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# Tracing the source of infection and virulence factors of Aeromonas hydrophila isolated from Tilapia nilotica.


**A Thesis Submitted by**

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# List of Abbreviations

| | |
|---|---|
| **MAS** | Motile Aeromonas Septicemia |
| **RAPD** | Random Amplified Polymorphic DNA |
| **ERIC** | Enterobacterial Repetitive Intergenic Consensus |
| **PCR** | Polymorphic Chain Reaction |
| ***act*** | Cytotoxic heat- labile enterotoxin |
| ***aero*** | Aerolysin  gene |
| ***hly*** | Haemolysin toxin |
| **RSA** | Rimler Shotts Agar |
| **AIA** | Aeromonas Isolation Agar |
| **CFU** | Colony Forming Unit |
| **R.S** | Rimler Shotts medium |
| **TCBS** | Thiosulphate Citrate Bile Sucrose |
| **MR** | Methyl Red |
| **VR** | Vogus Proskauer |
| **EUS** | Epizootic ulcerative syndrome |
| **AH*cytoen*** | A. hydrophila cytolytic enterotoxin gene. |
| **GCAT** | Glycerophospholipid Cholesterol acyltransferase |
| ***alt*** | Cytotonic heat-labile enterotoxin |
| ***ast*** | Cytotonic heat- stable enterotoxin |
| **omp** | Outer membrane proteins |
| **EMB** | Eosin Methylene Blue |
| **TSB** | Tryptone Soya Broth |
| **TSA** | Tryptone Soya Agar |
| **MST** | Microbial Source Tracking |

# I-INTRODUCTION

Tilapia farming is favored due to its multi-environmental tolerance, marketability, disease resistance, and easy production of marketable fish using multiple feeds ranging from natural organisms to artificial pellets ***(Huicab-Pech et al., 2016).***

Fish farms are liable to losses as a result of bacterial diseases such as Motile Aeromonas Septicemia (MAS), there have been epidemics. (***Monir et al., 2020).*** MAS is caused by members of the genus *Aeromonas*, such as *Aeromonas hydrophila, A. veronii, A. dhakensis, A. jandaei, A. sobria* and *A. caviae* ***(Amal et al., 2018).***

*Aeromonas hydrophila* is considered the main occasion of diseases including ulcerative syndrome leading to high mortalities also can be a problem for human consumers ***(Hanna et al., 2014).*** *Aeromonas hydrophila as one of the Aeromonas* spp. can cause severe inflammation in the gastrointestinal tract leading to chronic diarrhea and can infect other body systems such as urinary and respiratory systems ***(Szczuka & Kaznowski, 2004) .***

Among different *Aeromonas* spp., *A. hydrophila* is well-known as an opportunistic pathogen that results in many symptoms particularly diarrhea in both animals and humans ***(Hossain et al., 2018).***

The pathogenicity of A. *hydrophila* strains is related to several virulence factors that they produce, including structural features related to adhesion, cell invasion, and resistance to phagocytosis, as well as extracellular factors such as aerolysin, a cytolytic pore-forming toxin. and

enterotoxin ***(Abd El-Tawab et al., 2019).*** *Aeromonas hydrophila* produce virulence factors more frequently than other species ***(Kuhn et al., 1997).***

*Aeromonas hydrophila* which isolated from animal and human clinical cases and environment have the ability to produce many virulent related factors, such as haemolysins, enterotoxins, and proteases more than other *Aeromonas* spp.***(Roges et al., 2020).*** All *A. hydrophila* strains included in the study of ***González et al., (2002)*** produced typical zones of beta-hemolysis on sheep blood agar plates**.**

In most cases the origin of *Aeromonas* infection is suspected to be environmental. Interesting ***Kühn et al., (1997)*** have shown single strain from food and water. This data denied water borne-possible infection to fish from water. There are few reports documenting the source of the original gastrointestinal ***(Janda J.M., 1991);*** this after ***Altwegg M. and Geissk K.H.K., (1989)*** found few positive stool culture and patient bacteraemia at the same time.

In study and compare isolates of Aeromonas spp., various typing systems have been developed for pathogenesis screening and taxonomic characterization. Aeromonas might be a clonal property and only some clones may be responsible for disease. However, studies on the clonal structure and diversity within the strains are not uncommented the clonal relatedness of strains from diarrhoeal stool specimens with environmental (milk and fish) isolates by Random Amplified Polymorphic DNA (RAPD) PCR and Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR methods ***(Davin-Regli et al., 1998; Szczuka & Kaznowski 2004; Ramalivhana et al., 2010 and Subashkumar et al., 2014).***

It is generally believed that the bacterial genome is continuously reorganized into numerous families of short, interspersed repeatitive sequences. Enterobacterial repeatitive intergenic consensus sequences (ERIC) are described as units of intergenic repeats that compared to the majority of other bacterial repetitions in that they are common or exist in a larger number of species. ***(Bakhshi et al., 2016).***

None of Aeromonas strains were genetically similar and were forward confirmed by dendrogram. The dendrogram clearly exhibited that clinical fish and environmental isolates of *A. hydrophila* (mud and water) are not genetically similar. The combined RAPD and ERIC-PCR profiles also supported the co-existance of genetically varied *A. hydrophila* among the samples. Here also none of the strain have been observed identical profile as finding of ***(Davin-Regli et al., 1998 and Bauab et al., 2003).***

Isolation of *A. hydrophila* from fish meat poses a zoonotic public health risk. Therefore, further studies of the genetic relationships between isolates derived from several sources are needed, and the virulence factors of the isolates need to be studied in detail ***(Ahmed et al., 2018).***

**Aim of the work:**

Therefore, the purpose of this research is to understand wether any particular clone of Aeromonas strains was more often associated with fish disease, to investigate the genetical relatedness of *A.hydrophila* strains among samples collected from diseased Tilapia fish and compared with diarrhoeal stool patients and environmental (mud and water) samples. To achieve the study's goal the following point were came out through the following **A** & **B:**

**A. 1.** Isolation of Aeromonas hydrophila from Tilapia fish, environmental (water and mud) and diarrhoeal stool patient samples.

**2.** Identification of *A. hydrophila* from Tilapia fish, environmental (water and mud) and diarrhoeal stool patient samples with biochemical examination according to the schem of ***(Janda, J.M.,1991).***

**3.** Confirm identification of *A. hydrophila* isolates using Polymerase Chain Reaction (PCR techniques).

**B. 1.** Investigation the genetical relatedness of *A. hydrophila* strains epidemiological typing among samples collected from diseased Tilapia fish and compared with diarrhoeal stool patients and environmental (mud and water) samples by Enterobacterial Repetitive Intergenic Consensus (ERIC – PCR) and Random Amplified Polymorphic DNA (RAPD) techniques.

**2.** Comparison of RAPD and ERIC – PCR results.

# II-REVIEW OF LITERATURE

## 1. Prevalence of *Aeromonas hydrophila* in fish:

***Naylor et al., (2000)*** were recorded that experimental demonstration shows that infected fish in unsatisfactory environments have high risk of infection with A.hydrophila when the water contains nitrite, dissolved oxygen, or carbon dioxide (CO) or inproperly handeled, packed, transported 9 stress related disease).

***Cipriano and Bullock, (2001)*** reported that freshwater habitats around the globe host aeromonads like *Aeromonas hydrophila* and other aeromonads. Normally, these organisms can be found in the normal microflora and hydrobionates inhabiting fresh water fish, where, together with other bacteria, they form a natural bio filter.

**González et al., (2002)** noted that all *A.hydrophila* strains included in his study produced typical zones of beta-hemolysis on sheep blood agar plates**.**

***Karunasagar et al, (2003)*** told that *Aeromonas hydrophila* is often responsible for severe economic losses in both wild and farmed fish due to product degradation and high livestock mortality.

***Chu, et al., and Suomalainen et al., (2005)*** were mentioned that *Aeromonas hydrophila* is considered to be the most virulent within the Aeromonas complex and recorded that *A.hydrophila* is a pragmatic and zoonotically important primary pathogen in fish that causes MAS (Motile Aeromonads Septicemia).

***Edberg et al., (2007)*** showed in much epidemiological investigation that there is a clear association between water sources and Aeromonas-borne infections.

***LaPatra et al. (2009)*** cleared that, motile aeromonad disease or motile aeromonad septicaemia (MAS) can be latent with signs including acute, chronic or superficial lesions, bulging eyes, and abdomenal distension, muscle necrosis and dermal ulcers.

***Stojanov et al. (2010)*** examined 75 samples of diseased fish and the results revealed that, all samples were positive for *A. hydrophila* isolation. Sixty-two samples of them had skin without scales, inflamed, covered by ulcers filled with pus with bloody serous fluid and fin rot. Meanwhile, the other 13samples showed no clinical changes.

***Sarkar and Rashid (2012)*** investigated the pathogenicity of bacterial isolates of *A. hydrophila* against catfish, carp and sea bass. They reported mortality rates of 60-100% for all six species of catfish studied at 30°C water temperature.

***Uddin et al., (2012)*** reported that 10 bacterial genera and 14 species were found in water from a pond, carp and catfish gills, and intestines. The five species are represented by members (A. hydrophila, Shewanella putrefaciens, Vibrio cholera, Staphylococcus sp. and Vibrio vulnificus) were widespread to all populations. The most common bacterium is A. hydrophila, which makes up nearly a third of the overall bacterial population (32%).

***Ye et al., (2013)*** noted that MAS caused by *A. hydrophila* is one of the most common and complex freshwater fish diseases causing severe mortality.

***Noor El- Deen et al. (2014)*** reported that, the clinical picture of 40 examined *O. niloticus* samples showed signs of a lack of escape reflex; skin darkness; bilateral exophthalmia and ulcers differed in its severity, enlargement, congestion, and bleeding in internal organs were appeared in postmortem examination and the bacteriological examination revealed that, 10 *A. hydrophila* strains were isolated with a prevalence of 25%.

***Matter et al., (2018)*** recorded that O. niloticus was the highly susceptible fish species and the rate of isolation of *A. hydrophila* was 42.25% in Nile Tilapia. *A. hydrophila* was highest in liver followed by spleen and intestine in fresh water fish, also concluded that *Aeromonads* were the most prevalent bacterial species, were widely distributed in warm water aquaculture.

**Soni et al. (2021)** mentioned that Motile Aeromonas Septicemia (MAS) caused by *A. hydrophila,* is one of the most common and challenging diseases in freshwater fishes causing heavy mortality.

**2. Clinical findings of *A. hydrophila*:**

***Gugnani (1999)*** reported that Aeromonas pathogen emerging from water and food products. Furthermore, he noted that Aeromonas can also cause hemorrhagic septicemia in fish and is implicated in several outbreaks that have led to severe losses.

***Ibrahem et al., (2008)*** revealed that the A. hydrophila-caused motile Aeromonas septicemia (MAS) is seasonal in nature and in cultured O. niloticus; its prevalence in cultured fish is higher during the summer compared to wild fish.

***Enany et al., (2011)*** described the most prevalent clinical signs and symptoms of naturally bacterial infected Mugil Capito and They had a dark complexion, increased mucus output, haemorrhages, and gill cover congestion; anal opening, mouth and base of fins. The results of postmortem examination showed in some cases liver was pale and anemic and liver was congested and hemorrhagic in other cases. The bowel was inflammatory and hemorrhagic, and the kidneys and spleen were hemorrhagic and congested. and free from any food particles; excessive mucous secretion and hemorrhages were due to toxin and proteolytic enzymes of pathogenic strains.

***Al-Fatlawy & Al-Ammar*** **(2013)** mentioned that *A. hydrophila* which infected fish may show a diverse set of symptoms, including sudden death, lack of appetite, swimming abnormalities, pale gills, bloating and ulcerations on their skin. Fish with hemorrhagic septicemia from thisbacteria develop small superficial lesions, focal hemorrhages, especially in the gills and opercula, ulcers, abscesses, exophthalmia, and abdominal distention.

***Bannai (2013)*** reported that various signs of disease were observed on the collected fish according to the stage of the disease including dark coloration of the skin, loss of scales. Also massive irregular hemorrhages, a variety of different skin ulcers, erosions of the fins, inflamed vent, exophthalmia, and abdominal distention with hemorrhagic fluids exuding from the vent were present.

***Thomas et al., (2013)*** told that Aeromonas species caused the pathogen found in walking catfish ulcerative disease outbreaks.

***Sayed (2017)*** revealed that, outward clinical symptoms that are most important to 240 diseased fish with MAS (*A. hydrophila* infection) included scale loss, fin rot, and skin erosions on the head and trunk, as well as large or small areas of redness. Additionally, diseased fish postmortem examinations showed generalized swelling and congestive internal organs including the kidneys, liver, gonads, and spleen. Distended gall bladders and yellow blood-tinged mucoid exudates have been observed in some cases. Moreover, 20 out of 79 suspected Aeromonads were *A. hydrophila* isolates. Infection with A. hydrophila was found to be highest in the spring and summer seasons at 17.5, 10 & 13.75 percent and 25, 12.5 & 20 percent between cultured, wild O. niloticus and cultured C. gariepinus, respectively. In the studied A. hydrophila strains, the author detected 16srRNA, act/hlyA/aer, and aer-A genes.

**Mzula (2020)** there were severe mortalities and morbidities of cultured freshwater fish in several African countries including Egypt, the main cause of disease outbreaks in farmed fish is Aeromonas hydrophila.

## 3. Phenotypic and biochemical identification:

### 3.1. Phenotypic characterization of *Aeromonas hydrophila:*

***Andrade et al., (2006)*** reported that *Aeromonas* species are short, gram negative, facultative anaerobic, non-spore forming, motile bacilli with a single flagellum and can ferment glucose with or without producing of gas.

***Alperi et al., (2010)*** reported that *Aeromonas* species are Gram negative, short rod shape, facultative anaerobes resistance to O/129 vibrio static & non spore forming.

***Al-Maleky (2013)*** reported that Aeromonads are capable of growing at temperatures ranging from 5 to 44°C. They prefer a temperature range of 22 to 28°C for optimal growth. The most clinical significant isolates will grow readily at 37°C. The range of pH for growth is 5.5-9.0. Growth that is inhibited in 6.5% salt broth.

***Samal et al., (2014)*** found that *A. hydrophila* isolates (Ah1 Ah12) are characterized by forming small, round, smooth, convex, translucent, yellow colonies on Rimler Shott's agar (RSA) medium and Aeromonas isolation agar (AIA). Microscopically, A. hydrophila looked like a short rod with a pola flagella. It was motile by swarming, and its diameter was 0.5-1.2 m, and its length was 1.4-15.0 m.

**3.2. Culture condition of *A.hydrophila*:**

***Mary et al., (2001)*** reported that it has been shown that stressed and deprived Aeromonas can enter a non-cultivable condition, as most probable a result of sublethal injury mechanisms such as cell membrane damage. This could explain the reduced numbers of CFU ml1 found when stressed bacteria were injected in fresh media compared to viable cells.

***González et al., (2002)*** noted that all *A.hydrophila* strains included in the study produced typical zones of beta-hemolysis on sheep blood agar plates**.**

***Kirov, S. M. (2003)*** *A. hydrophila* is motile by a single polar flagellum. Its principal reservoir is the aquatic environment such as freshwater lakes and streams and wastewater systems. It grows optimally at around 28 C. It has the ability to grow at cold temperatures, reportedly as low as -0.1 C for some strain.

***Vivekanandhan et al., (2003)*** were investigated of the effect of salt concentration on *A.hydrophila* growth revealed that at 30 °C and 5 °C, NaCl concentrations of 0.5, 1.0, and 2.0 percent enhanced the organism's growth. The growth of *A.hydrophila* was slowed as the salt content increased, its growth in the medium was significantly promoted by 3 percent and 4 percent salt concentrations, but there was no growth at 5.0 percent NaCl concentration. It's interesting to see *A.hydrophila* at 5 degrees Celsius and this growth is moderate.

***Khalaf et al., (2005)*** pointed that three *Aeromonas species* appeared yellow on the TCBS media because they were able to ferment the sucrose present in the medium. Similarly yellow colonies emerged on R.S medium of the same species. The colonies growing on R.S medium are also characterized by concentrated pigmentations in the center. A dark-green center usually surrounds a light-green periphery. Therefore, this type of colony is commonly known as a Bulls-eye colony since it is able to ferment maltose in the medium.

***Daskalov (2006)*** Genus *Aeromonas* Members are Gram-negative, facultatively anaerobic, non-spore forming, rod-shaped bacteria.

***Pianetti et al., (2008)*** recorded that even at high (6%) NaCl concentrations, *A.hydrophila* display long-term halo tolerance. Colony Forming Units were fewer in quantity than viable cells, particularly in the presence of 6% NaCl, indicating the presence of stressed cells that sustain metabolic activity but are unable to develop on agar plates.

***Bhowmik et al., (2009)*** recorded that either yellow or bluish-green colonies were formed when all isolates grow on TCBS agar .

***Samal et al., (2014)*** reported that in the presence of 0 - 2 percent NaCl, *A. hydrophila* strains grow in nutritional broth and other basal media, but not in the presence of 4 - 8 percent NaCl.

***Wejdan et al., (2014)*** reported that *A.hydrophila* grow in culture media appeared 1-3 mm in diameter. *A.hydrophila* colonies showed a yellow shine color on TCBS agar and non-lactose fermenters on MaCcokey agar. And were smooth, convex, rounded, β-hemolytic colonies and pale white to grey color on blood agar.

### 3.3. Biochemical identification of *A.hydrophila*:

***Abbott et al., (2003)*** reported that the motile, mesophilic A. hydrophila, A. caviae, A. sobria, A. veronii, and A. schubertii, as well as the non-motile, psychrophilic A. salmonicida, are among the Aeromonas species.

***Rasmia Hanafi, (2005)*** reported that *A. hydrophila* isolates give positive reaction to oxidase, catalase, indol, glucose fermentation and gas production, while they give negative reaction for H2S production and lactose fermentation. She noted Variations in M.R, V. P, nitrate reduction, urease, citrate, sorbitol, arabinose, mannitol and trehalose fermentation as well as esculin hydrolysis.

***Saavedra et al., (2006)*** mentioned that Although the Aeromonas genus has usually been classified as a Vibrionaceae family member, there have been recommendations to designate it as a separate family, the Aeromonadaceae. Aeromonads have many biochemical similarities with Enterobacteriaceae members, but are distinguished principally by being oxidase-positive.

