Pathogenic characteristics and molecular identification of *Aeromonas hydrophila* isolated from some naturally infected cultured fishes

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Abstract

Nine isolates of *Aeromonas hydrophila* were isolated from four different fish farms of various water salinities located in Damietta, Egypt. The fishes were collected during mass mortality during 2007. The diseased tilapia and catfish were isolated from governmental fresh water farm, while diseased meager, grouper and mullet as well as sea bream were isolated from three private brackish water farms varied in salinity (2‰, 7‰, and 25‰, respectively). The bacterial isolates were characterized using various methods including selective media and biochemical tests as well as Polymerase Chain Reaction (PCR) technique based on three specific primers *Aer*, *Alt* and *Ast*. Also, gel electrophoresis proteins and pathogenicity testing have been performed. The antibacterial activity of some different herb extracts as well as a new Egyptian local produced prebiotic (T-protphyt 2000) against *A. hydrophila* isolates were investigated in this study. The results showed that, all bacteria isolates were PCR positive for the *Alt* and *Ast* genes; therefore, all tested isolates were *A. hydrophila* (SSU). While, six out of the nine isolates were positive for *Aer* gene (as a putative virulence factor), 8/9 isolates were positive for hemolytic activity and 2/9 were pathogenic *in vitro*. So, there was a close correlation between hemolytic activity and detection of *Aer* gene but didn't correlate well with the pathogenicity test for tilapia and the putative virulence factor. In general, the dendrogram of protein pattern for tested *A. hydrophila* were grouped according to their origin and environment, fish species and water salinity. T-Protphyt 2000, marjoram, rosemary, garlic and basil extracts exhibited pronounced in-vitro positive antibacterial effects against 100, 77.7, 77.7, 33.3 and 33.3% respectively, while parsley, onion and ginger extracts exhibited negative effects of all the tested isolates.

Keywords: A. hydrophila, fish, PCR, Specific primers, hemolysin, antibacterial extracts, electrophoresis.

1. Introduction

It is well established in the fish industry that bacterial infections are responsible for heavy losses in fish farms. Among the etiological agents of bacterial fish disease, the motile *Aeromonas* group, especially *Aeromonas hydrophila*. This organism is considered as an important pathogen causing primary skin infection or the secondary problem following stress from temperature change, handling, or poor water quality (Salyers and Whitt, 1994; Vivas *et al.,* 2004). *Aeromonas hydrophila* is a rod-shaped, gram negative and facultative aerobic bacterium (Popoff, 1984). This bacterium is responsible for hemorrhagic septicemia, a disease affecting a wide variety of fresh, brackish and marine water fishes (Pianetti *et al.,* 1998; Soler *et al.,* 2002).

The principal feature of the pathogenesis of *A. hydrophila* is generalized dissemination in the form of a bacteremia, followed by elaboration of toxins, tissue necrosis and bacterial hemorrhagic septicemia (Jeney and Jeney, 1995).

Aeromonas species secretes many extracellular proteins including amylase, chitinase, elastase, aerolysin (Pemberton *et al.,* 1997), heat–labile enterotoxin *(Alt*) and heat–stable enterotoxin (*Ast*) (Albert *et al.,* 2000). These proteins are known as virulence factors that cause disease in fish. Aerolysin (*Aer*) is a representative virulence factor of *Aeromonas* and was reported to function as hemolysins and cytolytic enterotoxins (Buckley and Howard, 1999), while Chopra *et al. (*1996) reported that both *Alt* and *Ast* consist of single polypeptide chains.

Aeromonas can be detected or diagnosed by using a variety of test methods, include culture on selective media such as ampicillin blood agar and *Aeromonas* medium base (Warburton *et al.,* 1994) followed by linotyping (Wang and Silva, 1999), in addition to nucleic acid detection by polymerase chain reaction (PCR) (Francis and Stewart, 1997) which are established methods generating DNA fingerprints and

allowing discrimination among strains of *Aeromonas* spp. (Aguilera-Arreola *et al.,* 2005). Also, SDS-PAGE, Sodium Doecyl Sulphate Polyacrylamide gel electrophoresis protein pattern braving a good level of taxonomic resolution at species or subspecies level (Devriese *et al.,* 1995). Identification of *Aeromonas* sp. by specific primers was detected in previous studies (Biscardi *et al.,* 2002; Yousr *et al.,* 2007; Jiravanichpaisal *et al.,* 2009).

Currently, bacterial infections in aquaculture, including motile aeromonad septicemia (MAS), are mainly controlled by antibiotics. Since the use of expensive chemotherapeutants for controlling diseases have been widely criticized for their negative impacts (Sahu *et al.,* 2008), probiotics have found use in aquaculture as a means of disease control, supplementing or even in some cases replacing the use of antimicrobial compounds (Abdel-Tawwab *et al.,* 2008; Abdel-Rahman *et al.,* 2009).In addition to many studies reported, the activities of spices and herbs on pathogenic bacteria; rosemary (*Rosmarinus officinalis*) (Yano *et al.,* 2006; Shan *et al.,* 2007), basil (*Ocimum basilicum*) (Yano *et al.,* 2006; Nedorostova *et al.,* 2009), marjoram (*Origanum marjorana*) (Busatta *et al.,* 2008; Nedorostova *et al.,* 2009), mint (*Mentha canadensis L.*) (Sagdıc and Ozcan, 2003; Shan *et al.,* 2007), parsley (*Petroselinum crispum*) (Wong and Kitts, 2006), ginger (*Zingiber officinale*) (Yano *et al.,* 2006), garlic *(Allium sativum L.*) and onions (*Allium cepa)* (Benkeblia 2004; Corzo-Martı´nez *et al.,* 2007). A new locally produced Egyptian prebiotic, T-Protphyt 2000; is a local product with a patent No. 23593. It consists of 15% zinc salts, 10% inorganic phosphorus, 5% dried fermentation products of *Aspergillus oryzae* growth, and starch as carrier up to one kilogram was tested as antibacterial by Abdelhamid *et al.* (2009).

