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Prevalence of aerolysin and hemolysin virulence genes and antibacterial resistance profile to control Motile *Aeromonas* Septicemia (MAS) isolated from *Oreochromis niloticus* in the Nile River, Egypt.

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ABSTRACT*: *The Nile River in Egypt holds paramount importance as a natural freshwater fishery, capable of providing substantial fish yields. Among the pathogens affecting Nile tilapia (Oreochromis niloticus), Aeromonas hydrophila is the most agent causing high mortality. In the June 2023 to September 2023, around 120 moribund and freshly dead Nile tilapia were gathered from the Nile River, Al- Munib, Cairo, Egypt. The clinical symptoms in these fish were consistent with Motile Aeromonas Septicemia (MAS), including indications of surface hemorrhage, erosions, loss of scales, and some samples even showed exophthalmia, eye cataract and skin discoloration. Postmortem assessments indicated internal organ deterioration, enlargement of gall bladder, pale liver, yellowish fluid in the abdominal cavity and pigmentation on surface of the liver. Identification of A. hydrophila was accomplished through phenotypic characterization, diagnostics using SRO, GN24, features and homology of 16S rRNA gene sequences. Antibiogram analysis revealed sensitivity to ciprofloxacin, nitrofurantoin, oxolinic acid, and ofloxacin. However, resistance was observed against amoxicillin, lincomycin, spiramycin, tetracycline, colistin and cephalothin. Furthermore, PCR tests confirmed the presence of the aerolysin and hemolysin genes in the identified A. hydrophila strains. Sequencing the genes and conducting phylogenetic analysis based on the 16S rRNA sequence demonstrated that the A. hydrophila strain with H/A (accession no. OQ687115) had a 100% match.

KEYWORDS: ***Oreochromis niloticus, Aeromonas hydrophila,* aerolysin gene, hemolysin gene, Pathogenicity.**

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I. INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) are two of the highest produced fish in Egypt either from natural fisheries (Nile River and Natural lakes) or aquaculture sector according to the Fish Statistical Yearbook for 2020, where Nile tilapia (*O. niloticus*) production from the natural lakes is estimated at 135,832 tons and from Nile River is 24,269 tons, while the total production from aquaculture sector is estimated at 954,164 metric tons, all together form 55.42% of total production of Egypt **(GAFRD, 2020).**

Nile tilapia (*O. niloticus*) possesses rapid growth, can reproduce successfully in controlled environments, has a short time between generations, exhibits resilience to stress and diseases, readily adapts to artificial feeds once it absorbs the yolk-sac, consumes food from lower trophic levels, and demonstrates tolerance to various environmental conditions **(Hamouda *et al.*, 2019).** Nonetheless, in recent times, particularly in the summer season, Tilapia has encountered elevated rates of mortality **(Abdel-Latif and Khafaga, 2020).**
Aeromonas spp., *Flavobacterium* spp., *Streptococcus* spp. and *Edwardsiella tarda* have all been responsible for the incidence of diseases in aquatic population. The primary cause of infections stems from these pathogens taking advantage of environmental stressors or pre-existing disorders that make fish more susceptible to invasion **(Abdelsalam *et al.*, 2021)**. *Aeromonas* spp. are facultative anaerobic bacteria, possess a Gram-negative characteristic, display motility, and are short, rod-shaped bacilli. They are non-spore-forming microorganisms and

have a broad host range, infecting various hosts including fish, domestic chickens, most vertebrates, and even humans **(Saleh *et al.*, 2021).**

Aeromonas species are abundant in freshwater and brackish water. Motile aeromonads such as *Aeromonas hydrophila* and *Aeromonas veronii* which are causative agents of Motile *Aeromonas* Septicemia (MAS) in fish **(Abd El-Salam *et al.*, 2017; Chandrarathna *et al.*, 2018; Hussein *et al.*, 2021).** Furthermore, *A. hydrophila* is responsible for severe economic losses in global freshwater fish in China, Egypt, and the USA. It may arise because of stress factors, such as poor water quality, and increased ammonia levels, giving rise to epidemics **(El-Bahar *et al.*, 2019).** Moreover, it is considered as an opportunistic pathogen that is commensal in fish gut, either marine or freshwater fish, that may be subjected to many stressors (inadequate PH, temperature, hypoxia, malnutrition). These stressors affect fish immunity and allow this pathogen to invade fish triggering MAS or hemorrhagic septicemia characterized by high morbidity and mortality thus leading to critical economic loss to fisheries **(Mansour *et al.*, 2019)**. The acute infection by *A. hydrophila* can occur via wounds, the gastrointestinal tract, and gills. Subsequently, the infection disseminates within the bloodstream, leading to hemorrhagic septicemia **(Fikri *et al.*, 2022).**