***Chuang et al., (2011)*** recognized that the API20E system was found to be accurate for *A. hydrophila* and *A. veronii* but not for *A. caviae*. In terms of accuracy, Vitek II was good for *A. hydrophila* and *A. caviae* was good but not for *A. veronii*. Based on the results of the BD-Phoenix system, the accuracy for *A. caviae* and *A. veronii* was good but not for *A. hydrophila*. The *Aeromonas* species which were identified by the commercial systems are necessary to confirmed by using these additional tests, like esculin hydrolysis, gas production from glucose, Voges-Proskauer reaction, ornithine decarboxylase, and arginine dihydrolase production. However, their recognition accuracy is still incompatible with molecular methods.

***Erdem et al., and Kivanc et al., (2011)*** reported that *A. hydrophila* gave a positive result to each of the oxidase, catalase, indole, methyl red, simmon citrate, motility test, vogas proskauer and gelatin liquefaction. These results are mostly found in the reports of other researchers.

***Thomas et al., (2013)*** carried out a bacterial analysis of several colonies associated with Aeromonas species. Counting, purification, and Gram staining of colonies were performed, and their identification was determined by the following tests: the oxidase test, the oxidation/fermentation test, and a resistance test to vibrio static agent O/129 and API 20E. A series of additional tests (to detect glucose gas production, hydrogen sulfide production from cysteine, esculin hydrolysis, and suicide) were run for identifying the species of catfish based on the organs obtained from infected fish at each sampling point. In the test of the pathogenicity of each of the bacterial isolates in the healthy catfish, two dominant isolates were obtained from each media. Several species of bacteria isolated from nutrient and Aeromonas agars caused mortality in healthy catfish and reproduced the clinical signs of ulcerative lesions.

**4.** ***Aeromonas hydrophila*** **importance (pathogenicity and virulence):**

***Albert et al., (2000)*** reported that both the alt and ast genes, which encode heat-labile cytotonic and heat-stable cytotonic enterotoxins, are found in isolates of *Aeromonas spp*., which are linked to watery diarrhoea.

***Rabaan et al. (2001)*** recorded thatenterotoxins, aerolysin, and hemolysin, as well as adhesions and mucinase synthesis, are the primary virulence factors of *A. hydrophila*.

***Sha et al. (2002)*** were molecularly identified three enterotoxins from diarrheal isolates of *A. hydrophila*. One type of enterotoxin is cytotoxic, while the other is cytotonic; one is heat labile, while the other is heat stable.

***Yokoyama et al., (2002)*** mentioned that extracellular serine protease and metalloprotease are also produced by *A. hydrophila*, and their characteristics have been examined. When the serine protease generated by *A. sobria* (ASP), which belongs to the kexin subfamily of serine proteases, was administered subcutaneously, it caused edoema at the injection site.

***Castro Escarpulli et al., (2003)*** noticed that because protease is necessary for host colonisation, clinical isolates produced more protease than environmental isolates. In the isolates, protease was identified as a significant pathogenicity enzyme. They measured caseinase and gelatinase production to assess the proteolytic activity of the A.hydrophila isolates. Only 61 percent of the strains produced caseinase, but they detected serine protease genes (97%) and gelatinase activity (96%) in them.

***Chacon et al. (2003)*** identified Aeromonas spp. biochemically and genetically (16S rDNA) which were isolated from 250 samples of frozen fish (*Oreochromis niloticus*). The results revealed that, 2.6% of isolated

strains were *A. hydrophila* and phenotypically they had hemolytic, proteolytic, lipolytic, nuclease and biofilm activities. Also, PCR results cleared that, these strains had aerolysin/hemolysin, lipases, including the glycerophospholipid-cholesterol acyltransferase (GCAT), serine protease, and DNases.

***Rahman et al. (2003)*** used PCR technique for virulence determination of four strains of bacteria connected to a newly discovered species. *Aeromonas spp.* were taken away from fish suffering from epizootic ulcerative syndrome (EUS) in Southeast Asian countries.

***Sechi et al. (2004)*** reported the first instance of bilateral contact lens keratitis caused by *Aeromonas caviae* and contamination of the contact lens case and enquired about the presence of pathogenicity factors such as cytolytic enterotoxin (AHCYTOEN) and Aeromonas pilus type IV (tap) genes, produced by Aeromonas species. The results cleared that; *The* conjunctiva, cornea, and contact lens case were used to cultivate *A. caviae* bilaterally. Protease and gelatinase synthesis were found in the organism. The AHCYTOEN and *tap* virulence genes were found in the *A. caviae* strain after PCR amplification.

***Xia et al., (2004)*** demonstrated that several genes were found to be involved in the presence of multiple virulence factors in Aeromonas species.

***Chu and Lu (2005)*** detected potentially pathogenic *A. hydrophila* strains and amplified the 16S ribosomal DNA and aerolysin genes of them by m-PCR method.

***Rajeswari-Shome et al. (2005)*** detected haemolysin and aerolysin genes in *A. hydrophila* caused outbreak in marigal cultured in Meghalay by PCR.

***Zhu-DaLing et al. (2006)*** noted that from *A. hydrophila* isolated from sick fish or tortoises with a soft shell, three virulence genes were discovered: aerolysin, hemolysin, and extracellular serine protease (aerA, hlyA, and ahpA, respectively).

***Khushiramani et al., (2007)*** showed that *A. hydrophila* protein layers, O-antigens, fimbriae, and outer membrane proteins are important for mechanism adhesion and contribute to fish tissue colonisation.

***Nam and Joh (2007)*** used PCR for identification of five virulence factors in isolated Aeromoneus species [aerolysin/ hemolysin gene (aer), serine protease gene (ser), GCAT gene (gcat), lipase gene (lip), and DNase gene (dns)]. The results revealed that, four genes (aer, dns, gcat, and lip) were detected in *A. hydrophila*, four genes (aer, gcat, ser, and lip) were detected in *A. sobria*, and five genes were detected in *A. caviae* and *A. salmonicida*.

***Yousr et al. (2007)*** cleared that, the potential virulence genes of *A. hydrophila* are aerolysin and hemolysin. Aerolysin is an extracellular protein generated by some strains of *A. hydrophila*, also soluble and a hydrophilic protein with hemolytic and cytolytic functions. The PCR analysis for the discovery of aerolysin (aer) and hemolysin (hly) genes among 85 *Aeromonas spp.* secluded from the outside world and sources of shellfish showed that, the *hlyA* gene was found in 20/38 *A. hydrophila* isolates, 13/38 *A. caviae* isolates, and 6/9 *A. veronii biovar sobria* isolates, while the *aero* gene was found in 20/38 *A. hydrophila,* 17/38 *A. caviae,* and 6/9 *A. veronii biovar sobria* isolates.

***Singh et al. (2008)*** studied 25 *A. hydrophila* isolates as a result of fish and pond water in order to detect aerolysin gene. The results revealed that, The gene for aerolysin was found in 85 percent of the isolates. It's

probable that a selection of isolates despite being positive for aerolysin gene did not result in hemolysis on blood agar either as a result of a gene disruption or as a result of a gene mutation.

***Ashok et al. (2009)*** detected that, Aeromonas isolates' culture filtrates were shown to have two types of enterotoxins: cytotonic and cytotoxic. Like cholera toxin (CT), cytotonic enterotoxins (heat-labile (alt) and heat-stable (ast)) did not promote crypt degeneration and the cytotoxic enterotoxin (act) caused significant epithelial destruction in small intestine villi. In addition to its enterotoxic effect, the act gene has hemolytic and cytotoxic properties.

***Yogananth et al. (2009)*** examined 15 fish samples for isolation of *A. hydrophila* using the modified Rimler-Shotts medium and Kaper's multi test medium, and isolated strains have two virulence factors that have been identified by using PCR assay. The results showed that, only seven samples were positive for *A. hydrophila*. Moreover, Aerolysin (aer A) and haemolysin (hyl H) are two of the virulence factors discovered. The molecular mass of aerolysin (aer A-416bp) and haemolysin (hyl H-597bp) can be seen in pathogeneic amplified genes of screened fish samples.

***Nawaz et al. (2010)*** isolated *A.veronii* from catfish samples and 96.0% of isolates were harbored the *aer*A gene and the cytotoxic enterotoxin *act* gene. Moreover, the presence of antibiotic resistant gene integrons was determined in them and 48.0 % of isolates contained class I integrons with different sizes ranging from 0.6 to 3.1 kb were discovered.

***Uma et al., (2010)*** found that bacteria secrete extracellular hemolysin and cytolytic enterotoxin, which are vital for inducing lytic activity in host cells.

***Samira- Oliveira et al. (2012)*** examined 140 bacterial isolates to detect the presence of the virulence genes aerolysin, hidrolipase, elastase, and lipase using PCR in isolates of *A. hydrophila* collected from fish in the Sao Francisco River Valley, virulence according to the existence of these genes was tested in Nile tilapia fingerlings. The factors of virulence were dispersed broadly in the isolates of *A. hydrophila*. The most common virulence factor found in the isolates investigated was aerolysin, *A. hydrophila* resulting in death of the Nile tilapia fingerlings.

***Hussain et al. (2013)*** a Multiplex PCR assay was invented for the discovery of virulence genes of *A. hydrophila* using 4 sets of primers. The primer set1, A16S1 amplifies a 953 bp fragment of 16SrRNA gene of *Aeromonas spp*. The primer set 2, amplified a 400 bp fragment of *act/hlyA/aer* complex gene. The primer sets 3 and 4 amplified 536 and 361 bp fragments for detection of *ahs* (heat stable entero-toxin) and *ahl* (heat labile entero-toxin) genes, respectively.

***Sarkar et al. (2013)*** studied the molecular characterization of fish isolated strains of *A. hydrophila* from several fish markets in West Bengal and samples of water from various rivers and ponds by polymerase chain reaction for the presence of cytolytic enterotoxin AHCYTONE gene, they detected cytolytic enterotoxin (AHCYTONE) gene in such isolates and considered them a potential risk to human health.

***Takahashi et al., (2013)*** reported that the metalloprotease generated by *A. hydrophila* (AMP) has been found to be involved in the degradation of elastin, a protein that is normally insoluble.

***Aravena et al. (2014)*** researchers looked on the possible pathogenicity of Aeromonas isolates from 98 clinical and 31 environmental samples by looking for 13 different virulence genes with

PCR (polymerase chain reaction)-based technique. At the very least one of the virulence genes was found in 96 percent of the strains. Overall, the distribution was *aer*A/haem, *alt*, *laf*A, *ast*, *fla*A, *asp*A, *vas*H, *asc*V and *aex*T (77, 53, 51, 39, 32, 29, 26, 16 and 13 %) respectively. There were no amplification items identified for the genes producing a pilus that forms bundles (*bfp*A and *bfp*G) or a toxin similar to Shiga (*stx*-1 and *stx*-2). In 42 percent of environmental isolates and 24 percent of clinical isolates, five or more virulence genes were found. In the most important species, *A. hydrophila* isolates containing 48 percent and *A. dhakensis* isolates containing 42 percent of the bacteria represented five or more virulence genes when compared to *A. veronii* isolates had 19 percent and *A. sobria* isolates also had 19 percent but *A. caviae* isolates had none.

***Cagatay and Şen (2014)*** were identified pathogeneic *A. hydrophila*, in which rainbow trout farms at the Mula-Fethiye region suffered from bacterial hemorrhagic septicemia. PCR assay was used on virulence-related genes. As a method of detecting harmful bacteria from examined samples, three targeted genes have been chosen.These genes were cytolytic enterotoxin (AHCYTOEN), haemolysin (*hly*) and outer membrane protein of bacteria (*omp*TS) genes. *A. hydrophila* that was found in infected fish samples, positive amplifications were used to demonstrate it. In addition, the reference amplification results strains independently verified these favourable results.

***Furmanek (2014)*** were tested the presence of genes for five pathogenicity factors was tested using PCR technique. The polar flagella *fla*A/*flab*, haemolytic toxins *aer*A and *ahh*1, the enterotoxin act and elastase *ahy* B were detected in *A. hydrophila* strains found in the Nile River. Resistance to β-lactam antibiotics and tetracycline was found in high numbers during drug testing. The Congo was in charge of slime

production. Test on a red agar plate. Two restriction enzymes were generated by the isolate named *aeh* I and *aeh* II .

***Grim et al. (2014)*** cleared that, *A. hydrophila* was discovered from the genomes of ten Aeromonas isolates and labelled with *A. hydrophila* WI, Riv3, and NF1-NF4, *A. dhakensis* SSU, *A. jandaei* Riv2, and *A. caviae* NM22 and NM33 were annotated and sequenced. NF1-NF4 isolates came from a patient suffered from necrotizing fasciitis (NF). River water from which the NF patient acquired the illness yielded two environmental isolates (Riv2 and -3). NF1 was genetically distinct from NF2-NF4 isolates, which were clonal. Several new genomic characteristics were discovered outside of the 10 isolates' conserved core genomes. One of the four virulence factors was present in the most virulent strains or any combination thereof; cytotoxic enterotoxin, exotoxin A, and type 3 and -6 secretion system effectors *aex*U and *hcp*.

***Stratev et al. (2015)*** studied to assess not only the β-haemolytic activity of 127 *Aeromonas spp.* strains obtained from common carps (Cyprinus carpio L.) but also activity, proteolytic, lipolytic and lecithinase activities, as well as their resistance to antimicrobials. According to the findings, 17 (13%) of them looked to have β-haemolytic activity and 14 of the strains were identified as *A. hydrophila/caviae*, while the other three were identified as *A. sobria*. Forteen strains were tested for caseinase, lipase, and lecithinase activity in the skin, blood, kidney, spleen, and musculature of carps, as well as water from the tanks where the carps were maintained. Caseinase activity was absent in one *A. hydrophila/caviae* and one *A. sobria* strain. In terms of antibiotic sensitivity, gentamicin resistance was found in 9 out of 10 *A. hydrophila/caviae* strains (> 4 µg/ ml) and EUCAST MIC clinical breakpoints were used as a reference for ofloxacin (> 1 g/ml). Tobramycin resistance was found in eight of the isolates

examined (> 4 µg/ ml) and also imipenem (> 4 µg/ml). *A. Hydrophila/caviae* had varying levels of resistance to the other antimicrobial medicines examined.

***Younes et al. (2015)*** reported that the *act* gene was the most prevalent virulence gene (81.8%) in *A. hydrophila* strains isolated from diseased *O. niloticus,* which has cytotoxic and enterotoxic activity.

***Stratev* et al. (2016)** by multiplex PCR examined 26 β-haemolytic *A. hydrophila* strains separated from separated from several types of samples included live carp, water, cooled horse mackerel, trout, silver carp cutlets, vacuum-packed trout filets and frozen trout to see if they have genes that code for aerolysin (aerA) and haemolysin (ahh1) using a strong β-haemolytic activity as a positive control (*A. hydrophila* strain isolated from a dead anaconda) and a reference *A. hydrophila* strain (ATCC 7965). The results showed that, 100% of all strains was in possession of the gene 16S rRNA (356 bp) with regard to *A. hydrophila*. The gene ahh1 was found in all (100%) A. hydrophila strains (130 bp.). In 26 A. hydrophila strains, the gene aerA (309 bp.) was confirmed (93 percent). Furthermore, the prevalence of enterotoxigenic strains of A. hydrophila in fish and fish items available for consumption by humans in grocery shops poses a health risk to consumers.

***Abd El Tawab et al. (2017)*** studied the *A. hydrophila* and *A. caviae* virulence genes identified from freshwater fishes at Qalubia Governorate. *Aero* gene was found in isolated Aeromonas strains based on PCR results for virulence genes. *Aero* gene was found in 9 out of 10 *A.hydrophila* studied strains and 3 out of 6 *A. caviae* strains; *hly* gene in 7 out of 10 *A.hydrophila* and 2 out of 6 *A. caviae* strains; *Ahcytoen* gene in 6 out of 10 *A.hydrophila* and 1 out of 6 *A. caviae* strains; *act* gene in 6 out of 10 *A.hydrophila* and 3 out of 6 *A. caviae* strains; and *ast* gene. Isolated isolates

producing a wide range of virulence factors indicate their ability to cause disease in fish and humans.

***Roges et al., (2020)*** revealed that *Aeromonas hydrophila* isolated from animal and human clinical cases and environment have the ability to produce many virulent related factors, such as haemolysins, enterotoxins, and proteases more than other *Aeromonas* spp.

**5. Antibiotic sensitivity pattern of *Aeromonas hydrophila* :**

***Vila et al., (2002)*** reported that Pathogenic *Aeromonas* species was considered highly drug resistance due to the common incidence of numerous antibiotic resistances in addition to beta-lactamic antibiotic resistance

***Naima I. Ferwana, (2007)*** reported that antibiotic resistance in *Aeromonas spp*. poses a potential issue in the treatment of infections caused by these organisms using antimicrobials. Broad-spectrum ampicillins, amoxycillin/clavulanate, co-trimethoprim, and first and second generation cephalosporins should not be used as first-line antibiotics, she advised, especially for invasive infections. Broad-spectrum ampicillins, amoxycillin/clavulanate, co-trimethoprim, and first and second generation cephalosporins should not be used as first-line antibiotics, she advised, especially for invasive infections. For the treatment of serious Aeromonas infections, fluoroquinolones such as ciprofloxacin, intravenous cefotaxime, and meropenem are indicated. Oral antibiotics such as ciprofloxacin and ofloxacin are effective.

***Bhowmik et al., (2009)*** recorded that resistance to furazolidone (93%) or tetracycline (93%) was substantially more common than resistance to nalidixic acid (14 percent).

***Durmazy and Turk, (2009)*** mentioned that ciprofloxacin works by inhibiting an enzyme called DNA gyrase, which is a crucial part of the system that transmits genetic information from a parent cell to daughter cells when a cell divides. Ciprofloxacin was shown to be the most efficient against *Aeromonas spp*. isolated from shrimp culture hatcheries and ponds in India.

***Rey et al., (2009)*** mentioned that penicillin resistance has long been thought to be universal among aeromonads. As a result, ampicillin was added to the culture media to allow for the selective isolation of aeromonads from contaminated samples.

***Sharma et al., (2010)*** reported a total of 38 Aeromonas isolates from chicken, fish, pork, and chevon were found to be very sensitive to antibiotic discs, according to an overall review of drug sensitivity tests that were recorded as Ciprofloxacin (97.36%), Streptomycin (86.84%), Amikacin (84.21%), Nalidixic acid (78.94%), Cephotaxime (73.68%), Gentamicin (63.15%), Cefuroxime (55.26%), Co-trimoxazole (18.42%), Chloramphenicol (13.15%), Sulphafurazole (13.15%), Carbenicillin (10.52%), Trimethoprim (7.89%), Tetracycline (5.26%), Chlortetracycline (5.26%), Ampicillin (2.63%), Cephalothin (2.63%), and Kanamycin (2.63%).