The objective of this study included the detection and identification of *Aeromonas hydrophila* isolated from some fresh and brackish water fishes (various salinities) using the PCR primer sets (*Aer, Alt* and *Ast,* genes) as well as protein electrophoretic pattern, pathogenicity testing and antibacterial activity of some herbs extracts and one commercial prebiotic were another objective.

2. Material and Methods

2.1. Fish and Isolation *Aeromonas* sp.

A total number of 30 naturally infected fishes (5 from each species) were collected during mass mortality in four different fish farms (various salinities) located in Damietta governorate, Egypt, during 2007 (Table 1). As well as fresh water fish, site 1, El-Serw, a governmental freshwater fish farm is supplied by River Nile water that has been pre-used in agriculture. Sites 2-4 are private farms located between the Mediterranean Sea (marine water) and Manzala Lake (brackish water). These farms have different water salinities which is dependent on their elevation. All Fishes were transported alive to the laboratory in El-Serw fish farm and were subjected to clinical and postmortem examinations according to Amlacher (1970). Isolated *Aeromonas* spp.; bacterial cultures of the liver, kidneys, and blood from the fish were taken aseptically and cultured on different selective media (MacConkey Agar, Ryan's *Aeromonas* media and Rimler-Shotts agar media [prepared after Shotts and Rimler (1973), with dehydrate ampicillin supplement]). The plates were incubated at 30ºC for 24 hr. Suspected pure colonies were subjected to Gram's stain and some biochemical tests such as oxidase, catalase, arabinose, galactose, manose and glucose fermentation, indol production and hemolysis.

Table 1: Types of fish examined and location and salinity ‰

Sites	Location and salinity ‰	Fish species	Number
1	El-Serw Fish Farm, Freshwater	1- Nile tilapia (*Oreochromis niloticus*) 2- African cat fish (*Clarias garipinus)*	**5** **5**
2	Brackish water farm, salinity 2‰	3- Meagre (*Argyrosomus regius*)	**5**
3	Brackish water farm, salinity 7‰	4- Grouper (*Epinephelus aeneus*)	**5**
4	Marine water farm, salinity 25‰	5- Mullet (*Mugil cephalus*) 6- Sea bream *(Sparus auratus*)	**5** **5**

2.2. DNA extraction of presumptive *Aeromonas* sp.

The presumptive *Aeromonas* strains, about 25 from all fishes were separately cultured overnight in tryptic soy broth (TSB, Merck, Germany) at 30ºC. Then the bacteria were washed 3 times with sterilized saline by centrifugation at 900 xg for 10 min at room temperature, cell concentrations were adjusted and verified by viable plate counts and were recorded as colony forming units (CFU) per milliliter. So, the genomic DNA of bacteria strains was extracted from each pure bacterial culture according to Frederick and Roger (1994). Purified DNA was stored at -20ºC until use. The genetically characteristics study was carried out at Department of Genetics, Faculty of Agriculture, Ain Shams University, Egypt.

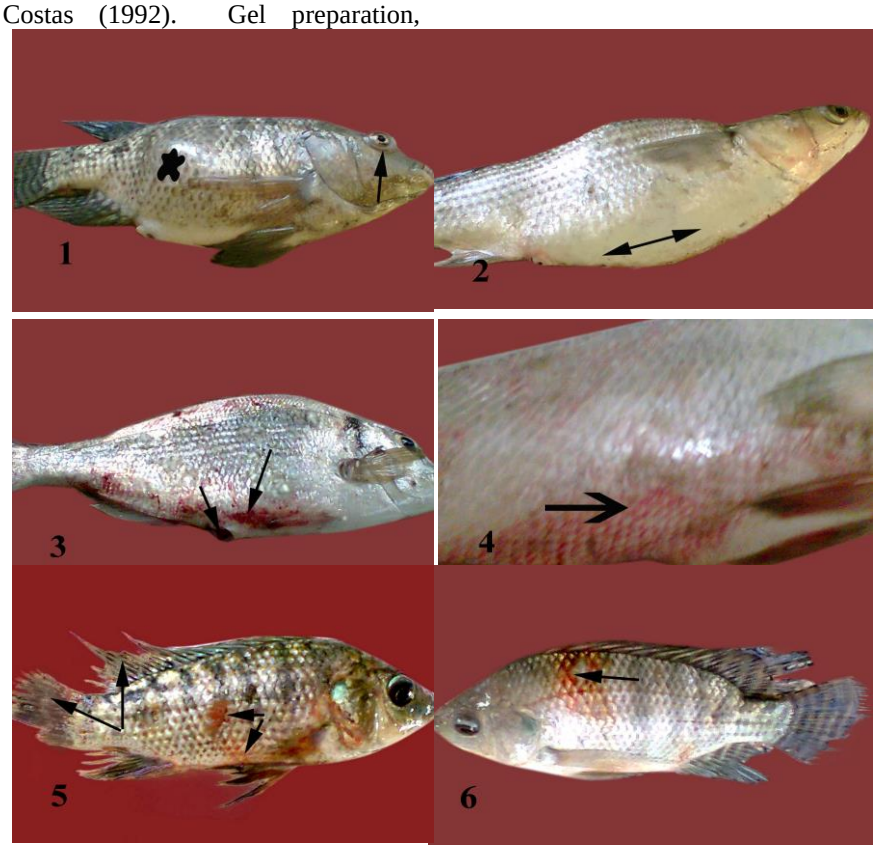
2.3. Specific primers design and PCR conditions