Naturally infected *Oreochromis niloticus* showed some abnormalities due to *A. hydrophila* infection represented in hemorrhages all over the body, skin darkness, detachment of scales and fin erosion **(Hamouda *et al.*, 2019; Saleh *et al.*, 2021; Enany *et al.*, 2022).** *A. hydrophila* is the primary bacterium found in warm water aquaculture in Egypt, leading to significant mortality rates even in the absence of noticeable symptoms during the early stages. However, during the advanced stages, it manifests as skin and fin ulcers, along with symptoms like ascites and exophthalmia. These lesions result in substantial economic losses due to the low survival rate of affected fish, impacting their overall recovery **(Abdel-Latif and Khafaga, 2020)**.

Visible indications commonly observed on the abdominal wall and fin bases include congestion, hemorrhage, and erosion of scales across the entire body. Significant postmortem findings often involve highly congested internal organs, the accumulation of ascitic fluid in the abdominal cavity, as well as enlarged internal organs **(Saleh *et al.*, 2021; Taha *et al.*, 2021).**

The development of disorder caused by *Aeromonas* spp. involves a sequence of elements that promote its virulence. These elements encompass hemolytic toxins such as the enterotoxin related to aerolysin (*Act*), the heat-sensitive cytotonic enterotoxin, hemolysin (*hly*A), heat-resistant cytotonic toxins (*Ast*), and aerolysin (*aer*A). Furthermore, the pathogenicity of *Aeromonas* is supported by additional factors, including the type III secretion system (TTSS), lateral flagella (*laf*), polar flagellum (*fla*), elastase (*ela*), and lipase (*lip*) **(Shuang *et al.*, 2020).** The aerolysin gene is identified as the potential virulence gene generated by certain *A. hydrophila* strains and acts as a cytolytic toxin that generates uncontrolled openings in the membranes of specific cells. When the toxin matures, it adheres to eukaryotic cells and clusters together, resulting in the formation of openings (about 3 nm in size). This process disrupts the membrane's permeability, ultimately leading to the breakdown of the membrane and osmotic lysis **(El Deen *et al.*, 2014).**

Bacterial infections are commonly managed through the regular use of antibiotics. However, this practice disrupts the ecosystem and lacks discrimination, as it eliminates both the beneficial normal flora and the harmful bacteria within the treated host. Furthermore, the excessive use of antibiotics has led to the emergence of antibiotic-resistant strains among bacteria **(Aly *et al.*, 2023)**. In turn, *Aeromonas* spp. undergo an adaptive reaction to specific antibiotics. The transmission of antibiotic resistance occurs through mechanisms like plasmids, integrons, prophages, and transposons. These mechanisms can also carry virulence genes, promoting the emergence of antibiotic resistance in *Aeromonas* strains possessing multiple virulence genes **(Sherif and Kassab, 2023)**.

Conventional approaches to identifying bacteria involve isolating them and observing their biochemical reactions. Nevertheless, molecular techniques, particularly the polymerase chain reaction (PCR), have demonstrated superior accuracy and speed in identification. The utilization of the 16S rRNA gene, a well-established method, plays a significant role in creating distinctive genetic sequences for the precise molecular identification of *Aeromonas* species **(Ahmed *et al.*, 2018).**

The aim of this research was to identify virulence genes and evaluate the effectiveness of various antibiotics against *Aeromonas hydrophila* isolated from wild Nile tilapia (*Oreochromis niloticus*) found in the Nile River, Al-Munib, Cairo, Egypt.

II. MATERIAL AND METHODS

2.1. Fish sampling:

A total number of 120 moribund and freshly dead Nile tilapia (*Oreochromis niloticus*) were randomly collected from Nile River, Al- Munib, Cairo, Egypt, with average weight of 110-200 g. during the period of June 2023 to September 2023. Samples transferred to the Fish Diseases Research Dept. at Animal Health Research Institute, Al-Doki, Cairo, Egypt.