***Ashiru et al., (2011)*** found the development of drug resistance is of clinical concern, both because this is most probably the consequence of increasing and often indiscriminate use of antibiotics and because this

organism may cause human infections. Given the severity of aeromonad infections in both fish and humans, correct identification of the infectious agent is essential for the rapid selection of antibiotic therapy. Ciprofloxacin, pefloxacin and ofloxacin are suitable antibiotics that can be used in the treatment of Aeromonas associated infections.

***Enany et al., (2011)*** tested *A.hydrophila* isolates from naturally infected Mugil Capito fish; they were sensitive to ciprofloxacin and nalidixic acid, intermediate to rifampicin while resistant to colistin sulfate, erythromycin, amoxicillin, amikacin and lincomycin.

***Ndi and Barton (2011)*** detected antibiotic resistant gene *aad*A2; *tet*A; *tet*C; *sul*1; *str*A-*str*B in 90 Aeromonas species isolated from fish of nine Trout fresh water farms in Victoria (Australia), sixty-nine (77%) *A. hydrophila*, ten (11%) *A. veronii bio sobria*, two (2%) *A. veronii bio veronii* and nine (10%) *A. caviae*. Moreover, Class 1 integrons were found in 28 of the 90 strains (31%), but not in class 2 or class 3. Furthermore, the aadA gene was found in 19 of the 27 streptomycin-resistant strains (70 percent). Also, sul1 gene was found in 13/15 (86.7%) sulphonamide-resistant strains, whereas the qac1 gene was found in 8/28 (28.1%) integron-bearing strains. In addition, tetC was found in all tetracycline-resistant strains, while tetA was found in 9/18 (50%) of them. In none of the strains, the strA-strB, blaTEM, or blaSHV genes were found.

***Sharma et al., (2011)*** mentioned that Antibiotics have proven to be extremely useful in the treatment of infectious disorders caused by bacteria, resulting in an increase in average life expectancy in the twentieth century. Bacteria that cause disease, on the other hand, have developed resistance to antimicrobial treatment and are becoming a growing public

health concern. For effective clinical control, an isolate's antibiotic susceptibility is frequently required.

***Igbinosa et al., (2013)*** noted that *Aeromonas species* could possess integron which makes it possible for them to receive and transmit resistance genes of antibiotic, increasing the likelihood of bacterial infections that are resistant to antibiotics. Periodic surveillance of drug resistance in these organisms in various geographical areas and from various sources is required in order to provide suitable guidance for the selection of antimicrobial agents for empiric therapy.

## 6. Molecular techniques for detection and identification of *A. hydrophila*:

### 6.1. PCR (Polymerase chain reaction):

Some immunological approaches have been utilised to detect *A. hydrophila* to date; however, there is significantly less data on PCR identification of virulent or pathogenic *A. hydrophila* isolates from fishes.

***Lantz et al. (1994)*** showed that, however, the presence of chemicals that block DNA polymerase, bind magnesium, and/or denature DNA reduced the utility of the PCR for detecting microorganisms in food and other complex samples. When a sample contains inhibitors, sensitivity is drastically reduced; as a result, extensive sample preparation and DNA extraction processes are normally necessary prior to the PCR.

***Olsvik et al. (1994)*** cited that because PCR can amplify particular DNA, it eliminates the requirement for huge amounts of test DNA in hybridization experiments. A single copy of the target gene should theoretically suffice for effective amplification.

***Rodriguez (1997)*** cited that PCR technique is widely used in veterinary research and this technique is predicted to have a significant impact on animal infectious disease epidemiology, treatment, and prevention. The emergence of PCR, on the other hand, has the potential to improve pathogen diagnosis in the lab.

***Kingombe et al., (1999) and Gonzales-Serrano et al., (2002)*** mentioned that using virulence-associated genes as detection markers is a very simple and quick way to identify organisms without having to go through the typical microbiological culturing process. Previous research has suggested that hemolysin and aerolysin production may both contribute to *A. hydrophila* pathogenicity.

***Peng et al. (2002)*** described the application of universal primer PCR to detect 16S rRNA genes from *A. hydrophila*. They explained that using molecular approaches such as PCR to identify virulence factors is a strategy for determining possibly pathogenic *Aeromonas spp*. isolates. Although many virulence factors have a role in the pathogenesis of Aeromonas-related fish and human diseases, none of them can be blamed for all disease signs. Because of the intricacy of Aeromonas spp. pathogenesis, which is multifactorial in nature, identifying several virulence factors of this genus is critical.

***Chacon et al., (2004)*** reported that pathogenic bacteria are detected and non-pathogenic strains are distinguished using nucleic acid amplification methods targeting virulence genes. PCR is an in-vitro amplification process that uses two oligonucleotide primers that hybridise to opposite strands and flank the region of interest in the target DNA to enzymatically synthesise particular DNA sequences. Template

denaturation, primer annealing, and extension of the annealed primers by thermostable DNA polymerase are all part of a sequence of cycles.

***Xia et al. (2004)*** studied the h-hemolysin genes of pathogenic *A. hydrophila* strains were initially identified using the PCR technique. PCR specificity is notable, and it can achieve great sensitivity. The h-hemolysin gene from *A. hydrophila* DNA had a detection limit of micrograms. However, more research is needed to determine whether the PCR can be used to detect all virulent *A. hydrophila* strains in the hosts. The h-hemolysin-specific PCR was used to identify virulent *A. hydrophila* strains. strains. The PCR approach will have a wide range of applications for detecting pathogenic *A. hydrophila* h-hemolysin genes. Aerolysin gene is a pore forming toxin and is regarded as the most important virulence factor in Aeromonas food poisoning. The Aerolysin gene encodes a pore-forming toxin that is thought to be the most critical virulence component in Aeromonas food poisoning.

***Yousr et al., (2007)*** mentioned that the most successful technique to find and characterize Aeromonas virulence factors is to screen hemolysin genes.

***Wen et al. (2008)*** developed and optimized the multiplex polymerase chain reaction to simultaneously detect and identify *A. hydrophila*. Specific primer was designed according to the sequences of 16S rDNA of *A. hydrophila*. The results revealed that, only 576 bp long DNA fragments from the *A. hydrophila* was amplified by the multiplex PCR using the primer.

***Kingombe et al., (2010)*** have used PCR technique to detect the strains of *A. hydrophila* (HG).

***Ottaviani et al., (2011)*** mentioned that using virulence-associated genes as detection markers is a very simple and quick way to identify organisms without having to go through the typical microbiological culturing process. Previous research has suggested that hemolysin and aerolysin production may both contribute to *A. hydrophila* pathogenicity.

### 6.2. ERIC-PCR (Enterobacterial repetitive intergenic consensus) and RAPD (Random Amplified Polymorphic DNA) techniques:

***Szczuka & Kaznowski, (2004)*** shown that *Aeromonas* spp. have a clonal character and only some clones could be pathogenic. They had screened 120 *Aeromonas* strains isolated from stool of diarrheic patients, animals, and the environment by RAPD, REP-PCR, and ERIC-PCR and found a clonal structure for only 6 spp. including *A. hydrophila*. Also they reported that RAPD and ERIC-PCR methods were more efficient than REP-PCR for distinguishing the different isolates of Aeromonas spp. Similarly.

***Ramalivhana et al., (2010)*** revealed that Enterobacterial Repetitive Intergenic Consensus (ERIC – PCR) and Random Amplified Polymorphic DNA (RAPD) techniques were extensively used to detect clonal identity among different bacterial species and strains. They found 12 unique RAPD figer prints revealed atendency of the human diarrhoria and environmental isolates to clusters according their origin of isolation.

***Diab & Al-Turk, (2011)*** observed that the 56-70% genetic similarities of the plasmid included in the same bacteria isolated from different sources using PCR- based molecular quantitative assay, such as ERIC and RAPD techniques, could be a good microbial source tracking (MST) approach. The bacterial isolates had been observed not only had the same identity, but also high genetic plasmid similarities, even when isolated from different ecosystems.

***Subashkumar et al., (2014)*** showed a remarkable heterogeneity among the animals and human clinical and environmental *A. hydrophila* isolates as revealed RAPD and ERIC-PCR methods.

***Anjay, Kumar, & Dubal, (2015)*** reported that various clusters generated in the ERIC- PCR dendrogram showed a similarity of isolates from diverse origin. The similar genotypes among human isolates and isolates from foods of animal origin, water, vegetables and sewage indicated the transmission of this important zoonotic pathogen through food and between human and animals.

***Sarkar et al., (2016)*** revealed that ERIC-PCR is the best suited method for studying the molecular characterization of *A. hydrophila* isolated from humans, animals and foods and other environmental samples. The study also confirmed the presence of different virulent Aeromonas strains and these techniques have wider and scopes of application in characterization and typing in molecular based diagnostic purpose of this pathogen.

***AL-Fatlawy et al., (2017)*** said that the ERIC band pattern is an adequate tool for epidemiological investigations of A. hydrophila, A. sobria and V. cholerae isolates and dicovered the new isolates in

Nucleotide/Blast which were recorded as the first sequencing in Gene-Bank/NCBI, DDBJ and ENA (INSDC). The frequency of A. hydrophila, A. sobria and V. cholerae isolates in Najaf were higher among clinical and environmental isolates. ERIC DNA Fingerprinting with ERIC primers generated amplification bands ranging in size (87 bp to 8000 kb). The 44 isolates of (34 A. hydrophila 2 A. sobria, 3 V. cholerae, 1 E. coli O 157, 1 E. coli, 1 Pseudomonas aeruginosa, 1 Enterobacter complex and 1 Acinetobacter manniuii) produced 93 different patterns by ERIC fingerprinting.

***Zarifi et al., (2020)*** obtained, A. baumannii strains consisted of 14 strains, based on the graph and Similarity coefficient value of⩾0.90 (Similarity thresholds of ⩾90%). Most strains in this typing also be-longed to clone 1. In ERIC-PCR, 49 strains out of 80 were typed and 31 strains could not be typed. Seven single-clones were observed in this typing.

# III-MATERIAL AND METHODS

## 1. Materials:

### 1.1. Samples:

The samples were collected from tilapia fish (*O. niloticus*) had signs of septicemia and suffered from MAS (n=56), environmental water (n=56), sediment of fish farms soil (n=56) and stool samples of patients suffering from gastroenteritis (n=56).

Fish and environmental samples were collected from different fish farms at Kafr El-Sheikh Governorate, Egypt. Human samples were collected from Endemic Diseases Department at Kafr El-Shiekh General Hospital from patients suffering from gastro-enteritis, some of them had diarrhoeal stool and the remaining had apparently abdominal pain as shown in Table (1).

Table (1): Distribution of the collected samples among Tilapia fish and human stool.

| Tilapia fish | | | | Human stool | | | |
|---|---|---|---|---|---|---|---|
| MAS | | Apparently healthy | | Diarrhoeal | | Apparently normal | |
| NO | % | NO | % | NO | % | NO | % |
| 24 | 42.8 | 32 | 57.1 | 37 | 66.1 | 19 | 33.9 |
| 56 | | | | 56 | | | |

Each examined sample was taken alone in a strong sterile plastic bag, kept in icebox and transferred to the lab. of Animal Health Research Institute, Kafr El-Sheikh branch, Agriculture Researches Center, Egypt for bacteriological examination.

### 1.2. Media for bacteriological examination:

#### 1.2.1. Media used for primary isolation:

a-Nutrient broth medium ***(Cruickshank et al., 1975):***

It was used for incubation of samples before their inoculation onto solid media.

b- TSB **(Oxoid- CM0129):**

It was used as pre-enrichment medium for the growth and multiplication of *Aeromonas Spp.*

c- TSA **(Oxoid):**

It was used as a medium for isolation of *Aeromonas Spp.*

d- Aeromonas agar (Ampicillin-dextrin agar medium):

It was used as a published selective media were evaluated for the isolation of *Aeromonas* spp.

e- EMB **(HI Media):**

It was used for the isolation of *A. hydrophila* and produce metallic chine colonies while the other Aeromonas strains do not produce this character, at pH 7.1±0.2.

f- Blood agar (***MacFaddin, 1980***)**:**

This medium was used to detect the haemolytic activity of the isolated *Aeromonas* spp. (β- Hemolytic activity will show lysis and complete digestion of red blood cell contents surrounding colony), at pH 7.4±0.2.

Medium was prepared by defibrinated sheep blood.

g- Starch Ampicillin agar (***Palumbo et al., 1985***)**:** Nutrient agar plus 0.4% starch and Ampicillin (10 ug), for detection of amylase enzyme of Aeromonas.

### 1.2.2. Media used for preservation:

a. Semi-solid agar medium (***McFadden, 1980***)**:**

This medium was used for preservation of the strains during the course of our study, it was used also for detection of motility.

b. TSB with 15% glycerol:

This medium was used for preservation of isolated strains.

### 1.2.3. Media used for characterization of the bacterial isolates:

1-One % peptone water broth ***(Cruickshank et al., 1975):***

This medium was used for detection of indole production.

2-Glucose phosphate broth ***(Cruickshank et al., 1975):***

It was used in methyl red and Voges-Proskauer (MR / VP) tests.

3-Simmon's Citrate agar medium ***(Koneman et al., 1992):***

This medium was used for citrate utilization test.

4-Stuart's urea broth ***(Koneman et al., 1992):***

It was used to detect urease activity of the isolated bacteria.

5-Sugar fermentation medium ***(Cruickshank et al., 1975):***

It was used in sugar fermentation test.

6-Nitrate broth medium ***(Koneman et al., 1992):***

It was used in detection of nitrate production.

7-Oxidation fermentation medium (OF) medium ***(Koneman et al., 1992):***

It was used to differentiate oxidative non fermenting bacteria.

### 1.3. Solutions and reagents :

1-Gram's stain ***(Quinn et al., 2002):***

It was used for staining films from bacterial isolates for morphological characters and staining reaction.

2-Hydrogen peroxide (3%) (H2O2) ***(Koneman et al., 1992):***

This solution was used for catalase test.

3-Kovac's reagent ***(Koneman et al., 1992):***

It was used for detection of indole production and oxidase test.

4-Methyl red indicator ***(Koneman et al., 1992):***

It was use as a pH indicator for methyl red test.

5-Voges-Proskauer reagents ***(Koneman et al., 1992):***

5 % α naphthol and 40 % KOH.

6-Phosphate buffer saline:

It was used as adiluent in some microbiological tests.

7-Phenol red reagent:

It was used in urease test.

8-Sodium citrate:

It was used to preserve sheep blood defibrinated.

**1.4. Antibacterial Sensitivity discs:**

By using the disc method of ***NCCLS (1992),*** Table (2), sensitivity test was done on each isolated Aeromonas strains to study their sensitivity for different antibiotics.

**1.4.1. Media used for Antibiotic sensitivity test:**

**1.4.1.a.** Muller Hinton broth (TM MEDIA -325), at pH 7.3±0.2.

**1.4.1.b.** Muller Hinton agar (HIMEDIA- M 173-500G), at pH 7.3±0.1.

Table (2). Antibiotic discs concentrations (***CLSI 2018***)

| **Antibiotic discs (oxoid)** | **Disc concentrations** | **Class** |
|---|---|---|
| Ampicillin | 10 µg | Penicillin |
| Amoxicillin | 25 µg | Penicillin |
| Penicillin-G | 10 µg | Penicillin |
| Cefotaxime | 30 µg | Cephalosporin |
| Gentamycin | 10 µg | Aminoglycosides |
| Streptomycin | 10 µg | Aminoglycosides |
| Norfloxacin | 5 µg | Quinolones |
| Ciprofloxacin | 10 µg | $2^{nd}$ Generation |
| Moxifloxacin | 5 µg | $3^{rd}$ Generation |
| Trimethoprim | (1.25/23.75) µg | Microlytes |
| Oxytetracycline | 30 µg | Tetracycline |
| Florphenicol | 30 µg | Chloramphenicol |
| Imipenem | 10 µg | Carbapenem |

## 1.5. Materials of Polymerase Chain reaction (PCR techniques):

### 1.5.1 Reagents and kits used for DNA extraction and polymerase chain reaction (PCR):

**a. Bacterial DNA extraction kit (Fermentas, K0721)** which includes the following:

- Proteinase K solution (to final concentration of 20 ug/ml)
- Lysis solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4).
- Wash buffer I

- Wash buffer II
- Elution Buffer (10 mM Tris-Cl, pH 9.0, 0.5 mM EDTA).

**b. PCR reaction kit (DreamTaq™ Green PCR Master Mix 2X, Fermentas, #K1071)** which includes the following:

- 2X DreamTaq™ Green buffer (10 ml mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5mM MgCl2, 0.1% Triton x-100, 0.2 mg/ ml BSA)
- Ten mM dNTPs, PH 7.0
- Four mM MgCl2,
- Taq DNA polymerase containing storage buffer (250 U Taq DNA polymerase, 20 mM TrisHCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM Dithiothreitol, 0.5% Tween 20, 0.5% Nonidet P40, 50% Glycerol, kept at -20°C)
- Two tracking dyes,
- Nuclease free water,

**c. Ethidium bromide** 0.5 mg/ml

**d. Agarose (bioshop, 1L22739)**, 1% (1.0 gm agarose in 100 ml of 1X TAE buffer).

**e. DNA loading buffer, 6x (Fermentas)**: (10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol, FF60% glycerol and 60 mM EDTA).

**f. One hundred bp ladder DNA marker** 0.5 µg/µl **(Fermentas, #SM0321)**

### 1.5.2. Solutions and buffers used for DNA extraction and polymerase chain reaction (PCR) :-

**a. Tris-HCl (1 M)** ***(Sambrook et. al., 1989):***

It was prepared by dissolving 121.1 g of Tris base in 800 ml of distilled water. The pH was adjusted to the desired value by adding concentrated HCl. The buffer volume was completed to one liter with

distilled water and then sterilized by autoclaving. It was used for preparation of Tris-EDTA buffer solution (TE).

**b. EDTA (0.5 M – pH 8.0)** ***(Sambrook et al., 1989):***

Di-sodium EDTA (186.1 g ) was dissolved in 800 ml of distilled water. The pH was adjusted to 8.0 with 10 N Sodium hydroxide. The solution was completed to one liter and autoclaved. It was used for preparation of Tris-EDTA (TE) and Tris-acetate EDTA (TAE) buffers.

**c. Tris-EDTA buffer (TE) (pH 8.0)** ***(Sambrook et al., 1989):***

| | |
|---|---|
| 1 M Tris – HCl | 1.0 ml |
| 400 mM EDTA (pH) | 0.2 ml |
| Double distilled water up to | 100 ml |

It was sterilized by autoclaving and used for dissolving the DNA pellet after ethanol precipitation.

