) (bioMérieux) was used to differentiate between aeromonads and Vibrio which it is negative to Aeromonas and positive to vibrios. So, for further studied these samples were picked up and stored by TSA media slant to be used for more specific analysis. The samples were store in growth medium with 15% glycerol at -20 °C, for additional characterization which includes specie identification and virulence factors determination through PCR technique to amplify the 16S rRNA gene.

2.5: Hemolytic test

Haemolytic test was done according to the methods as [1]. For hemolytic, the blood agar media (5% sheep blood and 5% horse blood) (Oxoid, England) were used to detect hemolysis. All the 28 samples were cultured on blood agar plates and incubated for 24-48 hours at 37 ^{0}C. The clear zones round the colonies called β-hemolysis (complete lysis of the red blood cell) while the green zones around colonies indicate α-hemolysis.

2.6: Proteolytic activity

Proteolytic activity was done according to the methods as with little changes [23]. The isolates was spot inoculated on nutrient agar with 2% skimmed milk and inoculated in 10% gelatine agar in test tube, the proteolytic activities were showed after 24hours of incubation. It was in the form of clear zone.

2.7: Extraction of bacteria DNA

Single colony from fresh (TSA) culture was transferred to 5 ml of TSB and grown overnight at 31 °C. Also, one ml of broth culture was used to extract DNA following the manufacturer instructions for Gram-negative bacteria. Genomic DNA (gDNA) was extracted according to manufacturer's manual from bacterial cultures using the Wizerd® Genomic DNA purification

kit (Promega, Madison). After extraction of the DNA, 5 µL of the DNA solution was used as a template for PCR amplification.

2.8: Oligonucleotide primers and PCR conditions

The polymerase chain reaction (PCR) was used to detect the presence of hemolysin and aerolysine in all Aeromonas isolates. Primer pairs used for PCR amplification present in (table 1).

Statistical analysis

The statistical analysis was conducted by SPSS and the Chi-square test was use to evaluate the significant difference between the difference phenotype of virulence factors and virulence genes in *A. hydrophila.*

Result

3.1 Aerobic plate count (APC)

A total of 150 strains were obtained from three types of fish ponds (50 Tilapia fish, 50 cat fish and 50 carp fish). Sample from Tilapia, cat and carp were cultivated on NA (Nutrient Agar), TSA and modified Rimler–shott (mRS) agar showed 1.2×10^6 cfu/ml, 8.7×10^5 cfu/ml, 6.5×10^5 cfu/ml; 1.2×10^6 cfu/ml, 2.7×10^7 cfu/ml, 1.5×10^4 cfu/ml; 5.5×10^5 cfu/ml, 2.5×10^6 cfu/ml, 6.7×10^4 cfu/ml respectively.

3.2 Phenotypic and biochemical characterization:

A total of 80 colonies showed a yellow color in mRS and then picked up sample selected were restricted on fresh media for purification. In the further study 28 colonies which were rod shaped, gram-negative, catalase-positive, oxidase-positive, H_2S production-negative, hemolysis-β, growth at 4 °C was negative, growth at 32 °C was positive, growth was 40 °C was negative, growth at (NaCl) 0% was positive, growth at (NaCl) 6% was negative, Esculin hydrolysis was positive and and 0/129 sensitivity was negative identified the characteristics

of *A. hydrophilla* present in (table 2). The results showed 35% of isolates which confirmed *A. hydrophila* from the all presumptive aeromonads.

3.3 Determination of virulence factors

A number of virulence factors derived from *A. hydrophila* have been proposed in an effort to explain the pathogenesis of infections to many organisms. Toxins with hemolytic, cytotoxic, and enterotoxin activities have been described in many *Aeromonas* spp [24]. Among mostly studied virulence factors in Aeromonas spp. are hemolysis, protease, lipases, aerolysis flagellation and biofilm production [17].

3.3.1 Haemolysin Production

In the present study the 68 % isolates of the *A. hydrophila* produced β-haemolysis which this strains were able to lyse sheep and horse erythrocytes and produced haemolysis on sheep and horse blood agar plates within 24 hours of incubation at 37 °C (Figure 1 d).

3.3.2 Protease Activity

In the present study the 43 % isolates of *A. hydrophila* exhibited significant protease production in the form of clearance zone and liquefaction. The isolates also showed the ability to produce protease to hydrolyze the protein when inoculated on skim milk agar for 24 hours at 37 °C (Figure 1 e). In the present study the chi-square test showed that there was no significant difference between the hemolytic and protease activity. Also there was no statistical difference found in the presence of genes.