Only nine isolates of *Aeromonas* spp. (Table 2) were studied using PCR, based on three specific virulent genes, Aerolysin (*Aer*), Altase (*Alt*) and Elastase (*Ast*). The primers designed for the detection and genotyping of *Aeromonas* spp. were done using a web-based tool, (Gen Bank database and BLAST network service). Table 2, showed the virulent genes, selected sequences, their nucleotide sequence accession number, primer site location and amplicon size. The PCR conditions were denaturation at 95ºC for 2

minutes followed by 30 cycles of denaturation (94ºC, 1 min) annealing (59, 54 and 55ºC, 1 min for *Aer*, *Alt*, and *Ast* genes respectively) and extension (72ºC, 1 min), and final extension (72ºC, 10 min). PCR products were running in 1.5% agarose gels, then stained with ethidium bromide and photographed using MV Tran illumination. A 50 – 10000 bp ladder (Sigma Chemical, MSA) was used as a molecular mass marker.

2.4. Bacterial protein extraction and electrophoresis

The method of protein extraction has been described by Costas (1992). Gel preparation, electrophoresis conditions, staining and destining gel were done according to Laemmli (1970). The protein extract samples of bacteria were analyzed using 10-well gel (9 samples and the standard). Each well was loaded with 26 µl of samples. Molecular weight standard was included in gel (205, 116, 97.4, 66, 45 and 29 KD_a) and the standard was applied to separate lane.



Figures (1-6): The gross pathology for naturally infected fishes; Figure 1: Showed exophthalmia and abdominal distension in tilapia. Figure 2: Abdominal distension of mullet. Figure 3: Inflamed vent and hemorrhage of sea bream. Figure 4: Hemorrhage on the body surface of meagre. Figure 5: Fin erosion and hemorrhage. Figure 6: ulcer on the skin of tilapia.

2.5 Hemolysin detection

Aeromonas strains were grown on blood agar (Oxoid), 5% sheep blood at 30ºC for 24 hr. The production of hemolysin was assayed by recording the lysis of sheep erythrocytes in agar blood.

2.6. Pathogenicity of isolated *Aeromonas* sp.

A total number of 100 apparently healthy *O. niloticus* with a mean body weight 40 g were obtained from El-Serw Fish Farm. The fish were divided into 10 groups each group was contained 10 fish. The fish were maintained in 50 L (70 × 40 × 30 cm) aquaria with aerated freshwater for 2 weeks and fed commercial pellets in order to adapt to laboratory conditions and prior experimental infection with the nine isolated *Aeromonas* isolates. Each of the ten fish in each group was injected intramuscularly (i.m.) below the dorsal fin using 21-gauge sterile needle with 0.1 ml of a suspension containing 10^5 CFU/ml in phosphate buffered saline (PBS). The 10 control fish were injected with PBS alone. The observation time was 7 days. The pathogenicity test was considered positive when more than 50% of the injected fish showed clinical signs and died within 96 hr.

2.7. Histological examination of fish liver

For the histological study, liver specimens were separated from freshly dead fish and were fixed in 10%

neutralized buffered formalin saline solution for histological study, embedded in paraffin, sectioned and stained with Hematoxylin and Eosin (H&E) according to Roberts (2004) , then examined microscopically.

2.8. Antibacterial activity of some extracts tested

Five herbs, basil, marjoram, mint, parsley and rosemary leaves were dried in the dark at room temperature after carefully washed with tap water, and 5 g of the ground leaves were extracted for 24 hr by soaking in 100 ml methanol (80%) then filtered through Whatman filter paper No. 1 and the filtrate was evaporated in a rotary evaporator (45ºC) till the solvent evaporated. Garlic and onion juice were freshly prepared because the active principles are very unstable and easily destroyed by heat (Roy *et al.,* 1992). While 2 grams powder of each the prebiotic (T-Protphyt 2000) and ginger were soaking in 5 ml sterile water as aqueous extract, the prebiotic is soluble in water but ginger was used as suspension.

The solvent extracts of herbs were tested by Disc diffusion method, where the extracts were adsorbed on discs of Whatman filter paper No. 1 (6 mm in diameter) and partially dried at room temperature under aseptically condition, while the aqueous extracts were tested by well method, where four wells were punched with cork borer (6 mm in diameter) in plates. In each case, the concentration was adjusted to be 50 µg per disc and well (each disc was impregnated with 25 µl of each solvent extract and each well contained 50 µl of each aqueous extract). Antibacterial activity was tested in NA plates which were inoculated with 0.1 ml of 10^5 CFU/ml and 24 h-old cultures of the tests *A. hydrophila* strains in nutrient broth, antibacterial-impregnated discs were placed on the solid medium. All plates were left one hr to allow diffusion, and then incubated at 30ºC for 24 hr. Zones of inhibition formed around the discs and wells were measured and considered positive results.