**d. Tris-acetate EDTA (TAE) buffer** ***(Sambrook et al., 1989):***

50 x TAE buffer : It was prepared as follows:

| | |
|---|---|
| Tris base | 242.0 g |
| Glacial acetic acid | 57.1 ml |
| 0.5 M EDTA (pH 8.0) | 100 ml |
| Distilled water up to | 1000 ml |

It was sterilized by autoclaving and stored at room temperature. It was used for preparation of agarose gel and running buffar for electrophoresis.

**e. Gel-loading buffer (6 x buffer) (*****Sambrook et al., 1989):***

| | |
|---|---|
| 0.25% | bromophenol blue. |
| 40% (w/v) | sucrose in water. |

This buffer was stored at 4 °C. This gel-loading buffer serves three purposes:

i- It increases the density of the samples, ensuring that the nucleic acid (PCR product) drops evenly into the gel well;

ii- It adds colour to the sample, theraby simplifying the loading process;

iii- It contains a dye that, in an electric field, moves towards the anods at a predictable rate.

It was used at a concentration of 0.5 µg/ml for staining the DNA in the agarose gel.

**2.Methods:**

**2.1. Collection of samples:**

**i-Fish samples:**

Fish samples were collected from different fish farms at Kafr El-Sheikh Governorate, Egypt. Each examined sample was taken alone in a strong sterile plastic bag, kept in icebox and transferred to the lab. of Animal Health Research Institute, Kafr El-Sheikh branch, Agriculture Researches Center, Egypt for clinical, post mortem and bacteriological examination.

**ii- Environmental samples (water and soil):**

Environmental samples (representative water and mud samples) were collected from fish farms waters (3 farms) and 3 farms their muds. Each examined sample was taken alone in a sterile plastic gar, kept in icebox and transferred to the same lab. for bacteriological examination. Water samples (100 ml) were collected and filtered through a 0.45 Mm membrane that was then placed on Tryptase soya agar (bio- Merieux) ***(Davin-Regli et al., 1998)***.

**iii- Human stool samples:**

Human stool samples were collected during the same period from Endemic Diseases Department at Kafr El-Shiekh General Hospital. These samples were collected from patients suffering from gastroenteritis, some of those samples were diarrhoeal stool. The samples were collected by sterile swabs and phosphate buffer saline were added to it until going to the same lab. for bacteriological examination.

### 2.2. Clinical and post mortem examination:

#### 2.2.1. Clinical examination:

Clinical examination was performed to investigate any clinical abnormalities according to the method described by ***Austin and Austin (2007).***

#### 2.2.2. Post mortem examination:

The fish surfaces were swapped with 70% ethyl alcohol for surface sterilization and then examination of internal organs was done according to the method described by ***Schaperclaus et al. (1992)*** through which the internal organs became visible and examined for detection of any abnormalities.

### 2.3. Bacteriological examination (Quinn *et al*., 2002 and Markey *et al.,* 2013) :

#### 2.3.1. Isolation of *Aeromonas spp.* from different samples:

After the fish skin surface was disinfected with 70% ethyl alcohol, sterilized swabs were taken from kidney and liver of 56 examined fish. A sterilized swab was taken from each human stool sample. Each swab was divided into two tubes. To one of them a nutrient broth was added and a Tryptone Soya Broth (TSB) was added to the other tube. The tubes were inoculated aerobically at 37°C overnight. The same was exactly happened with the environmental water and soil samples.

A loopful of the incubated nutrient broth was streaked onto Aeromonas agar plates. A loopful of the incubated Tryptone Soya Broth (TSB) was streaked onto Tryptone soya agar plates. Then all plates were incubated for 24 hours at 37ºC.

The suspected colonies were the creamy or the pale colonies on Aeromonas agar plates. The greenish colonies on Tryptone soya agar plates were the suspected colonies.

One separate suspected colony was picked up and streaked onto nutrient agar plate. The purified colony was studied morphologicaly.

### 2.3.2. Identification of the isolates (*Quinn et al., 2002; Austin and Austin, 2007 and Markey et al., 2013)*:

The bacterial isolates were subjected to characterization by studying their morphological, cultural, and biochemical characteristics as follows:

#### 2.3.2.1. Microscopical examination:

From pure suspected colonies the smears were prepared and stained with Gram's stain and examined microscopically.

According to staining reaction shape and cell arrangement under microscope isolates were selected for biochemical identification.

#### 2.3.2.2. Biochemical examination:

The selectad isolates were examined by different biochemical tests as following:

**i.Catalase test:**

Adding drop of hydrogen peroxide to the examined colony on a clean slide and mix. Formation of air bubbles indicate positive result.

**ii.Urease test:**

Bacterial isolates were cultured into Stuart urea broth and incubated at 37 $^{o}$C for 24 hrs., then examined. Development of red pink colour indicate hydrolysis of urea to ammonia and $CO_2$.

**iii.Oxidase test:**

Apiece of filter paper was soaked with few drops of oxidase reagent acolony of the test organism was then smeared on the filter paper. A deep purple color within few seconds (10sec) indicate positive result.

**iv. IMVIC:**

**1- Indole test:** one percent peptone water was inoculated with each culture and incubated at 37 c for 24-48h. Kovac's reagent was gently trickled down the side of the tube. Development of a rosy ring indicates indole producation.

**2-MR test:**

Five drops of methyl red reagent were added to 5ml of 48 hours glucose phosphate broth culture. Appearance of red color indicate positive reaaction.

**3-VP test**:

Three ml of alcoholic solution of &-naphthol and 1ml of 40% KOH solution were added to 5 ml of 48 hours glucose phosphate broth culture and incubated at 37c. The mixture was thoroughly shaken and examined after 15 minutes. Pink red color indicates positive reaction and yellow color indicated negative reaction.

**4-Citrate utilization test:**

The ability to utilize citrate was detected by making a single streak from the cuture at the surface of Simmons citrate agar slope and incubated at 37 c for 24-48h. Development of blue color indicates citrate utilizaiton while remaining green color means negative result.

**2.4. Antibacterial sensitivity test:**

Susceptability to 13 antimicrobial agents, 8 classes was determined by a standard disk diffusion method on Muller Hinton Agar (***NCCLS, 1992 and CLSI, 2018***). The isolated colonies were transferred into 5 ml

physiological saline. This was poured onto agar plate after mixing which allowed covering the entire surface of the agar. After it was drained off the nutrient agar plate, it was being left for a few second. Antibiotic discs were positioned on the agar surface then incubated at 37 C for 24 h. Diameters of inhibition zones was observed and measured in millimeters. The antibiotics sensitive reaction gives a zone of inhibition of 12 mm and above and a resistant reaction gives a zone of inhibition below 12 mm table (3). Calculation of the sensitivity as mentioned by ***(Idika, N., et al., 2011 and Magiorakos, S. et al., 2014).***

Table (3): Antibacterial zone diameter interpretive standards of their effect:

| Antibiotic discs (oxoid) | | Disc concentrations | Zone of inhibition(mm) | | |
|---|---|---|---|---|---|
| | | | **Resistant <mm (R)** | **Intermediate mm range (IS)** | **Sensitive > mm (S)** |
| Ampicillin | AM10 | 10 µg | ≤13 | 14-16 | ≥17 |
| Amoxicillin | AMX/25 | 25 µg | ≤14 | 15-17 | ≥18 |
| Penicillin-G | P10 | 10 µg | ≤10 | 11-12 | ≥13 |
| Cefotaxime | CTX/30 | 30 µg | ≤15 | 16-20 | ≥21 |
| Gentamycin | CN/10 | 10 µg | ≤12 | 13-14 | ≥15 |
| Streptomycin | S/10 | 10 µg | ≤11 | 12-14 | ≥15 |
| Norfloxacin | NOR/10 | 5 µg | ≤12 | 13-16 | ≥17 |
| Ciprofloxacin | CIP/5 | 10 µg | ≤15 | 16-20 | ≥21 |
| Moxifloxacin | MXF/5 | 5 µg | ≤12 | 16-20 | ≥21 |
| Trimethoprim | SXT/25 | (1.25/23.75) µg | ≤10 | 11-15 | ≥16 |
| Oxytetracycline | T/30 | 30 µg | ≤14 | 15-18 | ≥19 |
| Florphenicol | FFC/30 | 30 µg | ≤12 | 13-17 | ≥18 |
| Imipenem | IMP/10 | 10 µg | ≤12 | 13-16 | ≥17 |

## 2.5. Methods of Polymerase Chain reaction (PCR techniques):

### 2.5.1. Extraction of bacterial genomic DNA:

The genomic DNA was extracted using Gene JET genomic DNA extraction kit following the manufacturer protocol **(Fermentas, #K0721).** The principle of this reaction depends on digestion of the bacterial cell wall with Proteinase K. The lysate is then mixed with ethanol and loaded on the purification column where the DNA binds to the silica membrane. Impurities are effectively removed by washing the column with the prepared wash buffers. Genomic DNA is then eluted under low ionic strength conditions with the Elution Buffer.

**Technique:-**

1- Selected bacterial colony was added in 2ml tubes containing 400 µl of lysis solution and 20 µl of proteinase K was mixed well by vortexing or pipeting to obtain uniform suspension.

2- The samples were incubated at 56◦C.

3- Two hundreds µl of absolute ethanol (100%) was added and mixing well by pipeting. Then prepared lysate was transferred to Gene-JET™Genomic DNA Purification Columns inserted in a collection tube.

4- Centrifugation was performed for 1min at 8000xg, then flow through was discarded.

5- The columns were placed into a new 2ml collection tube and 500 µl of washing buffer II was added, centrifugation for 1min at 8000xg was performed, then flow through was discarded and purification columns were placed back into collection tube.

6- Five hundreds µl of wash buffer II was added to the Columns, centrifugation was performed for 3min at 1200xg, then collection tubes containing flow through solution were discarded and the Columns were transferred to a sterile 1.5 ml micro-centrifuge tube.

7- Two hundreds µl of elution buffer was added to the center of the Columns membrane to elute genomic DNA and incubation for 2min at room temperature then centrifugation for 1min at 8000xg was performed.

8- The column was discarded and purified DNA was stored at -20◦C.

**Alternative method used for extraction of bacterial DNA:**

DNA was also extracted from *Aeromonas spp.* isolates. *Aeromonas spp.* isolates were grown in TSA (Tryptone soya agar) overnight at 37 ºC. One colony was suspended in 100 ml sterile distilled water. The genomic DNA was extracted by boiling of the suspension for 13 minutes then following by freezing and subsequently centrifuged at 14000 rpm for 15 minutes to pellet the cell debris ***(Youssef et al.,2002)***. The supernatent was used as a template for PCR reaction.

**2.5.2. Polymerase Chain reaction (PCR techniques):**

The extracted DNA of 28 suspected isolates was used as a template for PCR identification using *16S rRNA* gene. The isolated DNA were amplified using dream taq green PCR master mix kit following the manufacturer protocol **(Fermentas, #K1071)**.

**a. Oligonucleotide primers used in PCR, ERIC-PCR and RAPD:**

Primers were dissolved in nuclease-free water to obtain 50 – 100 ml concentration ***(Fratemico et al., 2000)***. Table (4) shown that primers have specific sequence and amplify a specific product.

Table (4): Oligonucleotide primers sequences encoding for detection *A. hydrophila*.

Table (4.a): Primer sequences of *A. hydrophila* gene ***16S rRNA:***

| Micro-organism | Gene | Primer sequence (5'-3') | Length of amplified product | Reference |
|---|---|---|---|---|
| *Aeromonas hydrophila* | ***16S rRNA*** *F* | (5-GAAAGGTTGATGCCTAATACGTA-3) | 625 bp | ***(Gordon et al.,2007)*** |
| | *R* | (5-CGTGCTGGCAACAAAGGACAG-3) | | |

Table (4.b): Primer sequences of *A. hydrophila* for ***ERIC:***

| Micro-organism | Gene | Primer sequence (5'-3') | Reference |
|---|---|---|---|
| *Aeromonas hydrophila* | ***ERIC 1*** | (5-ATG TAA GCT CCT GGG GAT TCA C-3) | ***(Versalovic, J. et al., 1991)*** |
| | ***ERIC 2*** | (5-AAG TAA GTG ACT GGG GTG AGC G-3) | |

Table (4.c): Primer sequences of *A. hydrophila* for ***RAPD:***

| Micro-organism | Gene | Primer sequence (5'-3') | Reference |
|---|---|---|---|
| *Aeromonas hydrophila* | ***OPA-03*** | (5- AGTCAGCCAC -3) | ***(Sarkar, A.et al., 2012)*** |
| | ***OPA-10*** | (5- GTGATCGCAG -3) | |

**b. Preparation of PCR Master Mix Component**

The polymerase chain reaction mixture was carried out in a 25 µl which contain the component shown in Table (5.a), according to Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit. PCR mixture for ERIC and RAPD was carried out in a 40 µl which contain the component shown in Table (5.b), according to Perkin-Elmer/Cetus, Nonvalk, CT, USA.

Table (5.a): Component PCR Master Mix

| **Component** | **Volume/reaction** |
|---|---|
| Emerald Amp GT PCR mastermix (2x premix) | 12.5 *µl* |
| PCR grade water | 4.5 *µl* |
| Forward primer (20 pmol) | 1 *µl* |
| Reverse primer (20 pmol) | 1 *µl* |
| Template DNA | 6 *µl* |
| Total | 25 *µl* |

Table (5.b): Component PCR Master Mix for **ERIC-PCR** and **RAPD:**

| **Component for ERIC** | **Volume/reaction** |
|---|---|
| Emerald Amp GT PCR mastermix (2x premix) | 20 *µl* |
| PCR grade water | 10 *µl* |
| primer (20 pmol) | 4 *µl* |
| Template DNA | 6 *µl* |
| Total | 40 *µl* |

**c. The primers cycling conditions during PCR, ERIC and RAPD**

Temperature and time conditions of the primers during PCR, ERIC and RAPD are shown in Table (6) according to specific authors, Emerald Amp GT PCR master mix (Takara) kit and Perkin-Elmer/Cetus, Nonvalk, CT, USA.

Table (6): Cycling conditions of the different primers during cPCR, ERIC and RAPD:

| Target gene | Primary denaturation | Secondary denaturation | Annealing | Extension | No. of cycles | Final extension |
|---|---|---|---|---|---|---|
| ***A. hydrophila*** **16S rRNA** | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 40 sec. | 72˚C 45 sec. | 35 | 72˚C 10 min. |
| **ERIC** | 95˚C 5 min. | 95˚C 1 min. | 48˚C 1 min. | 72˚C 5 min. | 40 | 72˚C 5 min. |
| **RAPD** | 94˚C 5 min. | 94˚C 1 min. | 36˚C 1 min. | 72˚C 2 min | 45 | 72˚C 10 min |

At the end of cycling the tubes were stored at – 20 ºC until being used.

**2.5.3. Agarose Gel Electrophoresis (*Sambrook et al., 1989*):**

Agarose Gel electrophoresis was used to detect DNA fragments after DNA extraction and to determine the size of the PCR products. It was carried out according to the following:

1. Agarose 1% was prepared by weighting 1.0 gm agarose with 100 ml of 1X TAE buffer in a 250 ml flask. The mixture was cooked at microwave until agarose particles completely melted.
2. When agarose was cooled up to about 60 Cº, 35 µl ethidium bromide was added (to get concentration of 5 µl ethidum bromide / ml TAE buffer) with gentle agitation.

3. The agarose was then poured and any bubbles should be removed.
4. When the gel had completely solidified, it was placed into electrophoresis tank containing sufficient TAE buffer to cover the gel with 1mm and the gel comb was then withdrawn.
5. Three ul DNA sample was mixed with 2 µl loading buffer and the mixture was carefully loaded in each gel well using a micropipette.
6. Appropriate DNA molecular weight marker (100 bp ladder DNA marker) **(Fermentas, #SM0321)** or (1kb ladder DNA marker) **(Fermentas, #SM1331)** was loaded in the adjacent well which was as a ladder for reading of samples.
7. The power supply was connected and adjusted to 150 volts for 30 min.
8. The fragment patterns were visualized on the UV Trans-illuminator and photographed by gel documentation system **(UVDI major science).**

### 2.5.4. Quantification of DNA using Nanodrop (UV-vis spectrophotometer Q5000)

To quantify the concentration of extracted DNA and to be sure that the concentrations are high enough to complete other works and get high yield product. For very pure samples (without significant contamination from protein, phenol, free nucleic acids, organic solvents, carbohydrates, etc.), the absorption of ultra-violet (UV) light by the ring structure of purines and pyrimidines can be used to measure the amount of nucleic acids.

The Q5000 automatically performs all necessary measurements and calculations. The data are accumulated in the module data window and are easily exported to an Excel spread sheet and this method was used to quantify the concentration of extracted DNA.

1. The upper arm of instrument was lift and 1.5ul of blank buffer was first used to the lower surface to get the blank read by Closing the upper arm

and Clicking the “Measure” button to complete all measurements and calculations and then the upper arm was Lift and the blank was removed from both upper and lower surfaces with soft dry paper.

2. One and half ul of sample was measured by the same method.

3. Data were exported to an Excel worksheet by clicking the “Report” button.

4. The absorbance of the sample was read at 260 and 280 nm wavelength.

5. The concentration based on an optic density (OD) of 1 corresponding to approximately 50 µg/ml for double-stranded DNA was then automatically calculated.

The OD260/OD280 ratio for pure DNA is ≥ 1.8. Contamination by protein (which has an absorbance maximum of 280 nm) or phenol will cause the ratio to be significantly lower than these values.

