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**Aya F Matter**
Department of Aquatic Animal Diseases and Management, Faculty of Veterinary Medicine, Moshtohor, Benha University, Egypt

**Amel M El Asely**
Department of Aquatic Animal Diseases and Management, Faculty of Veterinary Medicine, Moshtohor, Benha University, Egypt

**Adel A Shaheen**
Department of Aquatic Animal Diseases and Management, Faculty of Veterinary Medicine, Moshtohor, Benha University, Egypt

**Eman A Abd El-Gawad**
Department of Aquatic Animal Diseases and Management, Faculty of Veterinary Medicine, Moshtohor, Benha University, Egypt

**Hiam El-Abd**
Department of Aquatic Animal Diseases and Management, Faculty of Veterinary Medicine, Moshtohor, Benha University, Egypt

**Amany A Abbass**
Department of Aquatic Animal Diseases and Management, Faculty of Veterinary Medicine, Moshtohor, Benha University, Egypt

**Correspondence**
**Aya F Matter**
Department of Aquatic Animal Diseases and Management, Faculty of Veterinary Medicine, Moshtohor, Benha University, Egypt

# Phenotypic and molecular characterization of bacterial pathogens isolated from diseased freshwater fishes

**Aya F Matter, Amel M El Asely, Adel A Shaheen, Eman A Abd El-Gawad, Hiam El-Abd and Amany A Abbass**

**Abstract**
Clinically diseased *Oreochromis niloticus*, *Claris gariepinus*, and *Mugil cephalus* were collected from different localities at El – Riah El- Tawfiki and its tributaries (Qalyubia), Kafr El-Sheik and Ismailia governorates. The clinical picture and gross lesions were recorded. Isolation and identification of the bacterial pathogens using traditional and molecular methods were demonstrated. It was found that the diseased fishes were showing general septicemic signs as skin hemorrhages and ulcerations, uni- and bilateral exophthalmia, congested internal organs with significant mortalities. Additionally, the total prevalence of the retrieved bacterial isolates was (55.27%) among the species and it was 62.5, 45, and 32.5 % in *O. niloticus*, *C. gariepinus*, and *M. cephalus* respectively. Summer season displayed the highest prevalence in the examined fishes. Based on the phenotypic and biochemical characterization using API20E, the isolated bacteria were identified as *Aeromonads* (*A. hydrophila*, *A. veronii*, *A. caviae* and *A. sobria*), *Pseudomonas* spp (*Ps. fluorescence*, *Ps. aeroginosa*, and *Ps. putida*), *Citrobacter* spp (*C. koseri*, *C. freundii*, and *C. braakii*), *Serratia* spp (*S. odorifera*, *S. liquefaciens*, and *S. marcescens*), *Enterobacter* spp (*E. sakazakii*and *E. cloacae*) and *Vibrio fluialis*.*O. niloticus* was the most infected fish species followed by *C. gariepinus* and *M. cephalus*. Almost all the detected bacterial isolates were isolated from kidneys and liver samples. The most prevalent bacterial isolates were *Aeromonads* (12 isolates, 48%), among them; *A. hydrophila* was the predominant species (5 isolates, 20%), and followed by *A. veronii* (3 isolates, 12%), *A. sobria* (3 isolates, 12%) and *A. caviae* (1 isolate, 4%). Molecular characterization using conventional PCR to confirm the biochemically identified *Aeromonads* using the16S rDNA region retrieved band at 900 bp. Together, these results suggest that several septicemic bacterial pathologies were widespread in the freshwater fishes, where their presence may lead to mortality.