2.2. Clinical and Postmortem examination:

Clinical and postmortem examinations were performed for the presence of any abnormal external and internal symptoms using the method described by **(Schäperclaus *et al.*, 1992).**

2.3. Bacteriological examination and Isolation of *A. hydrophila*:

Fish samples were subjected to bacteriological investigation under sterile conditions according to the methods described by **(Austin and Austin, 2016).**

Within a microbiological safety cabinet, bacteria were isolated from various organs of each fish (liver, kidney, spleen, gills, and any external lesions if present) using a sterile loop. These isolates were then inoculated in Tryptic Soy Broth (TSB) and incubated at 28°C for 24 hours. After incubation, a loopful from each tube was streaked onto agar plates such as TSA, MacConkey agar, and blood agar. These plates were subsequently incubated aerobically at 28°C for another 24 hours. Any suspicious colonies that appeared on the agar plates were streaked on Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar plates and Rimler-Shotts (RS) medium for further selection. The streaked plates were then incubated for 18 hours at 28°C. To identify the isolates, several methods were employed. Culture characteristics were observed, and microscopic examinations using Gram staining and motility tests were performed. Additionally, biochemical analyses were conducted, including tests for oxidase, catalase, indole, methyl red, Voges-Proskauer, citrate utilization, urease, H_2S production, and sugar fermentation, following the procedures outlined in **(Austin and Austin, 2016).** Furthermore, all the tested colonies were subjected to SRO. GN24 diagnostics (www.diagnostics.sk) for further biochemical identification.

2.4. Antimicrobial susceptibility of *A. hydrophila* isolate:

The disc diffusion technique was used for detecting the antimicrobial susceptibility of *A. hydrophila* isolates. Twelve different discs of antibiotics were chosen to cover different antibiotic groups as follows: gentamicin (CN) (10 µg), ciprofloxacin CIP (5 µg), lincomycin (MY) (10 µg), colistin (CT) (25 µg), spiramycin (SP) (100 µg), nitrofurantoin (F) (300 µg), amoxycillin (AML) (10 µg), nalidixic acid (NA) (30 µg), oxolinic acid OA (2 µg), ofloxacin (OFX)(10 µg), tetracycline (TE) (30 µg), cephalothin (KF) (30 µg). Mueller-Hinton agar was used for antibiotic sensitivity test, and inhibition zone diameters were interpreted as sensitive (S), intermediate (I), and resistant (R) according to **(CLSI, 2016)**

2.5. Phylogenetic analysis of *Aeromonas hdrophila:*

The *Aeromonas hydrophila* isolation was confirmed by 16S rRNA.

2.5.1.DNA extraction.

The isolates were inoculated into brain heart infusion broth (BHIB) and incubated for 24 h. According to the manufacturer's instructions, DNA was extracted using the using PathoGene-spin™ DNA Extraction Kit.

2.5.2. Genotypic confirmation by 16srRNA of *Aeromonas hydrophila*

PCR was carried out using oligonucleotide primer for the general **16SrRNA** *Aeromonas hydrophila* gene **(F:** GGGAGTGCCTTCGGGAATCAGA **and R:** TCACCGCAACATTCTGATTTG**) (Wang *et al.*, 2003)**. The PCR protocol was as follows an initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 72°C for 5 min. the primer used in the amplification study was obtained from **(Wang *et al.*, 2003).**

2.5.3. Genotypic detection of virulence genes:

PCR was used to detect the presence of virulence genes Aerolysin (*aero*) gene, and hemolysin (*hyl*) in the identified isolates of *A. hydrophila* isolated from fish. The *aero* amplified primers were "F:CACAGCCAATATGTCGGTGAAG , R:GTCACCTTCTCGCTCAGGC " and its cycling was an initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 2 min; 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s; 72°C for 5 min with a product size of 326 bp **(Singh *et al.*, 2008)**. while *hly* amplified primers were "F:GGCCGGTGGCCCGAAGATACGGG, R:

GGCGGCGCCGGACGAGACGGGG" and its cycling was an initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 72°C for 5 min with a product size of 592 bp **(Rahayu and Daruti, 2018).**

2.5.4. Sequencing:

PCR products of *Aeromonas hydrophila* 16SrRNA were purified using QIAquick PCR Product extraction kit. (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) **(Altschul *et al.*, 1990)** was initially performed to establish sequence identity to GenBank accessions. The phylogenetic tree was created by the MegAlign Phylogenetic analysis was done using neighbour joining in MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms **(Kumar *et al.*, 2018)**.