3.4 Molecular Identification of *A. hydrophila*

Molecular techniques is considered the most effective method for the identification [25]. During this study, 16S rRNA gene amplification of the representative strain using universal primers resulted in an amplicon with a product size of 1500bp (Figure 5). The product was partially sequenced and the 16S rRNA gene sequences of the isolate were submitted to the GenBank database and compared using the BLAST algorithm. The isolates were confirmed

as *A. hydrophila* with 98% similarity to the GenBank BLAST. The product size obtained in this study was comparable with previous studies such as [26].

3.5 Detection of aerolysin and haemolysin genes of *A. hydrophila* isolates

The major virulence factors of *Aeromonas* spp include protease, lipases, haemolysis, aerolysis, flagellation and biofilm production which was mentions in introduction [27]. In the present study, it was resulted that the 57% isolates of *A. hydrophila* possess haemolysin gene (*hlyA*), 130 bp (Figure 5). While 35% isolates were positive for aerolysin gene (*aer*) showing expected band at 309 bp (Figure 6). Polymerase chain reaction (PCR) was used to detection for these genes. [28].

Conclusion

There was a large bacterial flora found in fish ponds although the fish farms have good and optimal conditions in Qassim area of Saudia. The bacterial specie *A. hydophila* also identified from this flora. *A. hydophila* virulence showed haemolysis activity and proteases activity the genes of hemolysin and aerolysin confirmed its virulence.