### 2.5.5. Analysis of ERIC and RAPD fingerprinting data:

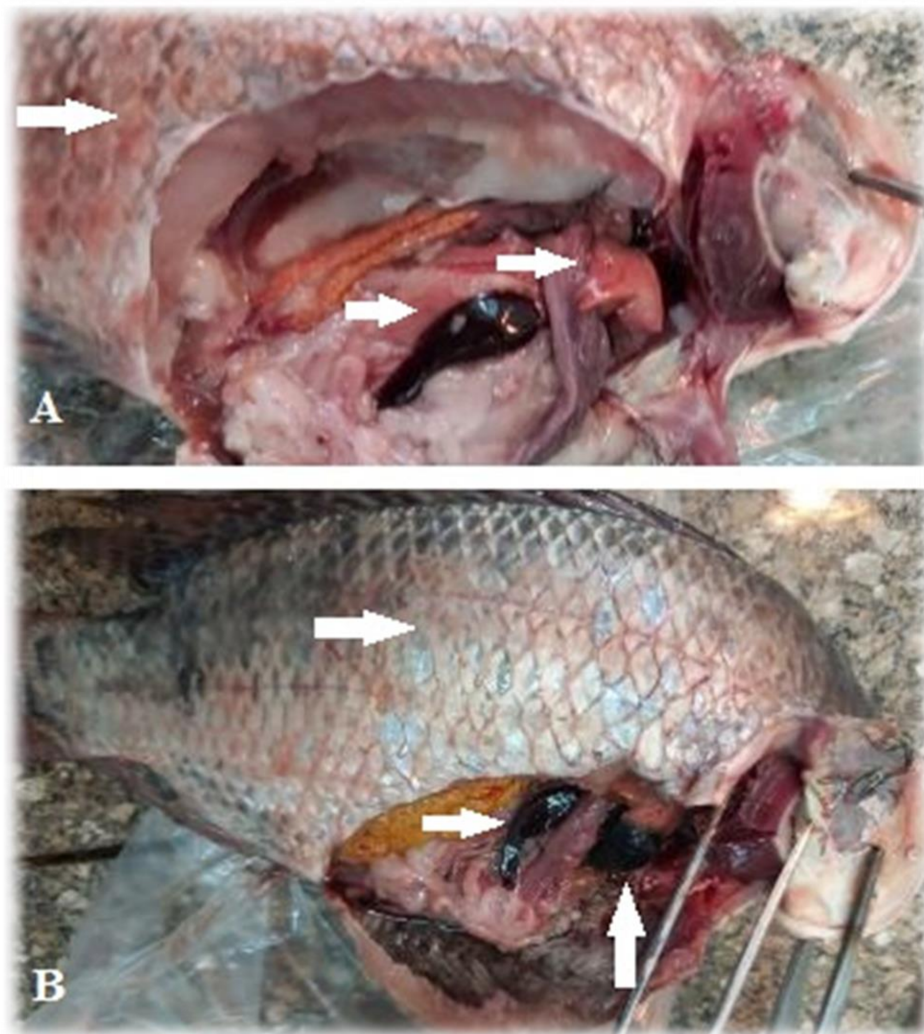
ERIC and RAPD fingerprinting data were transformed into a binary code depending on the presence or absence of each band. Dendrograms were generated by the unweighted pair group method with arithmetic average (UPGMA) and Ward’s hierarchical clustering routine. Cluster analysis and dendrogram construction were performed with SPSS, version 22 (IBM 2013) ***(Hunter, 1990).***

Among all samples were calculated using the online tool Similarity index (Jaccard / Tanimoto Coefficient and number of intersecting elements) (https://planetcalc.com/1664/).

# IV-RESULTS

**1. Clinical picture and postmortem lesions of examined fish:**

The clinical examination of the investigated fish revealed uneven redness throughout the body also particularly at base of the fins and around the anal opening with external hemorrhage. In some of the examined fish, there was abdominal dropsy with asceitis having exudates in red color, liver paleness and hypertrophy, splenomegaly, distended gall-bladder and congested intestine (Fig.1).




(Fig. 1) (A) external hemorrhage, splenomegaly, liver paleness and high abdominal fat deposition, (B) detached scales, external hemorrhage and distended gall-bladder.

**2. Bacteriological Isolation:**

Bacteriological investigation of the internal organs kidney and liver cultured on Aeromonas agar and Tryptone soya agar plates. All the other samples were cultured on the same plates giving different colors according the type of the medium.

On Tryptone soya agar plates, Aeromonas spp. grow in convex colonies yellow to green with black center.

The creamy or pale colonies on Aeromonas agar plates were the characteristic colonies for Aeromonas spp. as shown at Fig. (2)

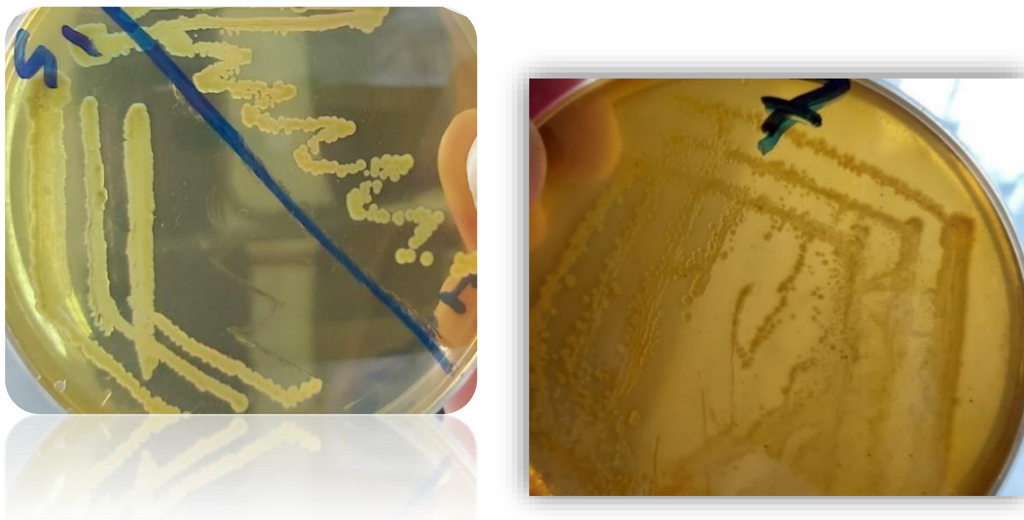


Fig. (2) Aeromonas spp. on Aeromonas agar plates.

**3. Bacteriological Identification:**

**3.1. Microscopical Identification:**

Gram negative, straight or slightly curved rods with round end, medium size and non-sporulated bacteria; that were selected for biochemical identification.

## 3.2. Biochemical Identification of Aeromonas species:

The results of biochemical examinations were as shown at Table (7).

Table (7): The biochemical characters of the isolates ***(Quinn et al., 2002)***

| **Biochemical tests** | **Aeromonas species** | |
|---|---|---|
| | ***A.hydrophila*** | ***A.caviae*** |
| Oxidase test | +ve | +ve |
| Catalase test | +ve | +ve |
| Indole test | +ve | +ve |
| Methyl red | -ve | +ve |
| Voges-Proskauer | +ve | - ve |
| Citrate utilization | +ve | +ve |
| Urease test | - ve | -ve |
| Sugar fermentation | | |
| Glucose | Ag | A |
| Mannitol | + ve | +ve |
| Sucrose | +/-ve | -ve |
| Lactose | + ve | -ve |

+ve =positive - ve = Negative Ag =Acid and gases

## 3. Prevalence of *Aeromonas* spp. in examined samples:

According to the phenotypic characterization a total of 87 positive *Aeromonas* spp. isolates were recovered from all samples. The prevalence of the positive isolates distributed based on their source was 55.3% (31/56) from tilapia fish, 21.4% (12/56) from water, 30.3% (17/56) from soil, and 48.2% (27/56) from human stool samples (Table 8).

Table (8). Prevalence of *Aeromonas* spp. in examined samples.

| Sample | No of samples | No. of *Aeromonas* spp. positive samples | Prevalence % |
|---|---|---|---|
| Fish | 56 | 31 | 55.3 |
| Water | 56 | 12 | 21.4 |
| Soil | 56 | 17 | 30.3 |
| Stool | 56 | 27 | 48.2 |

## 4. Antibacterial susceptibility profile of *Aeromonas* spp.:

The sensitivity tests revealed that, they were highly sensitive to ciprofloxacin (85.2%) followed by moxifloxacin and norfloxacin (82.4% and 80.6% respectively). Meanwhile, they were less sensitive to gentamycin, streptomycin and cefotaxime. Moreover, they were highly resistant to ampicillin (97.8%) followed by penicillin-G and amoxicillin (94.6% and 89.8% respectively).

## 5.Results of *A. hydrophila* molecular identification techniques:

### 5.1. Extraction of Bacterial DNA:

DNA was extracted from *A. hydrophila* isolates. Gel electrophoresis revealed the presence of intact bands near wells which means that they are bacterial genomic DNA and they are good enough to be used in the subsequent procedures (Fig.3).

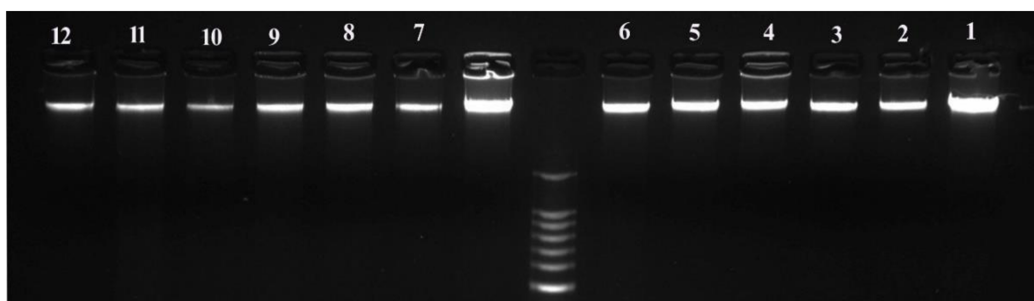



Fig. (3): Extracted bacterial genomic DNA on an agarose gel stained with ethidium bromide.

Nanodrop (UV-vis spectrophotometer Q5000, Quawell, USA) was used to measure DNA concentration and purity by measuring the absorption at 260 and 280 nm. The Nanodrop reading revealed that the isolated DNA is pure (the ratio of 260/280 and 230/260 ranged between 1.8 and 2.2) and with a considerable concentration (ranged from 500 to 1100 ng/µl) (Fig. 4**).** Therefore, it can be used successfully as a template for PCR.

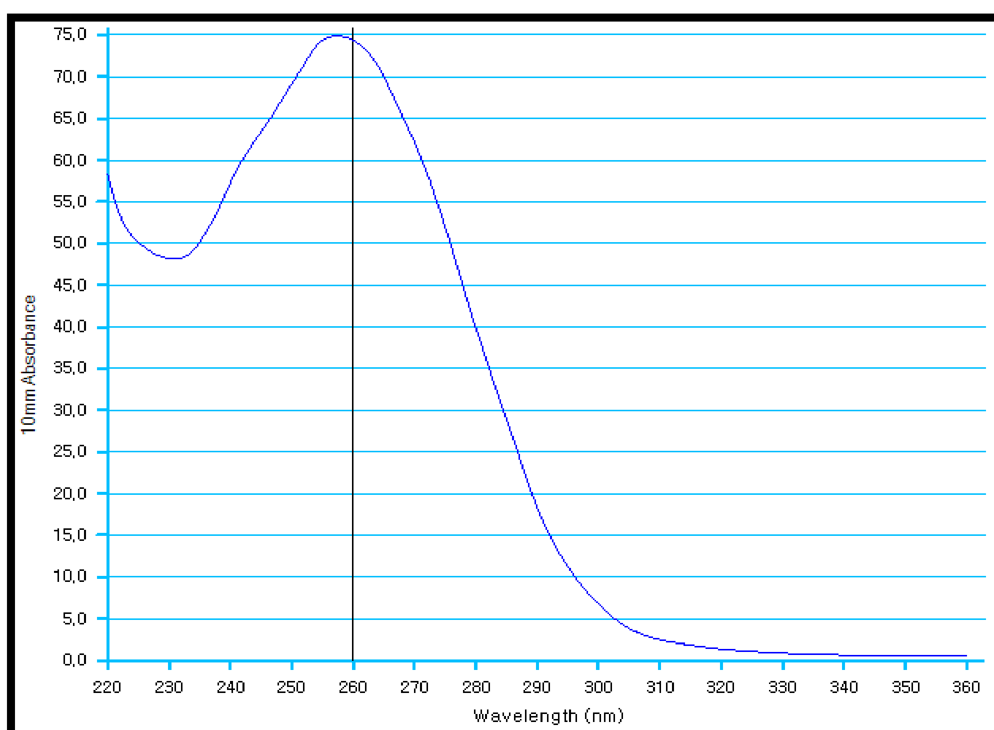



Fig. 4: Nanodrop curve showing concentration of extracted DNA from a representative sample which is 971ng/ul. The curve indicates the presence of pure DNA as the upper top of curve present at 260 and its bottom at 230.

## 5.2. PCR identification of *Aeromonas hydrophila* using *16S rRNA* gene:

The extracted DNA was used as a template for PCR identification using *16S rRNA* gene revealed the presence of a total of 18 positive *Aeromonas hydrophila* strains recovered from phenotypically identified as 28 *Aeromonas* spp. (Table 9 and Fig.5), which were distributed based on their source was 85.7% (6/7) from diseased fish, 42.3% (3/7) from water, 42.3% (3/7) from soil, and 85.7% (6/7) from stool swab of diarrhoeic patients.

Table (9). Results of PCR amplifications of 16S rRNA gene of *Aeromonas hydrophila* in the examined samples

| Sample | No of *Aeromonas* spp. positive samples | No. of *A. hydrophila* positive samples | (%*) |
|---|---|---|---|
| Fish | 7 | 6 | 85.7 |
| Water | 7 | 3 | 42.8 |
| Soil | 7 | 3 | 42.8 |
| Stool | 7 | 6 | 85.7 |
| Total | 28 | 18 | 64.2 |

%*: No of *A. hydrophila* positive samples / No of *Aeromonas* spp. positive samples

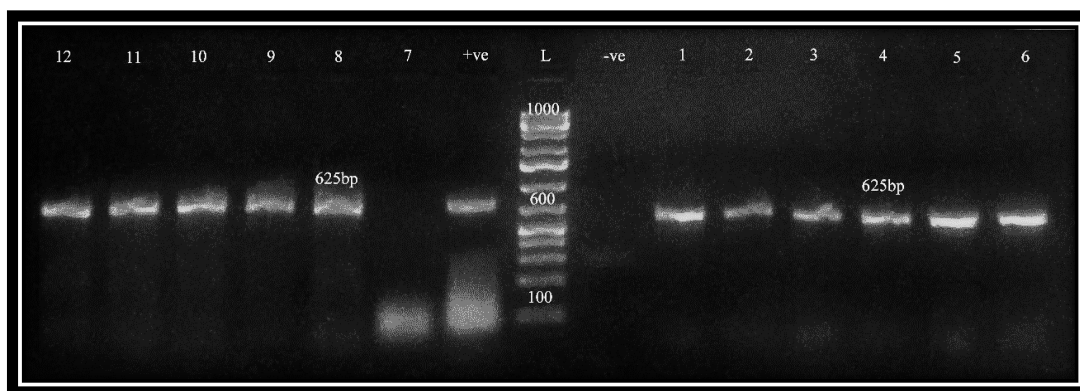



Fig.5. Electrophoresis shows PCR amplified products of the 16S rRNA gene on an agarose gel.

Lane L, DNA size marker (fragment sizes from down to up are 100, 200, 300, 400, 500, 600, 700, 800 bp………3kb);

Positive control is Lane +ve;

Negative control is Lane -ve (DNA free sample);

(lanes 3- 4 - 9 -10) *A. hydrophila* isolates from fish samples,

(lanes 5- 6 -11-12) *A. hydrophila* isolates from stool swab,

(lanes 2- 8) *A. hydrophila* isolates from water,

(lanes 1- 7) *A. hydrophila* isolates from soil.

The size in base pairs (625 bp) PCR product is indicated above the bands.

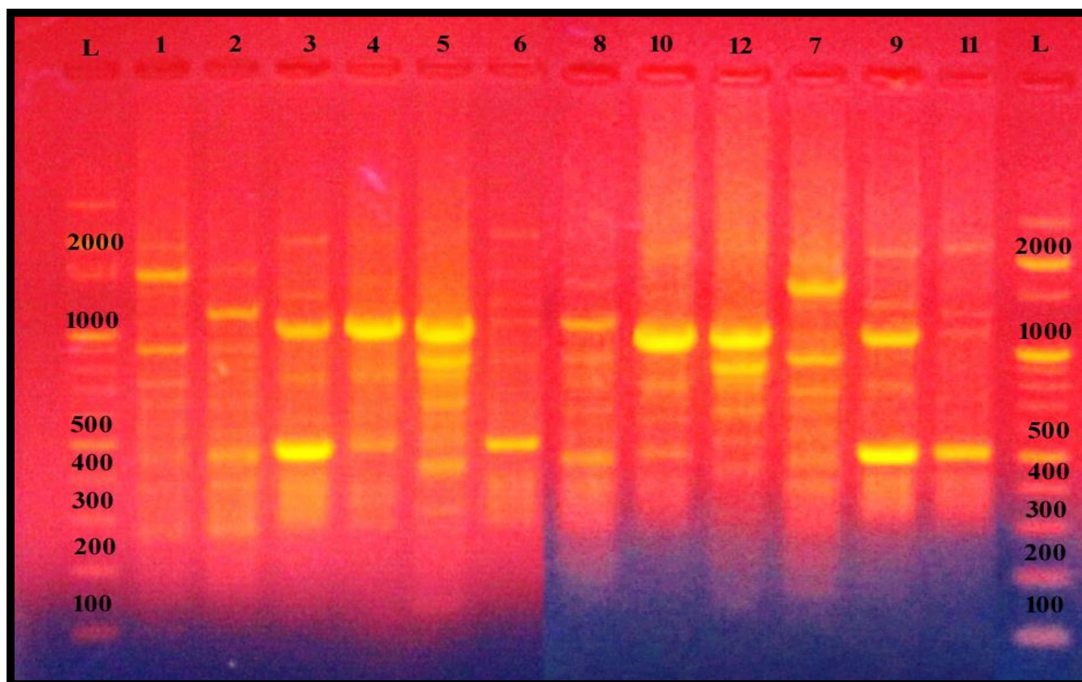
## 5.3. *Aeromonas hydrophila* strains typing by ERIC-PCR:

The ERIC-PCR genetic profiles of all positive *A. hydrophila* strains (n = 18) resulted in notable bands with size ranged from 0.27 to 2.73 kb (Table 10, Fig. 6).

The total number of ERIC-PCR bands was 14 in fish, 12 in water, 13 in soil, and 14 in stool swab strains. No identical band pattern was observed among the 18 isolates even among the isolates obtained from the same sources. This indicates the higher genetic variety among *A. hydrophila* isolates involved in this study.

The highest (largest size) band (2.73 kb) was noticed in 2 stool swab isolates (lanes 6 and 11), while the lowest (smallest size) band (0.27 kb) was found in fish (lane 4), water (lane 2) and soil (lane 1) isolates.

Bands with size of 0.35, 0.44, 0.51, 0.56, 0.76, 1.25, and 1.72 kb were common in fish, water, soil, and stool isolates (Table 11). Band with size of 0.63 kb were common in fish, soil, and stool isolates, while those with 0.67 kb were common in water, soil, and stool isolates. On the other hand, bands with size of 0.48 kb were common in water and stool isolates.