Biosafety measures:

This study applied biosafety measures according to Pathogen safety data sheets**:** Infectious substances *Aeromonas hydrophila,* Pathogen Regulation Directorate (**Public Health Agency of Canada, 2019).**

III. Results

3.1. Clinical and postmortem observations:

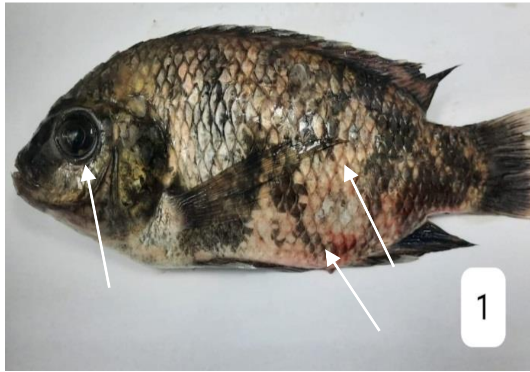
The fish displayed various severe symptoms, including extensive surface hemorrhage, erosions, loss of scales, erosion of fins and tail. Upon clinical examination, it was evident that the fish were diseased and exhibited signs consistent with hemorrhagic septicemia. These signs included surface lesions, hemorrhage at the base of the fin, and some samples even showed exophthalmia and eye cataract. Additionally, there were instances of skin discoloration as illustrated in **Figures 1** and **2**.

During the postmortem examination, further abnormalities were observed. Internal organs showed signs of deterioration, there was enlargement of gall bladder, pale liver and pigmentation on surface of the liver, as illustrated in **Figure 3** and **4**. These findings indicate the severity and extent of the infection or disease affecting the fish.

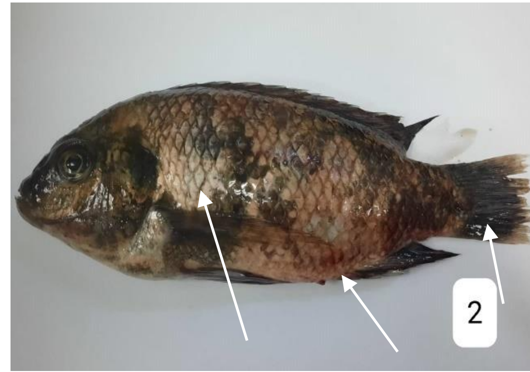
3.2. Phenotypic characteristics and prevalence of *A. hydrophila* in fish:

Phenotypic analysis of *Aeromonas* spp. colonies on trypticase soy agar (TSA) medium appeared round, convex, white, creamy and opaque while, on Rimler-Shotts (RS) medium, appeared deep creamy or light-yellow colonies with entire margin after 24 hrs. of incubation. On MacConkey's agar medium appeared pale colonies. Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS) medium changed to yellow color. For Triple Sugar Iron (TSI), *A. hydrophila* produces acid but without H_2S production. Urease -negative. By Gram staining, Gram-negative rod-shaped or coccobacilli bacteria. *Aeromonas hydrophila* is oxidase-positive, catalase-positive, Voges Proskauer-positive**,** diagnostics SRO. GN24 biochemical test confirmed for *A.hydrophila.*

3.3. Antibiogram of *A. hydrophyla* isolate*:*
Antimicrobial sensitivity testing was performed on all isolates of *A. hydrophyla* that isolated from *Oreochromis niloticus*. The tested isolates showed a notable sensitivity to ciprofloxacin, nitrofurantoin, oxolinic acid and ofloxacin, while they show intermediate effect to gentamicin and nalidixic acid and were highly resistant to amoxycillin, lincomycin, colistin, spiramycin, tetracycline, and cephalothin, as illustrated in **Figure 5** and **6**. Statistically, the tested *A. hydrophyla* isolates revealed a considerable difference in their susceptibility to different tested antimicrobial agents, as shown in **Table 1.**



Figures 1: Natural infected *O. niloticus* shows eye cataract, hemorrhage around anal opening and skin discoloration.



Figures2: Natural infected *O. niloticus* shows skin discoloration, desquamation of scales, erosion of tail and hemorrhage at the base of the fin.

Figure 3: Natural infected *O. niloticus* shows pale liver and pigmentation on surface of the liver.