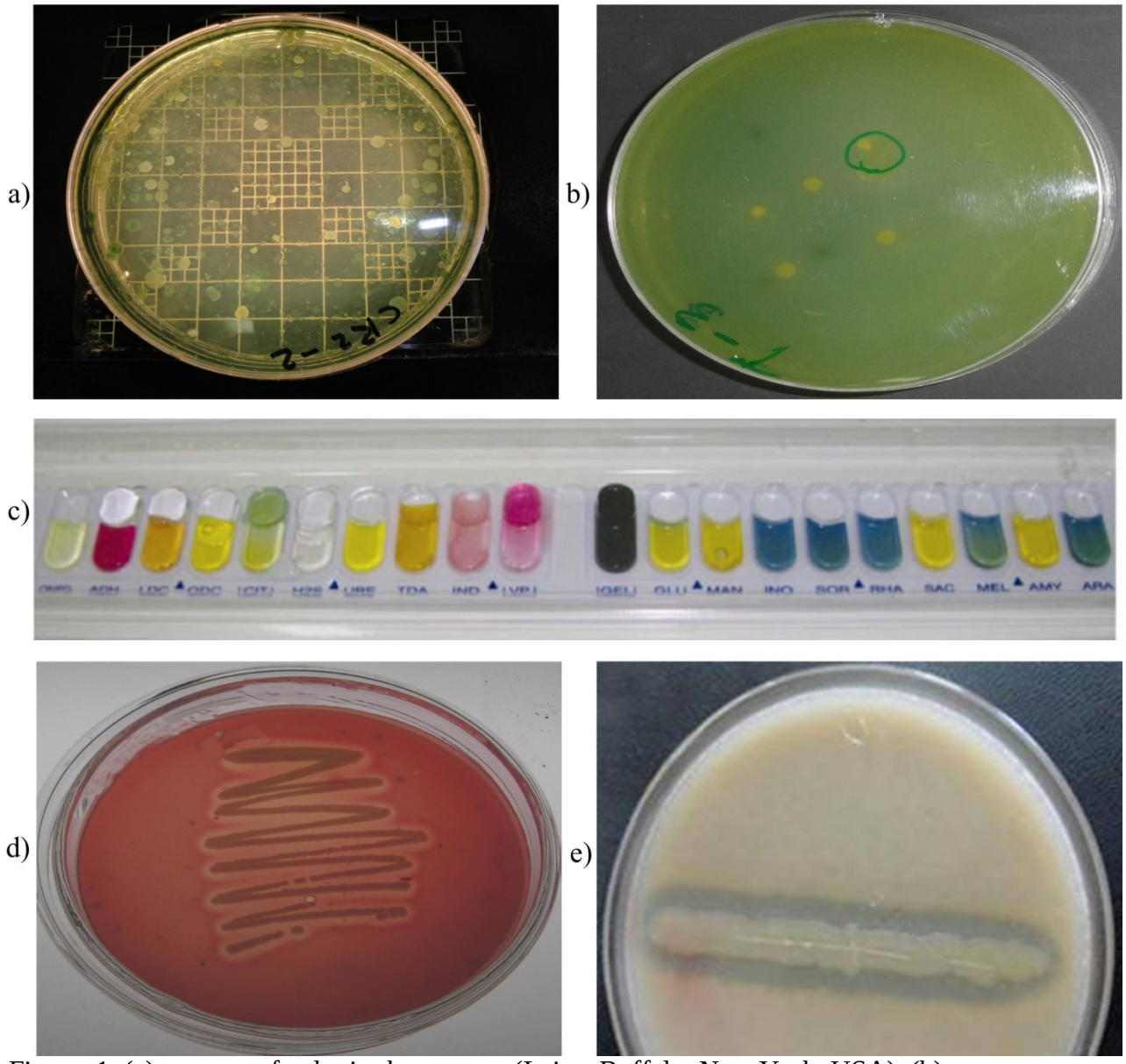

Figure. 1. (a) account of colonies by counter (Leica, Buffalo. New York, USA). (b) *Aeromonads spp.* on modified Rimler Shott–(mRS) (c) Identification of bacteria by API-20E kit (d) β Hemolytic activity shown by *A. hydrophila* (Ah8) (e) Protease activity shown by the *A. hydrophila*.