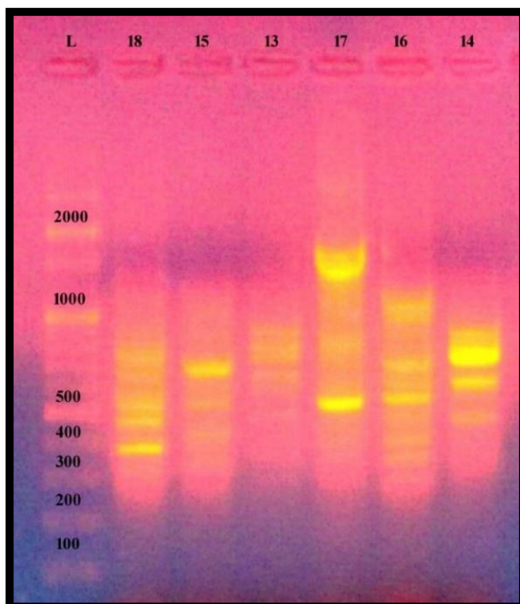




Fig. 6. Ethidium bromide-stained gel showing ERIC-PCR bands pattern generated with ERIC primers in 18 positive *A. hydrophila* isolates.

Lane L, DNA marker (100 bp),
(lanes 3, 4, 9, 10, 15, 16) fish samples,
(lanes 2, 8, 14) water samples,
(lanes 1, 7, 13) soil samples,
(lanes 5, 6, 11, 12, 17, 18) stool swabs.

Table (10). ERIC-PCR bands pattern in 18 positive *A. hydrophila* strains.

| kb | Fish | | | | | | Water | | | Soil | | | Stool | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | L3 | L4 | L9 | L10 | L15 | L16 | L2 | L8 | L14 | L1 | L7 | L13 | L5 | L6 | L11 | L12 | L17 | L18 | |
| **0.27** | - | + | - | - | - | - | + | - | - | + | - | - | - | - | - | - | - | - | 3 |
| **0.32** | - | - | - | - | - | + | - | - | - | - | - | - | + | - | - | - | - | - | 2 |
| **0.35** | - | - | - | - | + | + | + | - | - | + | - | - | - | - | - | - | - | + | 5 |
| **0.44** | - | - | - | - | + | + | + | - | - | - | + | + | - | - | - | + | - | - | 6 |
| **0.48** | - | - | - | - | - | - | - | + | - | - | - | - | + | - | - | - | - | - | 2 |
| **0.51** | + | + | + | + | - | - | + | - | + | - | + | - | - | - | + | - | + | + | 10 |
| **0.56** | - | - | - | - | + | + | - | + | - | + | - | + | + | - | - | + | - | + | 8 |
| **0.63** | - | - | - | + | - | - | - | - | - | + | + | - | - | + | - | - | - | - | 4 |
| **0.67** | - | - | - | - | - | - | + | + | - | + | - | - | - | - | - | + | - | + | 5 |
| **0.76** | + | + | - | - | + | + | - | - | + | + | + | - | - | - | - | - | - | + | 8 |
| **0.81** | - | - | + | + | - | - | + | - | + | - | + | + | - | - | - | - | - | - | 6 |
| **0.88** | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - | + | + | - | 4 |
| **0.94** | - | - | - | - | - | - | + | + | + | + | + | + | - | - | - | - | - | - | 6 |
| **1.25** | + | + | + | + | - | + | + | + | - | + | - | - | + | + | + | + | - | - | 12 |
| **1.34** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | 1 |
| **1.51** | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| **1.72** | - | - | + | - | - | - | + | + | - | + | + | - | - | + | + | - | + | - | 8 |
| **2.20** | + | - | + | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - | 4 |
| **2.50** | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| **2.73** | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - | - | - | 2 |
| **Total** | 5 | 4 | 6 | 4 | 4 | 6 | 9 | 6 | 4 | 10 | 8 | 4 | 5 | 5 | 5 | 5 | 3 | 5 | NO. |

+ present; - absent; L, lane.

Table (11) Results of *A. hydrophila* ERIC-PCR bands pattern among different sources:

| | Fish samples | Environmental samples | | Stool samples |
|---|---|---|---|---|
| | | Water | Soil | |
| Total band No. | 14 | 12 | 13 | 14 |
| Common band No. | 7 | 7 | 7 | 7 |
| Unique band No. | 2 | - | - | 1 |
| Lost band No. | 6 | 8 | 7 | 6 |

### 5.3.1. Results of ERIC fingerprinting analysis:

Based on the results of ERIC fingerprinting analysis Fig. (7) and Table (12), 18 *A. hydrophila* isolates were categorized into 6 clusters (C1 to C6) ERIC types. Isolates no. (3,4) isolated from fish, 2 isolated from water and (1) isolated from soil clustered in group (C1). Isolates no. (5) isolated from stool, (8) isolated from water and (7) isolated from soil clustered in group (C6). There was a high similarity among C1 and C6 from 60 % to 75% of identity. But there is no identity between isolate no. (17) isolated from human stool and C5 which has isolates no. (9 and 10) isolated from fish. High similarity was found between C3 and C4 reach to 67% of identity. C3 and C4 have isolates isolated from fish (15 in C3 and 16 in C4) and human stool (18 in C3 and 6 in C4) and human stool.

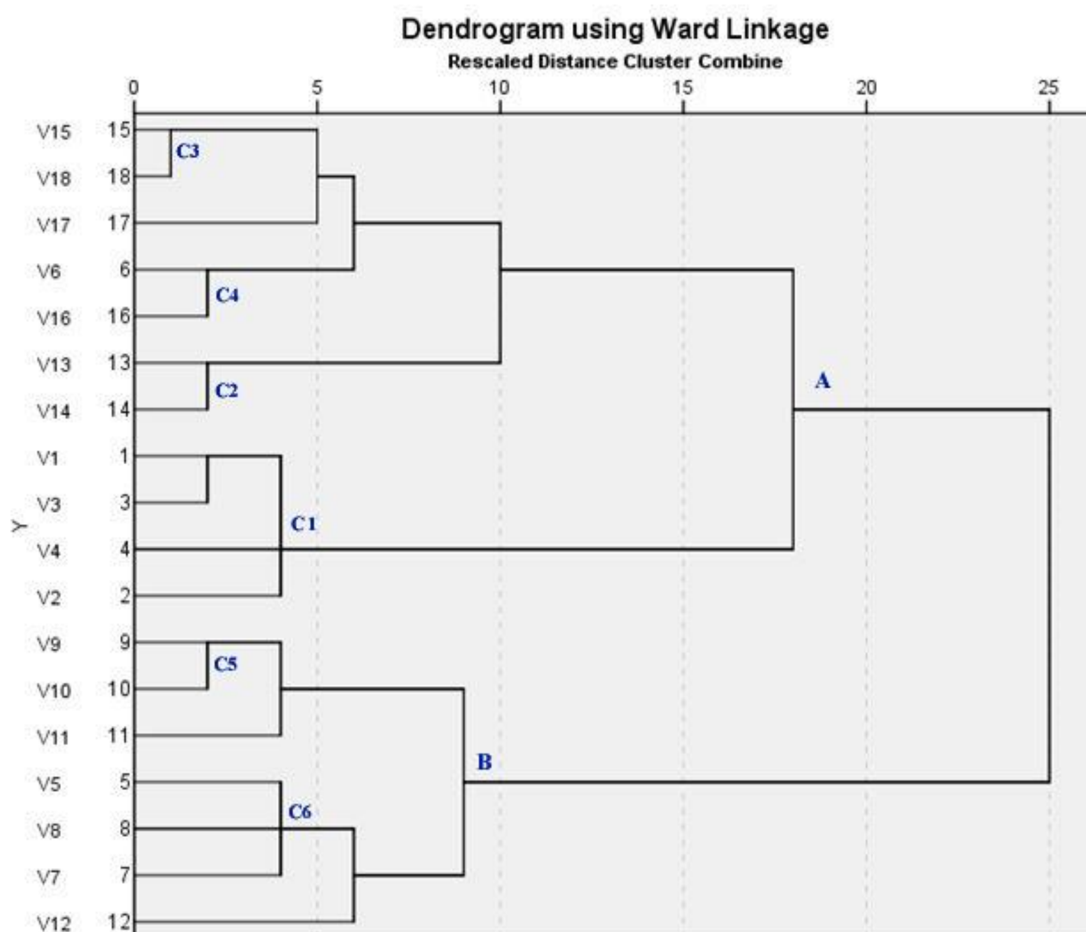
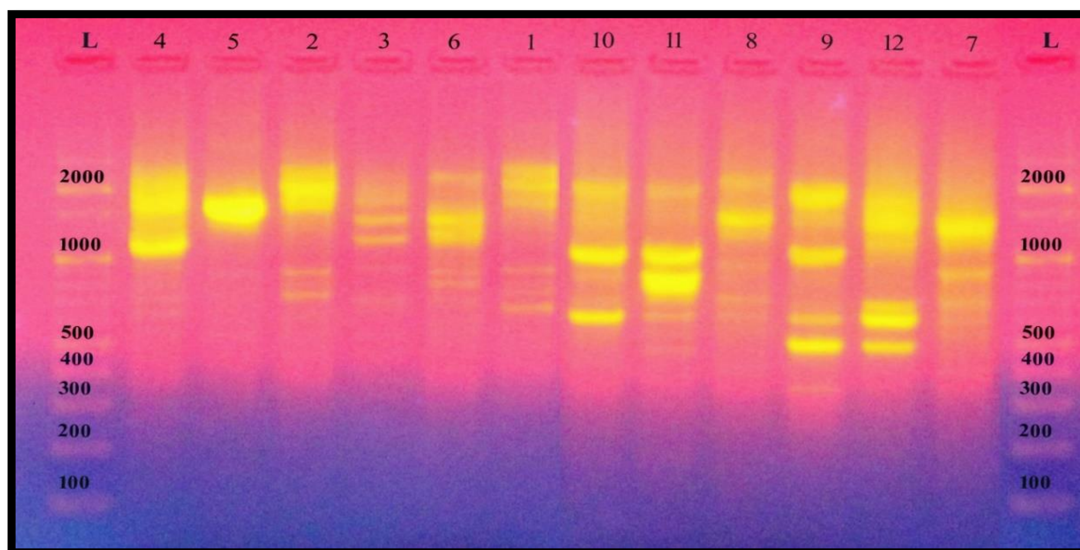



Fig. 7. Dendrogram showing genetic relatedness among *A. hydrophila* strains based on ERIC-PCR.

Table (12): Similarity index (Jaccard / Tanimoto Coefficient and number of intersecting elements) among *A. hydrophila* strains based on ERIC-PCR.

| | Jaccard / Tanimoto Coefficient | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
| 1 | | 0.75 | 0.73 | 0.58 | 0.57 | 0.5 | 0.64 | 0.36 | 0.31 | 0.29 | 0.21 | 0.36 | 0.38 | 0.36 | 0.23 | 0.46 | 0.27 | 0.23 |
| 2 | 9 | | 0.5 | 0.5 | 0.5 | 0.42 | 0.57 | 0.38 | 0.14 | 0.21 | 0.23 | 0.38 | 0.42 | 0.4 | 0.25 | 0.5 | 0.3 | 0.25 |
| 3 | 8 | 6 | | 0.6 | 0.46 | 0.67 | 0.43 | 0.14 | 0.4 | 0.25 | 0.17 | 0.23 | 0.25 | 0.2 | 0.18 | 0.45 | 0.22 | 0.3 |
| 4 | 7 | 6 | 6 | | 0.58 | 0.5 | 0.43 | 0.23 | 0.27 | 0.25 | 0.27 | 0.23 | 0.15 | 0.2 | 0.18 | 0.33 | 0.22 | 0.18 |
| 5 | 8 | 7 | 6 | 7 | | 0.5 | 0.64 | 0.58 | 0.42 | 0.64 | 0.42 | 0.46 | 0.38 | 0.25 | 0.33 | 0.58 | 0.17 | 0.33 |
| 6 | 6 | 5 | 6 | 5 | 6 | | 0.27 | 0.15 | 0.18 | 0.27 | 0.18 | 0.36 | 0.27 | 0.1 | 0.33 | 0.67 | 0.25 | 0.5 |
| 7 | 9 | 8 | 6 | 6 | 9 | 4 | | 0.54 | 0.5 | 0.46 | 0.5 | 0.54 | 0.27 | 0.23 | 0.21 | 0.33 | 0.15 | 0.13 |
| 8 | 5 | 5 | 2 | 3 | 7 | 2 | 7 | | 0.27 | 0.5 | 0.27 | 0.45 | 0.5 | 0.33 | 0.44 | 0.33 | 0.22 | 0.3 |
| 9 | 4 | 2 | 4 | 3 | 5 | 2 | 6 | 3 | | 0.63 | 0.5 | 0.27 | 0.18 | 0.11 | 0.1 | 0.17 | 0 | 0.1 |
| 10 | 4 | 3 | 3 | 3 | 7 | 3 | 6 | 5 | 5 | | 0.44 | 0.5 | 0.4 | 0.22 | 0.2 | 0.36 | 0 | 0.2 |
| 11 | 3 | 3 | 2 | 3 | 5 | 2 | 6 | 3 | 4 | 4 | | 0.4 | 0.08 | 0 | 0.1 | 0.17 | 0.13 | 0.1 |
| 12 | 5 | 5 | 3 | 3 | 6 | 4 | 7 | 5 | 3 | 5 | 4 | | 0.36 | 0.2 | 0.44 | 0.45 | 0.1 | 0.3 |
| 13 | 5 | 5 | 3 | 2 | 5 | 3 | 4 | 5 | 2 | 4 | 1 | 4 | | 0.57 | 0.5 | 0.5 | 0.11 | 0.5 |
| 14 | 4 | 4 | 2 | 2 | 3 | 1 | 3 | 3 | 1 | 2 | 0 | 2 | 4 | | 0.29 | 0.2 | 0 | 0.13 |
| 15 | 3 | 3 | 2 | 2 | 4 | 3 | 3 | 4 | 1 | 2 | 1 | 4 | 4 | 2 | | 0.44 | 0.14 | 0.67 |
| 16 | 6 | 6 | 5 | 4 | 7 | 6 | 5 | 4 | 2 | 4 | 2 | 5 | 5 | 2 | 4 | | 0.22 | 0.63 |
| 17 | 3 | 3 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 2 | | 0.33 |
| intersecting elements 18 | 23 | 3 | 3 | 2 | 4 | 4 | 2 | 3 | 1 | 2 | 1 | 3 | 4 | 1 | 4 | 5 | 2 | |

## 5.4. *Aeromonas hydrophila* strains typing by RAPD-PCR:



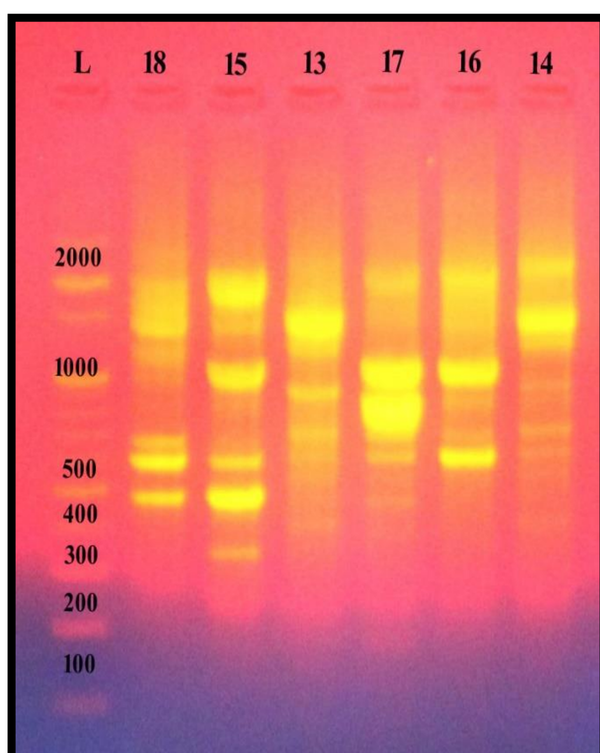




Fig. 8. Ethidium bromide-stained gel showing RAPD-PCR bands pattern generated with RAPD primers in 18 positive *A. hydrophila* isolates. Lane L, DNA marker(100 bp), (lanes 3, 4, 9, 10, 15, 16) fish samples,
(lanes 2, 8, 14) water samples,
(lanes 1, 7, 13) soil samples,
(lanes 5, 6, 11, 12, 17, 18) stool swabs.

### 5.4.1. Results of RAPD fingerprinting analysis:

The UPGMA cluster analysis on the basis of RAPD profile of *Aeromonas hydrophila* depicted all the strains having the several clusters and it suggests that their profiles are species specific. Visible RAPD fingerprinting profiles, Fig. (9) and Table (13), revealed the substantial

wide genetic diversity among the strains tested. Strains 12, 13, 10, 11, 2 and 9 formed a minor cluster (C3) with similarity average 43%, their sources were stool, soil, fish, stool, water and fish respectively, while 5 and 15 strains, their sources were stool and fish respectively, formed a separate minor cluster with similarity 25% against the cluster (C1) which consisted of 4 and 16 strain from fish source and 17 strain from stool source with similarity 57%. This indicates the genetic relationship among the strains within the clusters. It is clear that one can easily understand the percentage of genetic similarities between the strains. There was a high similarity among C4 (10,11 and 2 strains) and C5 (3 and 6 strains from fish and stool sources respectively) 71% of identity. But there is no identity between isolate no. (15) isolated from fish and isolate no. (2) isolated from water.