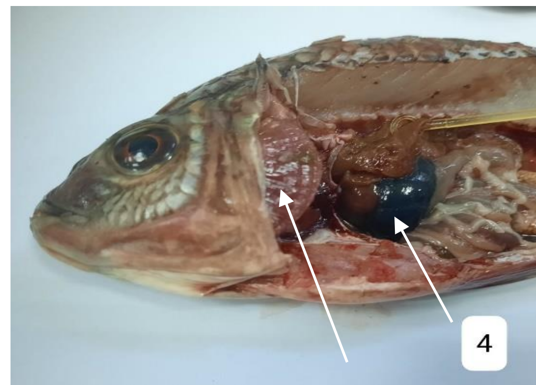


Figure 4: Natural infected *O. niloticus* shows enlargement of gall bladder and pale gills.

3.4. Genetic characterization of *Aeromonas hdrophila*:

The *Aeromonas hdrophila* isolate was confirmed by PCR giving a specific band at 356 bp. The *Aeromonas hydrophila* isolate was positive for aerolysin (*aero)* gene and hemolysin gene (*hly*) as shown in **Figure 7**.

3.5. Sequence analysis of *Aeromonas hydrophila* 16s gene:

From the sequence of *Aeromonas hydrophila.* 16s gene, **Figure 8**. The *Aeromonas hydrophila* isolate was submitted to the GenBank database under accession no. OQ687115.1 This isolate OQ687115.1 showed high nucleotide identity with other isolates MT447563.1 and OP941464.1 which were isolated from Mugil cephalus and Wild grasses, respectively in Egypt, OP787967.1, OP727559.1, OP002029.1 and ON203005.1 which were isolated from Alosa sapidissima, Tilapia, Gold fish and Red Swamp Crayfish, respectively, and MW940903.1, MW940902.1 and MW940898.1 that were isolated from Colossoma macropomum each.

V. Discussion

Protecting the fish industry from bacterial infections is of highest importance due to high economic losses caused during outbreaks, and the effectiveness of disease diagnosis results in the achievement of controlling, preventing, and treating these diseases **(EL-Makhzangy *et al.*, 2022)**.

In this current study, infected *O. niloticus* exhibited external signs consistent with hemorrhagic septicemia such as extensive surface hemorrhage, erosions, loss of scales, erosion of fins and tail, hemorrhage at the base of the fin, and some samples even showed exophthalmia and eye cataract and skin discoloration **(Austin and Adams, 1996; Thomas *et al*., 2013; El-Bahar *et al*., 2019; Hamouda *et al*., 2019; Abdelsalam *et al*., 2021; Bakiyev *et al*., 2022).** The postmortem examination displayed internal organs deterioration, and there was enlargement of gall bladder, pale liver, and pigmentation on surface of the liver as reported by **(Hamouda *et al*., 2019; Aboyadak *et al*., 2017; Austin *et al*., 2012).**

The bacteriological analysis of *O. niloticus* identified the presence of Gram-negative bacteria, specifically identified as *Aeromonas hydrophila*. The colonies observed on TSA medium displayed characteristics of being round, convex, and had a white, creamy, and opaque appearance. On RS medium, the colonies ranged from deep creamy to light yellow with smooth edges. MacConkey's agar medium produced pale colonies, while TCBS medium turned yellow. In TSI tests, *A. hydrophila* exhibited acid production without H_2S generation. It tested negative for urease and positive for oxidase, catalase, and Voges Proskauer, aligning with the results of **(Aboyadak *et al*., 2017; Ayoub *et al*., 2021; Mansour and El-Shaer, 2023)**.

Antibiotics have a significant role in managing diseases in both animals and humans, but their use can lead to bacterial resistance. Bacteria's capacity to withstand a broad spectrum of antibiotics can lead to the development of additional virulence traits. The global issue of Multiple Antibiotic Resistance (MAR) in *A. hydrophila* arises from the inappropriate use of antibiotics **(Ahmed *et al*., 2018)**. Quinolones are proven to be highly efficient antimicrobial agents when combating gram-negative bacterial infections. Both oxolinic acid and ofloxacin have received approval for treating columnaris and MAS in aquatic animals **(Thaotumpitak *et al.*, 2023)**. The current findings, based on the examination of 120 wild *O. niloticus* specimens, revealed a 20% prevalence of Motile *Aeromonas* Septicemia (MAS) within this study. It was observed that *A. hydrophila* displayed sensitivity to ciprofloxacin, nitrofurantoin, oxolinic acid and ofloxacin whereas it exhibited high resistance to penicillins and tetracycline. These results are in close alignment with findings from **(Austin *et al.*, 2012; Nasser *et al.*, 2022; Thaotumpitak *et al*., 2023)**. This research affirms that *A. hydrophila* displays resistance to β-lactam antimicrobials, and their efficacy has notably diminished due to the production of β-lactamases and other resistance genes. Nonetheless, they continue to be widely administered as the most effective treatments for bacterial infections in fish.