2.9. Statistical analysis

The gel of protein was analyzed using Total Lab Ver. 2.01, and SPSS Ver-15 software, and the similarity among samples was calculated as described by Lynch (1990).

3. Results

3.1. The clinical and postmortem findings for naturally infected fishes

The naturally infected fishes (to isolate *A. hydrophila*) showed one or more of the following signs according to disease stage; fluid accumulation in abdomen (Figures1and 2), hemorrhages on body surface (Figures 3 and 4), fin erosions (Figure 5), ulcers on the skin varied from shallow to deep necrotizing ulcer (Figure 6), increased darkness of the skin, thinning of musculature giving big head appearance, with congestion and enlargement of liver, spleen, kidneys and gall bladder.

3.2. Characterization of isolates

Only nine isolates of *Aeromonas* sp. were selected for characterization. These isolates were Gram negative, motile rods, grown on MacConkey Agar, nutrient agar (NA), Rimler-Shotts medium (RS), where colonies were yellow, and Ryan's *Aeromonas* media where colonies were dark green, opaque with a darker center. Also strains were oxidase and catalase positive, indol producted and fermented arabinose, galactose, manose and glucose with or without gas production.

3.3. Molecular analysis of *Aeromonas* isolates by PCR:

The data presented in Tables 2 and 3 showed that all tested *Aeromonas* isolates were *A. hydrophila* (SSU). All isolates were PCR positive for the *Alt* and *Ast* genes (which are specific of *A. hydrophila* SSU Table 2). While not all isolates were PCR positive for the *Aer* gene where amplification products were obtained in 6 out of the nine analyzed isolates. Some of the isolates didn't show reproducibility, i.e. very faint amplicon once and no product the next time and these were designated as inconclusive. The three *A. hydrophila* isolates not recognized (4Ah, 6Ah and 9Ah), were recorded in Figure 7, showed that the products of these strains (4Ah, 6Ah and 9Ah) were very faint banded or no amplicon found. On the other hand, the bands were observed in all isolates tested with primers *Alt* and *Ast* genes (Figure 8).

3.4. Hemolytic activity on blood agar and detection aerolysin gene by PCR

The PCR –based study revealed that at least 6 out of the 9 *A. hydrophila* isolates tested possessed the targeted *Aer* gene. A single PCR product (431 bp in size) was obtained from the aerolysin positive strains of *A. hydrophila* (6/9). Most of these isolates (8/9) were found to produce β-hemolysin on sheep blood agar media (Table 4).These results indicate that there is a very close relationship between hemolysin activity and cytotoxicity in all *A. hydrophila* isolates. So, the presence and frequency of *Aer* gene in the tested isolates was in agreement with their hemolytic activity in this study, where all isolates, except (No. 9Ah) showed positive hemolytic activity. Also, three isolates (3Ah, 6Ah and 9Ah., Figure 7) were deem inconclusive (Table 3) as they did not yield reproducible results, i.e. only a very faint amplicon once and no product the next time.

Table 2: Primers designed for the detection and genotyping of *Aeromonas* spp. virulent genes, selected sequences, their accession number, primer site location and amplicon size.

Gene	Reference Strain	Sequence Access. No.	Selected Primers (length)	References	Size bp
Aer	*A. hydrophila (SSU)* *A. hydrophila (28SA)* *A. hydrophila (Ah65)* *A. caviea (A1833)* *A. sobria (33)*	M84709 X65044 M16495 U40711 X65046	F. the forward primer CCTATGGCCTGAGCGAGAAG (20) R. The reverse primer CCAGTTCCAGTCCCACCACT (20)	Chopra, et al., 1993 Hirono,et al.,1992 Howard,et al.,1987 Wang,et al.,1996 Hirono,et al.,1992	431
Alt	*A. hydrophila (SSU)*	L77573	F. AAAGCGTCTGACAGCGAAGT (20) R. AGCGCATAGGCGTTCTCTT (19)	Chopra, et al., 1996	320
Ast	*A. hydrophila (SSU)*	AF419157	F. ATCGTCAGCGACAGCTTCTT (20) R. CTCATCCCTTGGCTTGTTGT (20)	Sha, et al., 2002	504

Table 3: Genotyping of *A.* spp. isolates based on three specific virulent genes.

	***Aeromonas* isoates**								
Gene	**1Ah**	**2Ah**	**3Ah**	**4Ah**	**5Ah**	**6Ah**	**7Ah**	**8Ah**	**9Ah**
Aerolysin *(Aer)*	+	+	Inconc.*	+	+	Inconc.*	+	+	Inconc.*
Altase *(Alt)*	+	+	+	+	+	+	+	+	+
Elastase *(Ast)*	+	+	+	+	+	+	+	+	+

* Inconclusive very faint band or no amplicon.

Table 4: Some criteria of the *Aeromonas* sp. isolates.