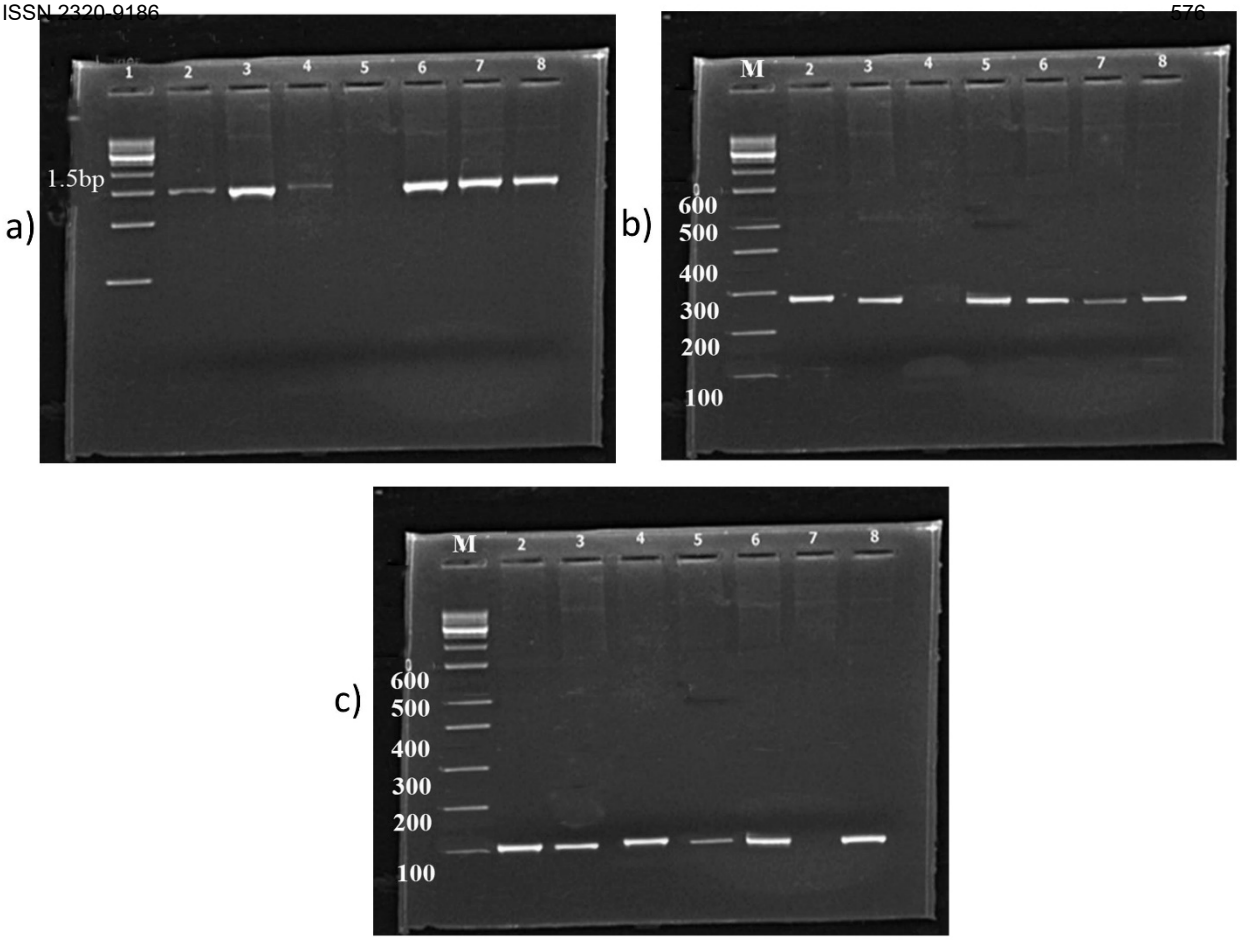

Figure. 2. (a) Agarose gel electrophoresis of amplified products of 16S rRNA of *A. Hydophila* yielding 1500 bp product {Lane 1: 1500 bp plus DNA ladder; Lane (2, 3, 4, 6, 7 and 8) were Ah (1, 11, 18, 19, 21 and 25) respectively}. Products of hlyA gene of *A. Hydophila* yielding 130 bp product {Lane M: 100 bp DNA ladder; Lane (2, 3, 4, 6, 7 and 8) were Ah (1, 3, 11, 20, 21 and 25) respectively}. (c) Products of aer gene of *A. Hydophila* yielding 130 bp product {Lane M: 100 bp DNA ladder; Lane (2, 3, 4, 6, 7 and 8) were Ah (4, 8, 11, 18, 25 and 28) respectively}.

Table (1). Primer pairs used for PCR amplification by [24]

Primer	Sequence (5to 3)	Target gene	Size of PCR amplicon (bp)
AHH1F AHH1R	GCCGAGCGCCCAGAAGGTGAGTT GAGCGGCTGGATGCGGTTGT	hlyA	130
AH-aerA F	CAAGAACAAGTTCAAGTGGCCA ACGAAGGTGTGGTTCCAGT	Aer	309
16S rRNA	5’-AGAGTTTGATCCTGGCTCAG-3’ 5’CGGTTACCTTGTTACGACTT-3’	16S rRNA	1500

Table 2. Average Bacterial Load (Colony Forming Unit) in Pond Water, for (Cat fish, Carp fish and Tilapia fish). Values are Given as Mean DS on enrichment media (NA Nutrient agar), (TSA tryptone soy agar media) and on Rimler Shott modified RSm media agar [29].

Type of fish	Type of media	Average of account
Tilapia	NA	1.2×10^{6} cfu/ml
	TSA	8.7×10^{5} cfu/ml
	RSm	6.5×10^{5} cfu/ml
Cat	NA	1.2×10^{6} cfu/ml
	TSA	2.7×10^{7} cfu/ml
	RSm	1.5×10^{4} cfu/ml
Carp	NA	5.5×10^{5} cfu/ml
	TSA	2.5×10^{6} cfu/ml
	RSm	6.7×10^{4} cfu/ml

Table 3: Morphological characteristics and biochemical tests for identification of *A. hydrophila* isolates

Test	ISOLATES	
	ATCC	Ah1-Ah28
Shape	Rod	Rod
Gram stain	-	-
Catalase	+	+
Oxidase	+	+
H_2S production	-	-
Hemolysis	β	β
Temperature 4 °C	-	-
Temperature 32 °C	+	+
Temperature 40 °C	-	-
Growth at(NaCl) 0%	+	+
Growth at(NaCl)6%	-	-
Esculin hydrolysis	+	+
0/129 sensitivity	-	-

Table (4) Percentage of phenotype of virulence factors of *A. hydrophila*

Isolates	*A. hydrophila*	*A. hydrophila*	Virulence Factors Percentage %
Hemolytic activity	19	28	68%
Protease activity	12	28	43%

$\chi 2 = 0.316$

Table (5) Percentage of virulence genes in *A. hydrophila*

Isolates	*A. hydrophila*	*A. hydrophila*	Virulence Genes Percentage %
haemolysin gene (hlyA)	16	28	57%
aerolysin gene (aer)	10	28	35%

$\chi 2 = 0.329$

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