The polymerase chain reaction (PCR) has demonstrated its superior accuracy and speed in identifying bacterial pathogens. Within bacteria, the 16S rRNA gene stands out as a highly conserved region with significant involvement in gene coding. It is widely recognized as the standard marker for distinguishing bacterial species in phylogenetic analysis **(Hamouda *et al*., 2019).** In this research, the 16S rRNA gene sequence was employed to identify *A. hydrophila,* showing a characteristic homology at 356 base pairs, which aligns with the findings of the authors **(Hamouda *et al*., 2019; Umutoni *et al*., 2020).**

Aeromonas hydrophila that has been isolated, exerts its effect using various virulence mechanisms, releases a range of virulence elements crucial for its ability to cause disease. These virulence components encompass outer membrane proteins, extracellular substances (cytotoxins and proteases), and enterotoxins **(Aboyadak *et al.*, 2017).** In addition to, hemolytic toxins that include a range of substances, including aerolysin-related cytotoxic enterotoxin (*act*), heat-labile cytotonic enterotoxin (*alt*), heat-stable cytotonic toxins (*ast*), hemolysin (*hly*A), and aerolysin (*aer*A). Additionally, contributing to the pathogenicity of *Aeromonas* are the type III secretion system (TTSS), polar flagellum (*fla*), lateral flagella (*laf*), elastase (*ela*), and lipase (*lip*) **(Zhou *et al.*, 2019).** This research detects the presence of hemolysin (*hly*A) at 592 bp, this result agrees with that reported by **(El-Bahar *et al.*, 2019; Mansour and El-Shaer, 2023)** the prevalence of *aerA* gene at 326 bp, this result matches with that of **(Abdel-Latif and Khafaga, 2020; Mansour and El-Shaer, 2023; Saleh *et al*., 2021; Ahmed *et al*., 2018).**

VI. Conclusion

Essentially, this data suggests that there may be highly multi- drug resistant (MDR) *A. hydrophila* strain present in *O. niloticus*. It highlights the significance of using antibiotics cautiously in the Nile tilapia fish industry. The presence of aerolysin and hemolysin, either individually or in combination, is crucial in how *A. hydrophila* becomes pathogenic. Utilizing 16S rRNA genotyping is informative and precise for accurately identifying *A. hydrophila* and conducting molecular epidemiology studies to assess its phylogenetic diversity.

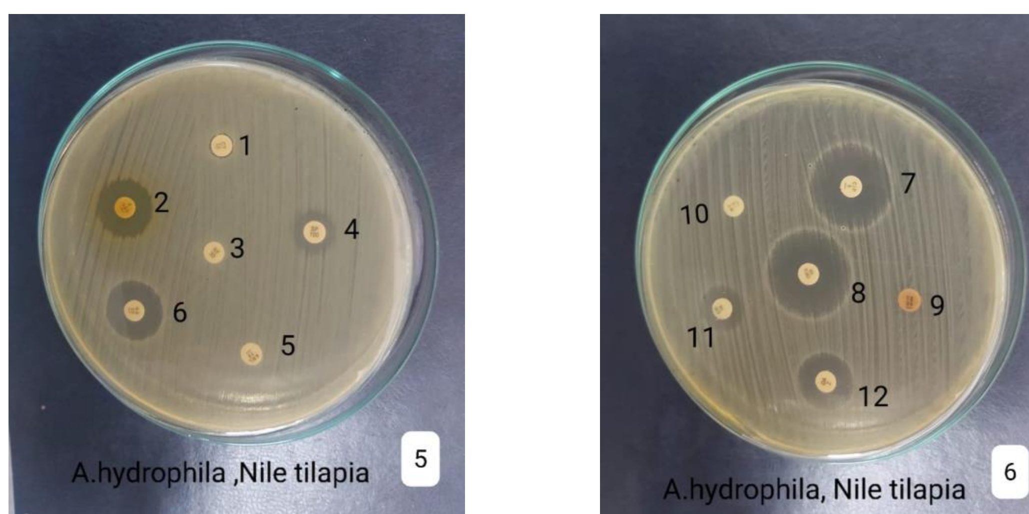


Figure 5 & Figure 6: Antimicrobial sensitivity test for A. hydrophila.(1= CT; 2= F; 3 = KF; 4= SP; 5= AML,6= NA; 7= CIP; 8= OFX; 9= TE;10= MY; 11= CN; 12= OA)