No.of isolate	**Isolation source**	**Hemolysis test**	**Fish mortality /isolate**	**Pathogenicity %**
1Ah	*S. auratus*	++	2	20
2Ah	*M. cephalus*	++	2	20
3Ah	*Cl. garipinus*	+	2	20
4Ah	*A. regius*	++	1	10
5Ah	*A. regius*	++	7	70
6Ah	*O. niloticus*	+	3	30
7Ah	*O. niloticus*	++	6	60
8Ah	*E. aeneus*	++	2	20
9Ah	*E. aeneus*	-	1	10

++ denote strong hemolysis + denote weak hemolysis - Negative

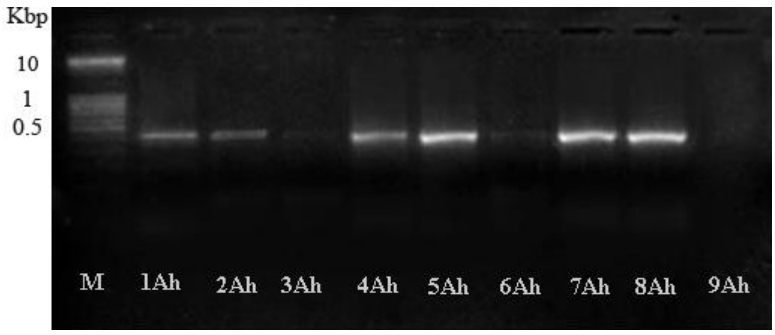


Fig. 7: Specific PCR products of 9 isolates of *A. hydrophila*. Amplification with primer *Aer*, lane (M) Amplize Molecular Ruler (10-0.5 kbp).1Ah -9Ah; *A. hydrophila* isolated from different fishes and sites.

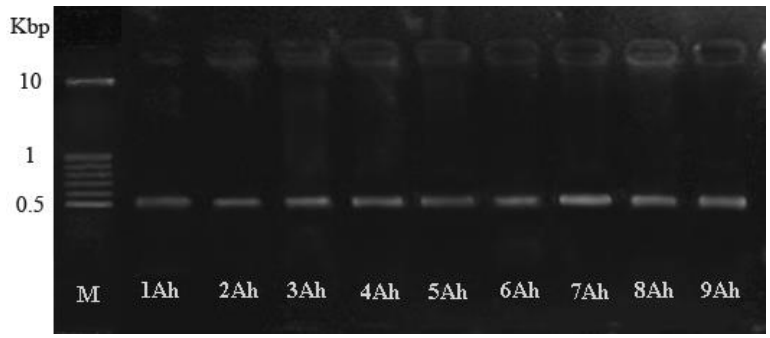


Fig 8: Specific PCR products of 9 isolates of *A. hydrophila* .Amplification with primer *Ast*, lane (M) Amplize Molecular Ruler (10-0.5 kbp). 1Ah -9Ah; *A. hydrophila* isolated from different fishes and sites.

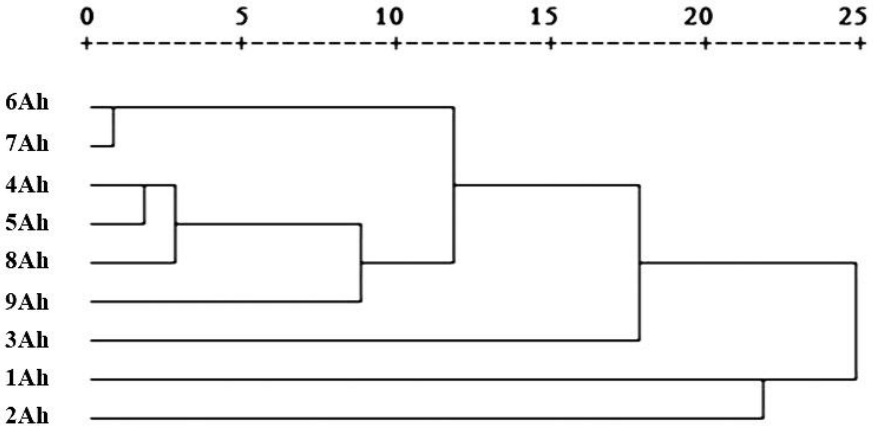


Fig 9: Dendrogram of protein similarity of 9 isolates of *A. hydrophila* (SSU) determined by SDS-PAGE protein banding pattern analysis.

3.5. Protein electrophoretic results of the different *A. hydrophila* strains

A differentiation of *A. hydrophila* (SSU) isolates was on the basis of genome homology. The phylogenetic tree represented in Figure 9 divided the isolate samples into one major cluster included 6 samples (6Ah, 7Ah, 4Ah, 5Ah, 8Ah and 9Ah), which grouped to two subclusteres (6Ah, 7Ah isolated from tilapia), and the second was No. 4Ah and 5Ah isolated from Meagre and 8Ah and 9Ah isolated from grouper. While the three remaining isolates of *A. hydrophila* (SSU) were not grouped in cluster because they exhibited singular protein patterns with no clear meaningful similarity to any of the other isolates used in this study. The isolates from different fish species and places showed difference in patterns, but the isolates within the same species and /or places had almost identical protein patterns (Figure 9) and there were tendency to cluster together (1Ah and 2Ah), (4Ah and 5Ah), (6Ah and 7Ah) and (8Ah and 9Ah) except sample No. 3Ah was genetically separated on samples 6Ah and 7Ah which isolated from the same place (El-Serw Fish Farm) but isolated from various sources of fish species. Also, in the present study, the isolates of *A. hydrophila* (SSU) isolated from various sources of fish in different places didn't constitute a single homogenous group.