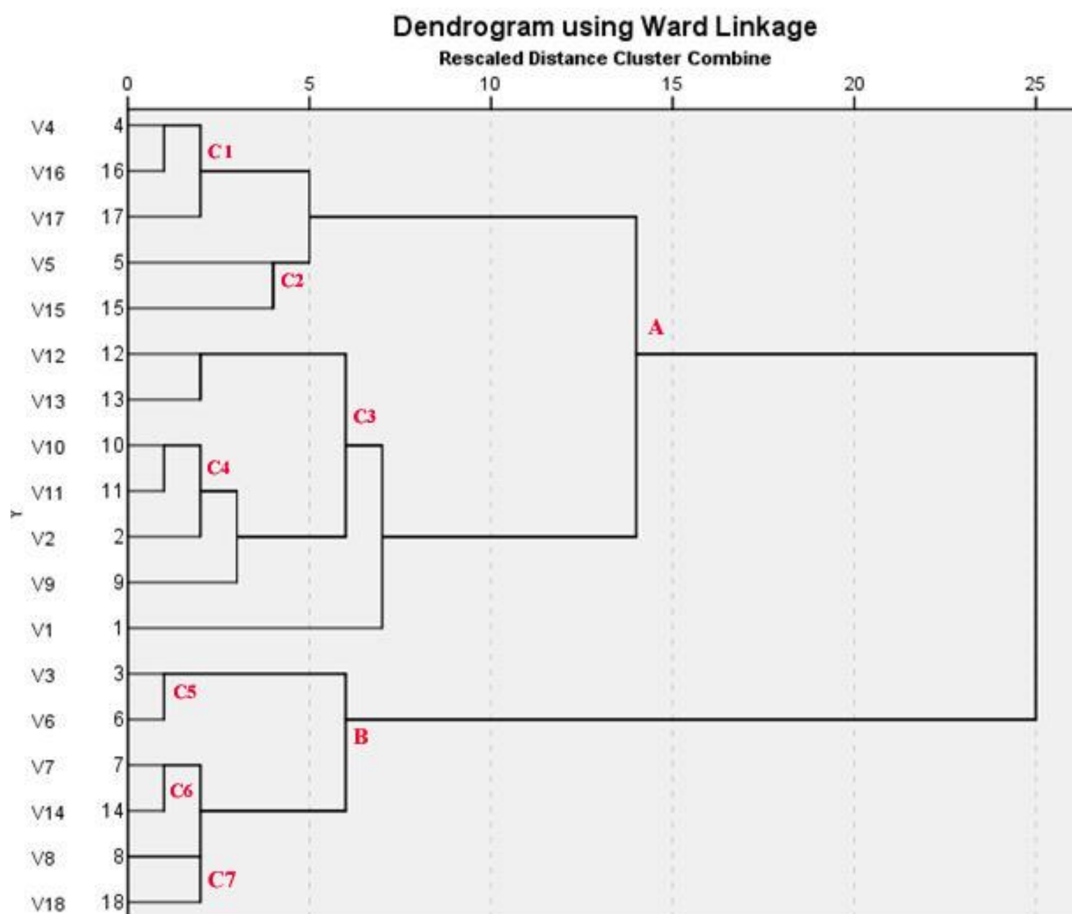



Fig.9. Dendrogram showing genetic relatedness among *A. hydrophila* strains based on RAPD-PCR.

Table (13): Similarity index (Jaccard / Tanimoto Coefficient and number of intersecting elements) among *A. hydrophila* strains based on RAPD-PCR.

| intersecting elements | Jaccard / Tanimoto Coefficient | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
| 1 | | 0.57 | 0.44 | 0.22 | 0.22 | 0.44 | 0.33 | 0.33 | 0.3 | 0.5 | 0.4 | 0.33 | 0.33 | 0.4 | 0.18 | 0.33 | 0.4 | 0.3 |
| 2 | 4 | | 0.43 | 0.14 | 0.14 | 0.43 | 0.29 | 0.29 | 0.25 | 0.5 | 0.38 | 0.13 | 0.29 | 0.22 | 0 | 0.13 | 0.1 | 0.11 |
| 3 | 4 | 3 | | 0.11 | 0.43 | 0.71 | 0.57 | 0.57 | 0.09 | 0.22 | 0.18 | 0.38 | 0.38 | 0.63 | 0.2 | 0.38 | 0.18 | 0.5 |
| 4 | 2 | 1 | 1 | | 0.14 | 0.11 | 0.29 | 0.29 | 0.43 | 0.5 | 0.57 | 0.29 | 0.29 | 0.22 | 0.43 | 0.5 | 0.57 | 0.43 |
| 5 | 2 | 1 | 3 | 1 | | 0.25 | 0.29 | 0.13 | 0 | 0.13 | 0.22 | 0.29 | 0.13 | 0.38 | 0.25 | 0.13 | 0.22 | 0.43 |
| 6 | 4 | 3 | 5 | 1 | 2 | | 0.57 | 0.57 | 0.09 | 0.22 | 0.18 | 0.22 | 0.38 | 0.44 | 0.09 | 0.22 | 0.18 | 0.33 |
| 7 | 3 | 2 | 4 | 2 | 2 | 4 | | 0.67 | 0.22 | 0.43 | 0.33 | 0.43 | 0.67 | 0.71 | 0.22 | 0.43 | 0.2 | 0.57 |
| 8 | 3 | 2 | 4 | 2 | 1 | 4 | 4 | | 0.22 | 0.25 | 0.2 | 0.43 | 0.43 | 0.5 | 0.22 | 0.43 | 0.2 | 0.57 |
| 9 | 3 | 2 | 1 | 3 | 0 | 1 | 2 | 2 | | 0.57 | 0.63 | 0.38 | 0.38 | 0.3 | 0.33 | 0.38 | 0.44 | 0.2 |
| 10 | 4 | 3 | 2 | 3 | 1 | 2 | 3 | 2 | 4 | | 0.71 | 0.25 | 0.43 | 0.33 | 0.22 | 0.43 | 0.33 | 0.22 |
| 11 | 4 | 3 | 2 | 4 | 2 | 2 | 3 | 2 | 5 | 5 | | 0.33 | 0.5 | 0.4 | 0.3 | 0.33 | 0.56 | 0.3 |
| 12 | 3 | 1 | 3 | 2 | 2 | 2 | 3 | 3 | 3 | 2 | 3 | | 0.43 | 0.71 | 0.22 | 0.43 | 0.5 | 0.57 |
| 13 | 3 | 2 | 3 | 2 | 1 | 3 | 4 | 3 | 3 | 3 | 4 | 3 | | 0.71 | 0.22 | 0.43 | 0.33 | 0.38 |
| 14 | 4 | 2 | 5 | 2 | 3 | 4 | 5 | 4 | 3 | 3 | 4 | 5 | 5 | | 0.3 | 0.5 | 0.4 | 0.63 |
| 15 | 2 | 0 | 2 | 3 | 2 | 1 | 2 | 2 | 3 | 2 | 3 | 2 | 2 | 3 | | 0.57 | 0.44 | 0.5 |
| 16 | 3 | 1 | 3 | 3 | 1 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | | 0.5 | 0.57 |
| 17 | 4 | 1 | 2 | 4 | 2 | 2 | 2 | 2 | 4 | 3 | 5 | 4 | 3 | 4 | 4 | 4 | | 0.44 |
| 18 | 3 | 1 | 4 | 3 | 3 | 3 | 4 | 4 | 2 | 2 | 3 | 4 | 3 | 5 | 4 | 4 | 4 | |

## 6. Comparison of ERIC-PCR and RAPD techniques results:

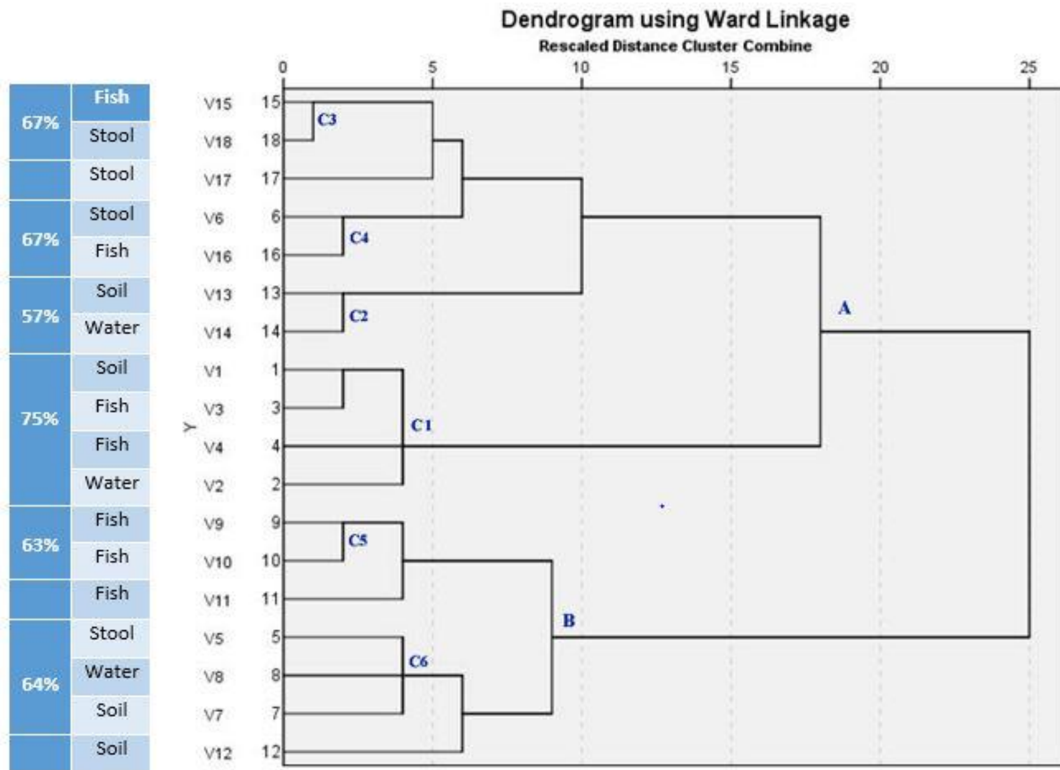



**Fig. 10 showing analysis of ERIC-PCR dendrogram results.**

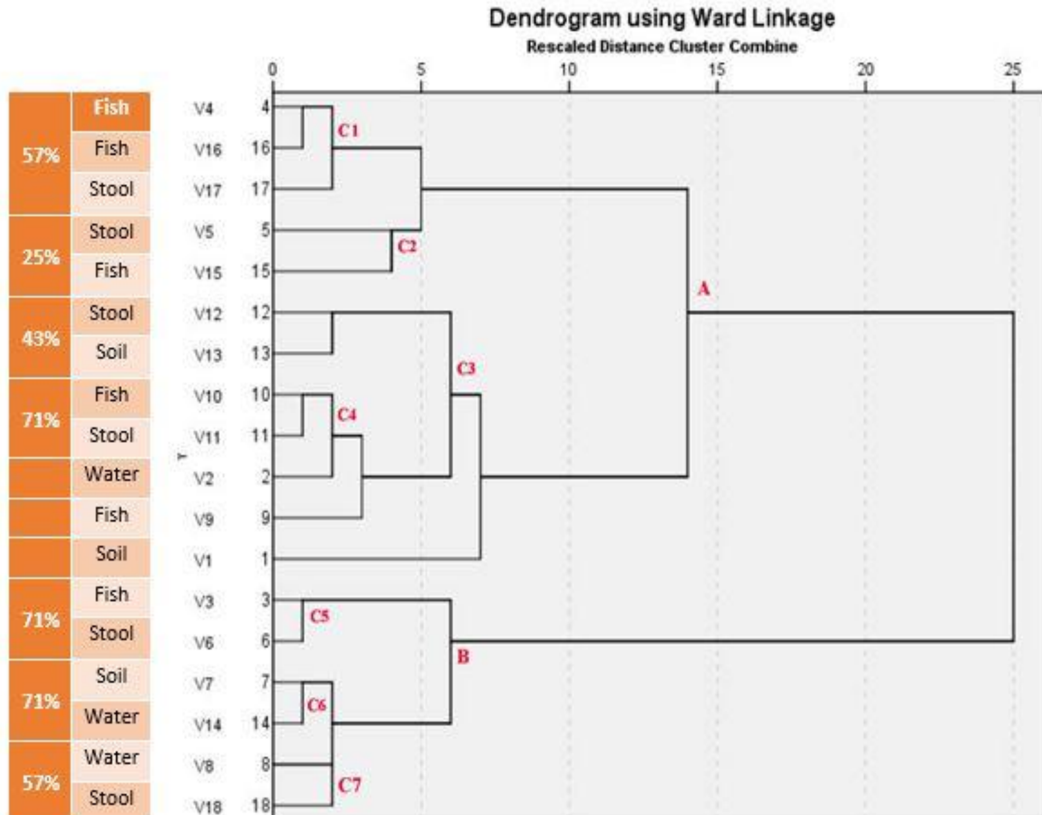



**Fig. 11 showing analysis of RAPD dendrogram results.**

# V-DISCUSSION

Aeromonads motile, are adapted to environments have a wide range of conductivity, turbidity, PH, salinity and temperature found in fresh water and in sediment than centimeter ***(Bannai, M.A, 2013);*** present in normal microflora and hydrobionats, inhabiting fish as reservoirs, acting with other microorganisms as water bio-filter in fresh water fish ***(Saad and Atallah, 2015).*** Exclusive, *A. hydrophila* is frequantely isolated pathogen from diarrheal illness patients, accompanied by abdominal pain and nausea ***(Wejdan et al., 2014)*** and the main causative organism isolated from outbreaks in farm fish suffered clinically from Motile Aeromonads Septicemia (MAS) ***(Mzula, 2020)*** associated with heavy mortality ***(Son et al.,2021)***.

One of the challenges in managing the risk to fish farms bacterial infection is identifying possible sources of pathogens. The need for this is becoming more acute in prevalent trend occurring every year as outbreaks in many fish farms in Egypt. Microbial source tracking methods and tools like traditional methods of bacterial identification and classification phenotypically; are instable and will lead to wrong identification and classification. Recently, molecular methods such as ERIC-PCR and RAPD are widely powerful tools in microbial typing and tracking ***(Diab & Al-Turk, 2011; Shao-wu, et al., 2013 and Subashkumar et al., 2014).*** Both RAPD and ERIC–PCRs were stated to be powerful tools for differentiating the strains and nucleotide sequence similarities of *A. hydrophila* ***(Davin-Regli et al., 1998; Aguilera-Arreola et al., 2005; Thayumanavan, 2005; Subashkumar et al., 2006b);*** which hybridize with sufficient affinity to chromosomal DNA sequence at low annealing temperatures.

The Random Amplified Polymorphic DNA (RAPD) technique is based on the PCR using short (usually to nucleotides) primers while the Enterobacterial Repetitive Intergenic Consensus (ERIC–PCR) technique relies on the amplification of genome DNA fragment using single primer pain, which is complementary to the short repetitive sequence and generation of reproducible and complex figerprints ***(Houf et al., 2002).*** The targets are highly conserved central inverted repeats of 126=bp located at extragenic regions of bacterial genomes.

The amplification products in both are separated by agarose gel electrophoresis to generate bacterial fingerprint and the banding patterns are used to compare the relatedness of bacterial strains ***(Reza-Ranjbar, etal., 2014).***

Briefly, the two methods are DNA based typing methods used in the molecular epidemiology of microbial pathogen. There has been considerable interest in the so-called microbial source tracking to ascribe host source to environmental isolation of *A. hydrophila*.

Our results collectively coincide with all these issues and reports but still little is known wither this pathogen genetic diversity and if it is water borne disease or not.

Taxonomic identification within the genus Aeromonas has been subject to considerable debate and differentiation at species level is somewhat perplexed ***(Blair et al., 1999)***. The need for a good system to enable identification and classification of Aeromonas is justified because of their ecological and clinical importance. Plasmid carriage is infrequent (20 to 58 %) in A. hydrophila ***(Janda, 1991)***. Although there are some arguments exist against the reproducibility of PCR–based typing methods ***(van Belkum et al., 1997; Perez et al., 1998).*** There are reports with excellent correlation and statistically validated between RAPD and ERIC-PCR methods ***(Davin-Regli et al., 1998; Szczuka and Kaznowski, 2004).***

One the other hand ***Anjay, Kumar, & Dubal, (2015)*** reported that various clusters generated in the ERIC- PCR dendrogram showed a similarity of isolates from diverse origin. The similar genotypes among human isolates and isolates from foods of animal origin, water, vegetables and sewage indicated the transmission of this important zoonotic pathogen through food and between human and animals.

Unfortionately, in this study the PCR identification using *16S rRNA* gene revealed presence a total of 18 positive *Aeromonas hydrophila* strains recovered from 28 *Aeromonas* spp. positive samples, their distribution based on their sources (Table 9). All of 18 positive *Aeromonas hydrophila* strains resulting in a 625 bp output, as illustrated in Fig. (7). A similar finding was made by ***Chu and Lu (2005); Abd El Tawab et al. (2019) and Mansour et al. (2019).*** But, in the sometimes the coming results revealed the presence of a notable genetic diversity (heterogeneity) among *A. hydrophila* isolated from fish, water, soil, and stool of patients. I did not find any identical ERIC-PCR band profile among the tested isolates; even sporadic within samples of the same source. In agreement with ***Moyer et al., (1992)*** who found a different fingerprints between *A. hydrophila* isolated from stool of diarrheic patients and environment. In further support, ***Davin-Regli et al., (1998)*** who also found a different ERIC–PCR profiles for *A. hydrophila* isolates obtained from diarrheic patients and treated drinking water. ***Thayumanavan et al., (2003)*** also found a notable genetic diversity among *A. hydrophila* strains isolated from freshly caught and marketed seafood. ***Szczuka & Kaznowski, (2004)*** reported that there was no common *A. hydrophila* clone causing diarrhea in patients. These results were consistent with those obtained by ***Ramalivhana et al., (2010)*** who did not also find any identical ERIC-PCR profile for *A. hydrophila* isolated from stool of diarrheic children and environment (fish and cow milk). Other studies on *A. hydrophila* isolated from river water and raw

milk ***(Subashkumar et al., 2014)*** have also confirmed my results. . Besides, they also found there is little genetic resemblance between clinical and environmental strains of *Aeromonas spp*. separated from various geographic regions or even from the same region.

Acually, in the present study, ERIC-PCR profiles of *A. hydrophila* isolates showed bands with size ranged from 0.27 to 2.73 kb and a total band number of 14 in fish, 12 in water, 13 in soil, and 14 in stool swab strains. The highest band (2.73 kb) was noticed in stool swab isolates, while the lowest band (0.27 kb) was found in fish, water and soil isolates.

Consistent with my results, ***Davin-Regli et al., (1998)*** observed 6 distinct ERIC-PCR band patterns among 10 *A. hydrophila* isolated from diarrheic patients and ***Ramalivhana et al., (2010)*** also found the highest band (5.8 kb) in children stool isolate and the lowest band (0.3 kb) in environmental isolates.

No common band found in all the strains, while some strains shared some common bands may be for the genus. Bands with size of 0.35, 0.44, 0.51, 0.56, 0.76, 1.25, and 1.72 kb were common in fish, water, soil, and stool isolates. Band with size of 0.63 kb were common in fish, soil, and stool isolates, while those with 0.67 kb were common in water, soil, and stool isolates. On the other hand, bands with size of 0.48 kb were common in water and stool isolates. Similarly, ***(Ramalivhana et al., 2010)*** reported common band (0.9 kb) among some *A. hydrophila* isolates.

This genetic diversity suggests the need for frequent observing of *Aeromonas* strains for infective potentials and for the probability of epidemic strains that may spread among people. There was a superior intra-specific genetic variation within *A. hydrophila* clinical and environmental isolates, suggesting that *A. hydrophila* is a microorganism with a genetically diverse population which have various pathogenic capacities to both humans and animals ***(Aguilera-Arreola et al., 2005).***

Some researchers showed that fish could be infected with *Aeromonas* spp. from contaminated water in the fish farm ***(Kühn et al., 1997).*** Nevertheless, my findings indicated that *A. hydrophila* isolated from infected fish did not have a close genetic similarity with those isolated from water samples of fish farm, i.e similar in phenotypic but differ in genetic.