Table 1: Results of susceptibility test for *A. hydrophila*

Antibiotic agent	Susceptibility results
Gentamicin (CN) 10 µg	I
Ciprofloxacin CIP 5 µg	S
Lincomycin (MY) 10 µg	R
Colistin (CT) 10 µg	R
Nitrofurantoin (F) 300 µg	S
Amoxycillin (AML) 10 µg	R
Nalidixic acid (NA) 30 µg	I
Oxolinic acid OA 2 µg	S
Tetracycline (TE) 30 µg	R
Spiramycin (SP) 100 µg	R
Ofloxacin (OFX) 10 µg	S
Cephalothin (KF) 30 µg	R

R = Resistant, **I** = Intermediate, **S** = Sensitive

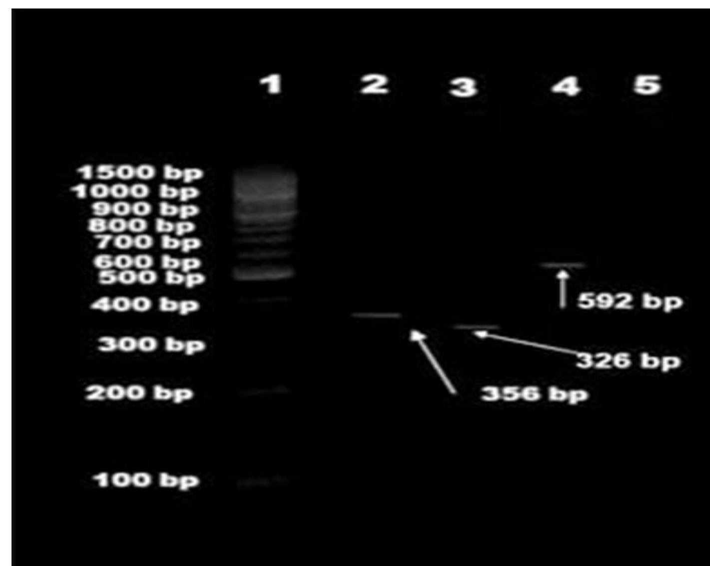


Figure 7. Agarose gel electrophoresis showing amplification of 16srRNA *Aeromonas hydrophila* using specific primer. Lane 1: 1 kb Ladder, Lane 2: Sample 356 bp fragment for 16SrRNA *Aeromonas hydrophila*; Lane 3: Sample 326 bp fragment for *of aero* gene, Lane 3: Sample 592 bp fragment for *of hly* gene.

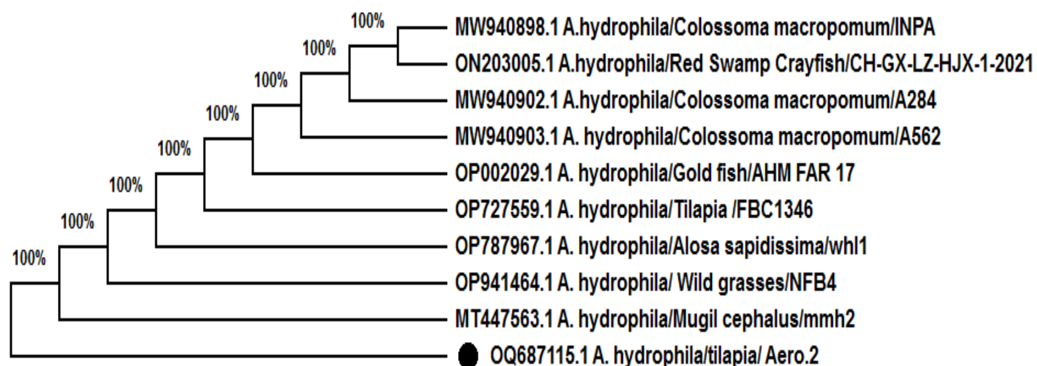


Figure 8. shows the phylogenetic tree of *Aeromonas* spp. 16s gene black circle**:** this research isolate.

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