3.6. Pathogenicity for tilapia

There was no death or signs of disease in control fish. Table (4) presents the pathogenicty % of *A. hydrophila* (SSU) isolates. Only two isolates (5Ah & 7Ah) proved to be virulent against tilapia causing greater than 50% mortality within 96hrs (Table 4). These fish had hemorrhages and ulcers externally and congestion and swelling of internal organs.

3.7. Histopathology of liver

The histopathological lesions detected in the liver of *O. niloticus* fish injected with the two pathogenic isolates of *A. hydrophila* (SSU) in the present study, were necrosis of hepatocytes and some hepatocytes lost their normal polygonal structure and showed disappearance of hepatocyte wall and karyolitic necrosis Figure 10a), intravascular haemolysis between hepatocytes and shrinkage of hepatocytes (Figure 10b), vacuolar degeneration in the hepatocytes with some picnosis in hepatocytes (Figure 10c). Moreover, hemorrhage between hepatocytes (Figure 10d).

Table 5: The diameters of the inhibition zones (in mm) and susceptibility status of isolates *A. hydrophila* (SSU)

No. of isolate	Methanol extracts of herbs					Aqueous extracts			
	Basil	Marjoram	mint	parsley	Rosemary	Garlic	ginger	onion	T-Protphyt
1Ah	11	25	11	R	15	R	R	R	25
2Ah	24	30	20	R	22	23	R	R	27
3Ah	23	30	22	R	22	30	R	R	15
4Ah	10	32	10	R	25	15	R	R	27
5Ah	R	21	10	R	R	R	R	R	24
6Ah	10	10	10	R	15	25	R	R	22
7Ah	10	R	R	R	R	11	R	R	23
8Ah	15	14	R	R	15	13	R	R	25
9Ah	14	22	10	R	30	R	R	R	24

R= Resistant (diameters of the inhibition zones from 0-<10 mm)