In the present study, based on the results of ERIC fingerprinting analysis, the high similarity among C1 and C6 from 60 % to 75% of identity. But there is no identity between isolate no. (17) isolated from human stool and C5 which has isolates no. (9 and 10) isolated from fish. High similarity was found between C3 and C4 reach to 67% of identity. C3 and C4 have isolates obtained from fish (15 in C3 and 16 in C4) and (18 in C3 and 6 in C4) from human stool. In agreement with ***Subashkumar et al., (2014)*** found that RAPD profiles by coefficient simple matching dendrogram were represented various groups of *A. hydrophila* that clearly clustered according to the origin of isolation (diarrhoea, milk and fish). All the *A. hydrophila* strains have formed most variable groups, which composed of at least 12 groups. The highest similarity index was observed in two clusters with 0.94 between two diarrhoeal isolates (Ah-D15 and Ah-D24) (cluster 1) and fish (Ah-F1) and milk (Ah-M1) strains (cluster 2). The similarity index value 0.87 was observed with diarrhoeal isolates, Ah-D3 and Ah-D6 which formed a new cluster (cluster 3).

A greater heterogenecity among the clinical (human & fish) and environmental isolates of *A. hydrophila* were also included. A greater heterogenecity among all isolates has been demonstrated by RAPD and EIRC-PCR methods. All strains of *A. hydrophila* were having genetically heterogenous as previous concluded by ***(Subashkumar et al., 2014).***

ERIC–PCRs were used as an effective method to distinguish among different strains of *A. hydrophila* **(Aguilera-Arreola et al., 2005).**

***Thayumanavan et al., (2003); Szczuka & Kaznowski (2004)*** **and** ***Ramalivhana et al., (2010)*** have found a similar powerful differentiation efficiency for both RAPD and ERIC–PCRs when used in comparing between *Aeromonas* spp. Genetic typing by these two tools was superior to the repetitive extragenic palindromic (REP) PCR. However, ***(Subashkumar et al., 2014)*** reported that RAPD–PCR was not able to differentiate *Aeromonas* spp. Therefore, in the present study I preferd used RAPD and ERIC-PCR to compare the genetic relationship among different *A. hydrophila* isolates obtained from different sources, and unfortionately not answered the main question is the bacterium a water borne pathogen?

Finally, this study clearly confirms the broad genetic diversity of *A. hydrophila* clones of different origin (fish, human and environment). On public health point of view, the significance of checking and assessing infection-control procedures for better hygiene and to prevent cross-contamination are necessary, unfortionatily the difference did not show any obvious or cluster specific branches to each source of bacterium however ERIC-PCR would be useful for epidemiological investigation, it has simple operation and low cost. And, the combined RAPD and ERIC-PCR efficiency can be used in *A. hydrophila* epidemiological studies.

# VI- SUMMARY

*Aeromonas* spp. are considered the main occasion of diseases including ulcerative syndrome leading to high mortalities also can be a problem for human consumers.

The samples were collected for my study from tilapia fish (*O. niloticus*) had signs of septicemia and suffered from MAS (n=56), environmental water (n=56), sediment of fish farms soil (n=56) and stool samples of patients suffering from gastroenteritis (n=56), where:

1. *Aeromonas spp.* were isolated from tilapia fish suffering from MAS with a percentage of 55.3 % (31/56).
2. *Aeromonas spp.* were isolated from stool samples of patients suffering from gastroenteritis either diarrhoeal stool or apparently had abdominal pain at a percentage of 51.3 % (19/37) and 42.1 % (8/19) respectively with a total percentage of 48.2 % (27/56) and were isolated from environmental samples water and soil at a percentage of 21.4 % (12/56) and 30.3 % (17/56) respectively.
3. A total of 87 positive *Aeromonas* spp. isolates recovered from all samples. The prevalence of the positive isolates distributed based on their source was 55.3% (31/56) from tilapia fish, 21.4% (12/56) from water, 30.3% (17/56) from soil, and 48.2% (27/56) from human stool samples.
4. The sensitivity tests for the isolated *Aeromonas* strains revealed that, they were highly sensitive to ciprofloxacin (85.2%) followed by moxifloxacin and norfloxacin (82.4% and 80.6% respectively). Meanwhile, they were less sensitive to gentamycin, streptomycin and cefotaxime. Moreover, they were highly resistant to ampicillin (97.8%) followed by penicillin-G and amoxicillin (94.6% and 89.8% respectively).

5. PCR identification using *16S rRNA* gene confirmed identification of a total of 18 positive *Aeromonas hydrophila* strains recovered from phenotypically identified 28 *Aeromonas* spp. positive samples obtained from total 87 *Aeromonas* spp. positive samples. The prevalence of the positive isolates distributed based on their source was 85.7% (6/7) from diseased fish, 42.3% (3/7) from water, 42.3% (3/7) from soil, and 85.7% (6/7) from stool swab of diarrheic patients.
6. In typing those strains the total number of ERIC-PCR bands was 14 in fish, 12 in water, 13 in soil, and 14 in stool swab isolates. No identical band pattern was observed among the 18 isolates even single band not significant among the isolates of the same sample types may be due to the genus. This indicates the higher genetic variety among *A. hydrophila* isolates involved in this study. The highest (largest size) band (2.73 kb) was noticed in 2 stool swab isolates (lanes 6 and 11), while the lowest (smallest size) band (0.27 kb) was found in fish (lane 4), water (lane 2) and soil (lane 1) isolates.
7. Based on the results of ERIC fingerprinting analysis, 18 *A. hydrophila* isolates were categorized into 6 clusters (C1 to C6) ERIC types. Isolates no. (3,4) isolated from fish, 2 isolated from water and (1) isolated from soil clustered in group (C1). Isolates no. (5) isolated from stool, (8) isolated from water and (7) isolated from soil clustered in group (C6). There was a high similarity among C1 and C6 from 60 % to 75% of identity. But there is no identity between isolate no. (17) isolated from human stool and C5 which has isolates no. (9 and 10) isolated from fish. High similarity was found between C3 and C4 reach to 67% of identity. C3 and C4 have isolates isolated from fish (15 in C3 and 16 in C4) and human stool (18 in C3 and 6 in C4) and human stool.

8. The UPGMA cluster analysis on the basis of RAPD profile of *Aeromonas hydrophila* depicted all the strains having the several clusters and it suggests that their profiles are species specific. Visible RAPD fingerprinting profiles revealed the substantial wide genetic diversity among the strains tested. Strains 12, 13, 10, 11, 2 and 9 formed a minor cluster (C3) with similarity average 43%, their sources were stool, soil, fish, stool, water and fish respectively, while 5 and 15 strains, their sources were stool and fish respectively, formed a separate minor cluster with similarity 25% against the cluster (C1) which consisted of 4 and 16 strain from fish source and 17 strain from stool source with similarity 57%. This may be insufficient to indicates the genetic relationship among the strains within the clusters. It is clear that one can easily understand the percentage of genetic similarities between the strains. There was a high similarity among C4 (10,11 and 2 strains) and C5 (3 and 6 strains from fish and stool sources respectively) 71% of identity. But there is no identity between isolate no. (15) isolated from fish and isolate no. (2) isolated from water.

However, not infer the possibility of water borne infection of *Aeromonas hydrophila* from water and need in my opinion to correlate these results of clinical (fish and human) and environmental water and soil (ecotype) and virulence gene, in a wide scale.

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# الملخص العربى

ان اهمية الاسماك ليست فقط لقيمتها العالية من البروتينات والمعادن والفيتامينات الا انها أيضًا أحد القطاعات الأساسية التي توفر الدخل لبعض البلدان .

سمك البلطي من الاسماك المفضل استزراعها بسبب قدرته على الصمود في العديد من الظروف البيئية ، وإمكانية التسويق ، ومقاومة الأمراض الجرثومية من بكتريا و فطريات و فيروسات الا ان الاصابة بميكروب الايروموناس هيدروفيلا تلعب دورًا مهمًا في أمراض الأسماك ، فهي تعتبر السبب الرئيسي للأمراض بما في ذلك المتلازمة التقرحية التي تؤدي إلى ارتفاع معدل الوفيات كما يمكن أن تكون مشكلة للمستهلكين من البشر حيث تسبب التهابًا حادًا في الجهاز الهضمي مما يؤدي إلى الإسهال المزمن ويمكن أن يصيب أجهزة الجسم الأخرى مثل الجهاز البولي والجهاز التنفسي .

احد العوامل المهمة فى مقاومة اصابة الاسماك بالبكتريا الممرضة هى معرفة مصدر تلك العدوى من اجل ذلك اجريت هذه الدراسة للتخلص من المشاكل التى يسببها هذا الميكروب من خلال تتبع مصدر العدوى بميكروب الايروموناس هيدروفيلا حيث اجريت على 224 من العينات المختلفة من اسماك البلطى الموجودة بمزارع محافظة كفرالشيخ والبيئة المحيطة بها من ماء الاحواض وتربتها وايضا من براز الانسان المصاب بمشاكل فى الجهاز الهضمى من قسم المتوطنة بمستشفى كفرالشيخ العام.

*** و على هذا كان الهدف من الدراسة :-**

1. توضيح مدى تواجد ميكروب الايروموناس هيدروفيلا فى عينات الاسماك والبيئة المحيطة (من ماء و تربة ) و ايضا عينات براز الانسان اللتى تم تجميعها و زراعتها على الأوساط الملائمة .

2. تحديد الخصائص البيو كيميائية وعمل اختبارات الحساسية لميكروب الايروموناس هيدروفيلا.

3. تأكيد تصنيف ميكروب الايروموناس هيدروفيلا بواسطة تفاعل البلمرة المتسلسل .

4. تتبع مصدر العدوى من خلال اجراء ERIC-PCR على معزولات من التى تم فحصها و مقارنة التشابه الجينى بينها وتأكيد تلك النتائج باستخدام اختبار RAPD ايضا ومقارنة نتائج كل منهما بالاخرى.

*** اوضحت الدراسة النتائج التالية :**

1- أن Aeromonas spp تم عزلها من أسماك البلطي التي تعاني إما من MAS أو اعراض مرضية بنسبة 75٪ و 40.6٪ على التوالي. علاوة على ذلك ، أشارت إلى أن Aeromonas spp. تم عزلها من جميع أسماك البلطي بنسبة 55.3٪.

2 - أن Aeromonas spp. تم عزلها من عينات براز مرضى يعانون من التهاب المعدة والأمعاء إما براز إسهالي أو طبيعي ظاهرياً بنسبة 51.3٪ و 42.1٪ على التوالي. علاوة على ذلك ، أشارت إلى أن Aeromonas spp. عزلت من جميع عينات البراز بنسبة 48.2٪.

3 - أن Aeromonas spp. عزلت من عينات المياه والتربة البيئية بنسبة 21.4٪ و 30.3٪ على التوالي. علاوة على ذلك ، أشارت إلى أن Aeromonas spp. تم عزلها من جميع العينات البيئية بنسبة 27.6٪.

4 - اوضحت اجمالا وجود مجموعه 87 إيجابية من Aeromonas spp. تم الحصول على عزلات من 224 عينة بمعدل انتشار إجمالي قدره 38.8٪. كان انتشار العزلات الإيجابية الموزعة على أساس مصدرها 55.3٪ (56/31) من أسماك البلطي ، 21.4٪ (56/12) من الماء ، 30.3٪ (56/17) من التربة ، 48.2٪ (56/27) ) من عينات البراز البشري.

5 - أظهرت اختبارات الحساسية للسلالات المعزولة من الاسماك المريضة ان الأيروموناس كانت شديدة الحساسية للسيبروفلوكساسين (85.2٪) يليها موكسفلوكساسين والنورفلوكساسين (82.4٪ و 80.6٪ على التوالي). في هذه الأثناء ، كانوا حساسين للغاية للجنتامايسين والستربتومايسين والسيفوتاكسيم. علاوة على ذلك ، كانت مقاومة عالية للأمبيسيلين (97.8٪) تليها البنسلين G والأموكسيسيلين (94.6٪ و 89.8٪ على التوالي).

6 - كشف التعرف على تفاعل البوليميراز المتسلسل باستخدام جين 16S rRNA عن وجود ما مجموعه 18 عترة إيجابية من *Aeromonas hydrophila* تم استعادتها من 28 *Aeromonas* spp تم اختيارها نتيجة لانها كيميائيا ايجابية. عينات إيجابية بنسبة انتشار إجمالية قدرها 64.2٪ و الموزعة على أساس مصدرها 85.7٪ (7/6) من الأسماك المريضة ، 42.3٪ (7/3) من الماء ، 42.3٪ (7/3) من التربة ، 85.7٪ (7/6) ) من مسحة براز مرضى الإسهال.

7 - عند تصنيف هذه السلالات كان العدد الإجمالي لنطاقات ERIC-PCR في الأسماك 14، و 12 في الماء ، و 13 في التربة ، و 14 في عزلات مسحة البراز. لم يلاحظ نمط نطاق متطابق بين العزلات الـ 18 حتى بين العزلات من نفس أنواع العينات. يشير هذا إلى تنوع وراثي أعلى بين عترات *A. hydrophila* المشاركة في هذه الدراسة.

لوحظ النطاق الأعلى (الأكبر حجمًا) (2.73 كيلو بايت) في عترتين من مسحات البراز (المساران 6 و 11) ، بينما تم العثور على النطاق الأدنى (الأصغر حجمًا) (0.27 كيلو بايت) في الأسماك (الممر 4) ، والمياه (المسار 2). ) وعزلات التربة (الممر 1).

8 - بناءً على نتائج تحليل البصمة ERIC ، تم تصنيف 18 عزلة من نوع *A. hydrophila* إلى 6 مجموعات (C1 إلى C6) من أنواع ERIC حيث ان عترة رقم (3،4) معزولة عن الأسماك ، (2)معزولة عن الماء و (1) معزولة عن التربة تم جمعهم في (C1). و ان عترة رقم (5) معزول عن البراز ، (8) معزول عن الماء و (7) معزول عن التربة تم جمعهم فى (C6) وكان هناك تشابه كبيرفى الهوية بين C1 و C6 من 60٪ إلى 75٪ بينما لا يوجد تطابق بين العزلة (17) المعزولة عن البراز البشري و C5 التي تحتوي على عزلات (9 و 10) معزولة عن الأسماك , الا انه وجد تشابه كبير بين C3 و C4 تصل إلى 67 ٪ وقد عزل من الأسماك (15 في C3 و 16 في C4) والبراز البشري (18 في C3 و 6 في C4) والبراز البشري.

9 - كشفت ملفات البصمات المرئية RAPD الخاص بـ *Aeromonas hydrophila* عن التنوع الجيني الواسع بين السلالات المختبرة حيث شكلت السلالات 12 , 13 , 10 , 11 , 2 و 9 كتلة رئيسية (C3) بمتوسط تشابه 43٪ ، مصادرها كانت البراز ,التربة ,الأسماك ,البراز , الماء والأسماك على التوالي ، بينما( C2) وتتكون من 5 و 15 سلالتان كان مصدرهما البراز والأسماك على التوالي و شكلت كتلة صغيرة منفصلة مع نسبة تشابه 25٪ ضد العنقود (C1) والذي يتكون من 4 و16 سلالتان من مصدر سمكي و 17 سلالة من مصدر براز مع تشابه 57٪.

قد يكون هذا غير كافٍ للإشارة إلى العلاقة الجينية بين السلالات داخل العناقيد الا انه من الواضح امكانية سهولة فهم النسبة المئوية للتشابه الجيني بين السلالات حيث كان هناك تشابه كبيرمن الهوية يصل الى 71 ٪ بين C4 (10،11 و 2 سلالات من مصادر الأسماك والبراز والماء على التوالي) و C5 (3 و 6 سلالات من مصادر الأسماك والبراز على التوالي) بينما لا يوجد تطابق بين العزلة (15) المعزولة عن الاسماك والعزلة (2) المعزولة عن الماء.

***  نستخلص من هذه الدراسة الاتى :**

تؤكد هذه الدراسة بوضوح التنوع الجيني الواسع للميكروبات المستنسخة *A. hydrophila* من أصل مختلف (الأسماك والبشر والبيئة) لذلك ،

فضلت فى هذه الدراسة استخدام RAPD و ERIC-PCR لمقارنة العلاقة الوراثية بين عزلات *A. hydrophila* التي تم الحصول عليها من مصادر مختلفة ، وللأسف ، لم يظهر الاختلاف أي فروع محددة واضحة أو عنقودية لكل مصدر للبكتيريا ولكن ERIC-PCR سيكون مفيدًا للتحقيق الوبائي ويمكن استخدام كفاءة RAPD و ERIC-PCR المدمجة في الدراسات الوبائية *A. hydrophila*.

و من ثم لم أجد الاجابة على السؤال الرئيسي ،ان هذه البكتيريا هي احد مسببات الأمراض المنقولة بالماء.

حيث تم عزل *A. hydrophila* من عينات بيئية وسريرية مختلفة و يشكل عزل *A. hydrophila* من الأسماك مصدر قلق للصحة العامة لذلك ،

هناك حاجة في رأيي إلى ربط هذه النتائج السريرية (الأسماك والبشر) والمياه البيئية والتربة (النمط البيئي) من خلال العديد من الدراسات حول العلاقة الجينية بين العزلات من مصادر مختلفة  الى جانب الدراسة التتبعية لمصدر العدوى على نطاق واسع اكبر واشمل .

بسم الله الرحمن الرحيم

فَأَمَّا الزَّبَدُ فَيَذْهَبُ

جُفَـاءً ۖ وَأَمَّـا مَـا يَنْـــفَعُ

النَّـــاسَ

فَيَمْكُثُ فِي الْأَرْضِ

صدق الله العظيم

سورة الرعد الآية رقم ﴿١٧﴾

جامعه الاسكندرية
كلية الطب البيطري
قسم الميكروبيولوجي

# تتبع مصدر العدوى و عوامل الضراوة لميكروب الايرومونس هيدروفيلا المعزول من سمك البلطى.

رسالة مقدمة من

**ط.ب/ سالي أحمد محمد جادالله**

**(بكالوريوس العلوم الطبيه البيطريه, جامعه طنطار 2004)**

**(ماجستير العلوم الطبيه البيطريه, جامعه الاسكندريه, 2013)**

للحصول علي
درجه دكتوراه الفلسفه في العلوم الطبيه البيطريه في الميكروبيولوجي
تخصص
البكتريولوجيا والفطريات والمناعة

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بكفر الشيخ

(2022)

To

My

Father's Soul

Mother

Sister

Lovely 3R

Brothers

To All my Close Friends.

# Acknowledgment

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