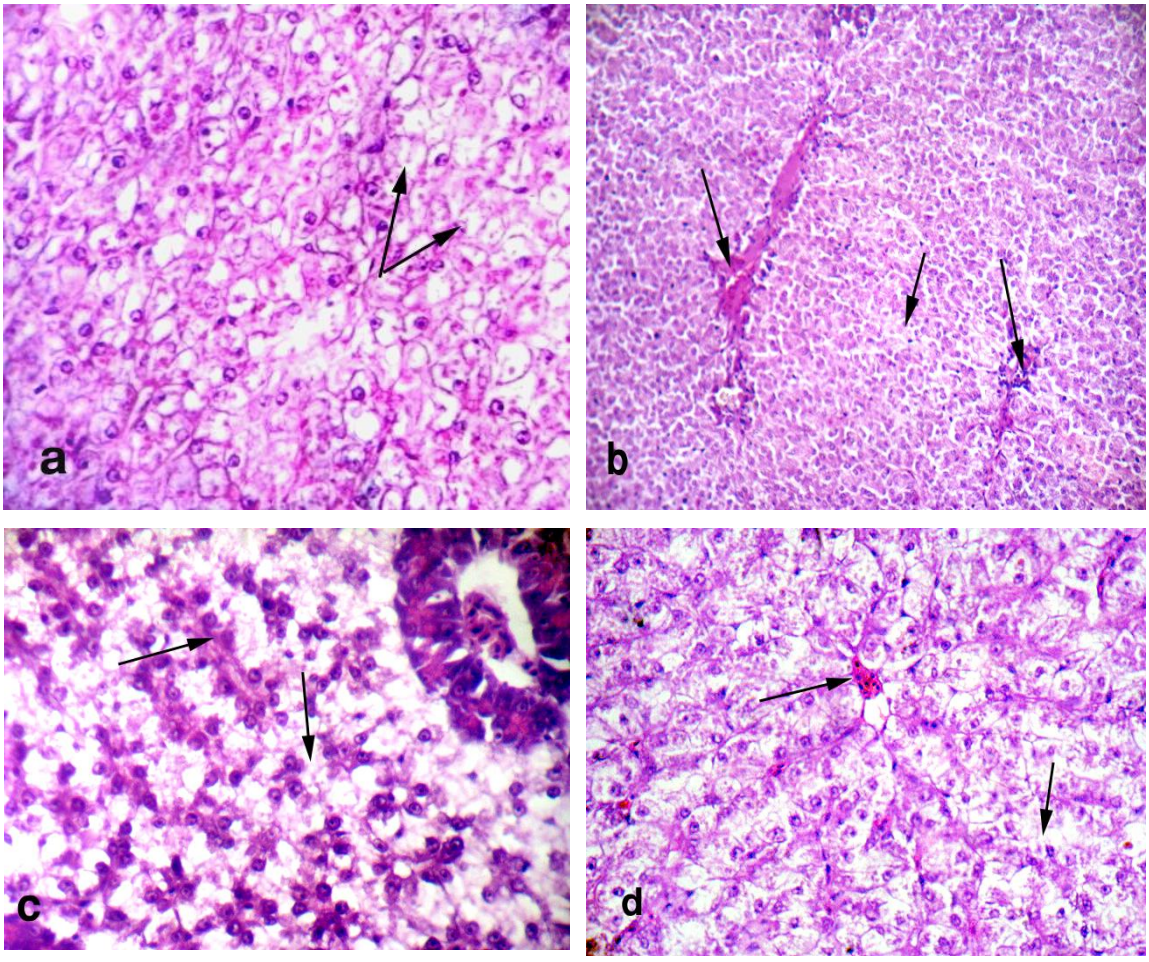


Figure 10 (a-d): Sections of liver of *O. niloticus* fish stained with H &E; (a) liver of fish injected with pathogenic *A. hydrophila*, showing necrosis of hepatocytes (x330), (b) showed intravascular haemolysis between hepatocytes and shrinkage of hepatocytes (x200), (c) showed vacuolar degeneration in the hepatocytes and necrosis (x350), (d) showed hemorrhage between hepatocytes and vacuolar degeneration in the hepatocytes (x300).

3.7. Antibacterial tests

The results (Table 5) showed that all isolates of *A. hydrophila* (SSU) were resistant to parsley, onion and ginger while the two pathogenic isolates, Ah5 and Ah7 were resistant to all tested antibacterial except with prebiotic (T-Protphyt 2000). The aqueous extract of this prebiotic was also recorded, as the most effective extract against all *A. hydrophila*. T-Protphyt 2000, marjoram, rosemary, garlic and basil exhibited pronounced positive antibacterial effects against 9, 7, 7, 3 and 3 of the nine isolates, respectively.

4. Discussion

The PCR results showed that not all isolates were PCR positive for the *Aer* gene which agrees with Yousr *et al.* (2007) who found that *Aer* gene was mainly associated only with 44.7% of *A. hydrophila*. Pollard *et al.* (1990) and Lior and Johnson (1991) showed that the *Aer* gene was only detected in hemolytic, cytotoxic and enterotoxic strains of *A. hydrophila*. Howard *et al.* (1987) reported that aerolysin is an extracellular, soluble and hydrophilic protein exhibiting both hemolytic and cytolytic properties. Also, it is considered a putative virulence factor related to the pathogenicity of several *Aeromonas* strains (Biscardi *et al.,* 2002) where it is able to alter cell permeability, so clouding disease (Albert *et al.,* 2000). In the present study, absence of *Aer* gene in some isolates which had a hemolytic activity may be due to unfulfilling the virulence criterion of presence of both hemolysin and aerolysin genes in all virulent *A. hydrophila* strains. Wang *et al.* (2003) reported that the hemolysin gene was observed in all the clinical *A. hydrophila* strains that were studied while Noterdame *et al.* (1991) reported that only 55% of *A. hydrophila* isolates from clinically affected fish and 3.3% of *A. hydrophila* isolates from healthy fish carry the *Aer* gene. The *A. hydrophila* (SSU) isolates from different fish species and places showed difference in Dendrogram of protein similarly`, Szczuka and Kaznowski (2007) reported that probably microorganisms adapt to very specific environmental conditions by turning on the expression of genes that allows survival specific habitat. Also, in the present study, the isolates of *A. hydrophila* (SSU) isolated from various sources of fish in different places didn't constitute a single homogenous group. Similarly, Garcia *et al.* (2000) found that there were genetic heterogeneity among strains of *A. salmonicida* subsp *salmonicida* isolated from different species of fish. So, the dendrogram showed that *A. hydrophila* (SSU) isolates were grouped according to their origin and environment, fish species and water salinity. The postmortem lesions in this study were agreed with Noga, (1996); AL-Dughaym, (2000) and the clinical signs of *A. hydrophila* in *O. niloticus* were agreed with those previously reported by Okpkowassili and Okpkowassili (1994) who reported that septicemia, erosion, ulceration, detachment of scale and muscular necrosis are the most predominant clinical signs of *A. hydrophila* in the Nile tilapia.

The Correlation of putative virulence factors and pathogenicity and hemolytic tests showed that, the genes encoding the putative virulence factor in toxicity of fish, *Ast* and *Alt* (Kanai and Wakaboyashi, 1984; Sakai, 1985) were present in all isolates 9/9 of *A. hydrophila* (SSU), whereas *Aer* gene was present in 6/9 *A. hydrophila* and 8/9 isolates were positive for hemolytic activity. So, these results indicated that there was a close correlation between hemolytic activity and putative virulence factors but did not correlate well between the pathogenicity test for tilapia at the laboratory conditions and the putative virulence factors, where only two isolates (Ah5 and Ah7) were pathogenic, because A. *hydrophila* secretes two cytolytic toxins, α and β hemolysin (Thune *et al.,* 1986). Pollard *et al.* (1990) demonstrated that β-hemolysin genes hold in human virulent isolates of *A. hydrophila*, it is not clear that the important virulent factor is significant for fish isolates.

The histopathological alterations in liver agree with some lesions described by Grizzle and Kiryu (1993) and EL-Barbary (2009). These lesions due to the virulence of *A. hydrophila*, where the pathogenesis of *A. hydrophila* has been reported to involve a variety of biologically active extracellular products and enzymes including toxins (cytotoxic and cytotonic); endotoxin, hemolysin, enterotoxins and protease (Cahill, 1990 and Zhang *et al.,* 2000). These Toxins apparently cause irreparable systemic damage to the hematopoietic system and liver which leads to death (Brenden and Huizinga, 1986).

The positive effect of T-Protphyt 2000 as antibacterial agrees with finding of Abdelhamid *et al.* (2009) who reported that *A. hydrophila* is sensitive to T-Protphyt 2000. The positive effect of garlic as a antibacterial agrees with previous works of Saxena and Vayas (1986) and Roy *et al.* (1992), they reported that garlic (*Allium sativum*) has shown that its bulb extract inhibits the growth of certain Gram-positive and Gram negative bacteria isolated from man and animals. Also, marjoram, rosemary, and basil exhibited pronounced positive antibacterial effects on pathogenic bacteria in previous studies (Yano *et al.,* 2006; Shan *et al.,* 2007; Nedorostova *et al.,* 2009).

The difference in antibacterial susceptibility might be due to their usage at low therapeutic level; furthermore, resistance of bacteria to some extracts could be a reflection of misuse of prebiotics, since easy access to various prebiotics is the case. Because, the use of antibiotics in aquaculture has received considerable attention because their use can lead to the development of drug resistant bacteria, thereby reducing drug efficacy. Moreover, the accumulation of antibiotics both in the environment and in fish can be potentially risky to consumers and the environment (Alderman and Hastings, 1998). So that, usage natural prebiotic, herbs and spices which have antibacterial

effects may be safed than the use of antibiotics in aquaculture.

In conclusion, this study showed that for the identification of *Aeromonas* at the species level clearly indicated that *A. hydrophila* could only be confirmed by electrophoresis after preliminary biochemical tests. Identification to the species level using PCR technique to detect specific aerolysin gene was found to be useful for direct detection of pathogenic isolates of *A. hydrophila* (SSU). Also, T- Protphyt 2000 and marjoram, rosemary, garlic and basil herbs extracts exhibited positive antibacterial effects against *A. hydrophila* (SSU).

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الخصائص المرضية والتعريف الجزئيى للايروموناس هيدروفيلا المعزول من بعض الأسماك المستزرعة والمصابة طبيعياً

منال ابراهيم البربري

معمل امراض الاسماك - المعهد القومي لعلوم البحار والمصايد

تم عزل 9 من عزلات ميكروب الايروموناس هيدروفيلا من 4 مزارع سمكية مختلفة في درجة ملوحة المياه والتي تقع في محافظة دمياط وقد تم جمع 6 أ نواع من الأسماك خلال النفوق الجماعي عام 2007. تم تجميع أسماك البلطي النيلي والقرموط من مزرعة مياه عذبة حكومية (محطة الأسماك بالسرو) بينما اللوت والوقار وكذلك كلا من البوري والدنيس تم عزلهم من 3 مزارع سمكية خاصه مختلفة في درجة الملوحة (2‰ و7‰ و 25‰ علي التوالي). العزلات البكتيريه المعزوله من تلك الأسماك تم تعريفها بإستخدام طرق مختلفه كالبيئات المتخصصه والإختبارات البيوكيميائيه ، وكذلك بإستخدام تكنيك التفاعل المتسلسل للبوليمريز (PCR) (باستخدام ثلاثه من الجينات المختلفة المتخصصة في تعريف الايروموناس هيدروفيلا Aer, Alt, Art) ،وكذلك استخدام التفريد الكهربي للبروتين .

كما تم إجراء إختبار سميه (القدره علي إحداث المرض) هذه العزلات معملياً علي أسماك البلطي النيلي كما تم إختبار كفاءة بعض المستخلصات النباتيه كمضادات للنشاط البكتيري ضد الايروموناس هيدروفيلا وكذلك بروبايوتك منتج محليا يسمي T- Protphyt-2000 في هذه الدراسة. أظهرت النتائج أن جميع عزلات الايروموناس هيدروفيلا كانت ايجابيه مع كل من Ast , Alt وبالتالي فإن كل العزلات المختبرة كانت ايروموناس هيدروفيلا في حين كانت 6 عزلات من أصل 9 ايجابيه لجين Art (كعامل من عوامل قدره الميكروب علي إحداث المرض) ،كما أن 8 عزلات من العزلات التسع كانت ايجابيه لاختبار تحلل الدم و عزلتان منهم كانت ممرضه للاسماك في تجربه معمليه ، لذلك كان هناك ارتباط وثيق بين قدره العزلات علي تحلل الدم وبين وجود جين Aer. ولكن لم يكن هناك ارتباط واضح بين وجود هذه الجينات وبين القدره المرضيه لهذه العزلات علي سمك البلطي النيلي تحت الدراسه المعمليه.

وبشكل عام ، فلقد أظهر التفريد الكهربي لبروتين العزلات البكتيرية أن بروتين العزلات تتجمع في مجاميع وفقا للمصدر البيئي ونوع الأسماك المعزولة منها وأظهر كل من T - Protphyt 2000، بردقوش ، روزماري ، الريحان والثوم قدرة إيجابيةعلي وقف نشاط 100 ، 77.7 ، 77.7 ، 33.3 و 33.3 ٪ من البكتريا تحت الدراسه على التوالي ، في حين أن كل من البقدونس والبصل والزنجبيل لم يكن له نشاط إيجابي علي وقف نمو هذه العزلات.