**Keywords:** *Aeromonads* -freshwater fishes -molecular characterization -prevalence

## Introduction
Egyptian aquaculture industry has been grown in the last few years by 45% which were mostly produced by fish farms [4]. Egypt ranked the first in fish farm production in theAfrican and Mediterranean Sea countries [44]. Fish and fish products are the hope to solve human nutritional problems in Egypt, according to reports of Agriculture World Journal [2]. Nile tilapia is considered as one ofthe most important freshwater species for commercial aquaculture in Egypt, due to its high nutritional values, rapid growth rate and resistance to diseases leading to high level of production [12]. Semi-intensive fish culture in earthen ponds is by far the most important farming system in Egypt, in addition; recent years have witnessed a rapid development of intensive systems in both tanks, cages and in the desert led to a boom in this aquaculture industry [44]. The health keeping of fish depends on the relationship between fish, environment, and pathogens. Increasing intensification of fish production and lack of health management measures have led to many disease problems of bacterial, viral, fungal and parasitic origin lead to mass mortalities in fish farms during the last five years in Egypt [6]. Most bacterial pathogens are a serious problem in tilapia production causing 80% of fish mortalities [39, 50, 21]. The most recorded gross signs were hemorrhagic septicemia and fin rot [3].Numerous general and selective media have been developed for the isolation and presumptive identification of the etiological agents [37]. API20E test strip is used widely for identification of Enterobacteriaceae and this system is characterizing by fast detection of bacteria without the need for many of culture media [34]. Both Aeromonads

(*A. hydrophila, A. sobria, and A. caviae*) and *Pseudomonas* spp (*Ps. fluorescens, Ps. putida, and Ps. aeruginosa*) were incorporated in severe outbreaks in fish farms,[3]. Because the biochemistry, genetics, and serology of the motile Aeromonas taxon are heterogeneous, the taxonomic position of this genus has been unstable [40]. As a common method, polymerase chain reaction (PCR) is used to identify Aeromonas genomo species. PCR developed here was successfully applied for the identification of *Aeromonads* from diseased fish samples [19, 28]. This work was aimed to study culture, biochemical characterization of bacterial pathogens isolated from the examined freshwater fish species, determine their prevalence and molecular identification of the most prevalent bacterial isolates using conventional PCR.

## 2. Materials and Methods

### 2.1 Fish collection and sampling

A total number of (360) farmed freshwater fishes; including (240) *O. niloticus*, (80) *C. gariepinus* and (40) *M. cephalus* were collected from El – Riah El- Tawfiki and its tributaries (Qalyubia), Kafr El-Sheikh, and Ismailia governorates during the period from January to December 2016. Clinically diseased and freshly died fishes were transferred to the laboratory of the Department of Aquatic Animal Diseases and Management for clinical, postmortem and bacteriological examinations were carried out according to Austin and Austin [8].

### 2.2 Clinical examination

All the collected fishes were subjected to clinical and postmortem examinations according to Austin and Austin and Conroy and Herman [10, 22].

### 2.3 Bacteriological examination

Samples for bacteriological examination were taken from skin lesions, gills, liver, kidneys, spleen, heart, brain and intestine of the clinically diseased fishes and inoculated onto Brain heart infusion broth (BHIB) and incubated at 28°C for 24 hrs then streaked over Brain heart infusion agar (BHIA) and incubated at 28°C for 24 hrs [48]. Gram staining and motility test were performed according to Cruickshank *et al*, [23]. Pure colonies streaked on to Rimler's - Shotts medium (RS medium), Aeromonas selective agar base with Ampicillin supplement, XLD media, and MacConkey agar plate for detection the fermentation activity, all incubated at 28 °C for 24 hrs, culture characters of isolated bacteria were recorded. The isolated bacteria were stored at -80 °C in BHIB containing 20% glycerol for further molecular studies[26]. Phenotypic characterization of the bacterial isolates was demonstrated according to Bergey's, Elemar *et al*., Madigan and Martinko, and Schäperclaus *et al*, [15, 24, 31, 43]. All isolates were identified biochemically by streaking bacterial colonies over tryptic soy agar and incubated at 28°C for 24 hrs then identified at the genus level by using API20E strips [16] (biochemical profiling test was performed according to manufacturer's instructions) to identify the isolated bacteria [36]. The prevalence of bacterial isolates among the examined fishes was recorded. The most prevalent bacterial isolates were selected and subjected to molecular identification using PCR.

### 2.4 Molecular characterization of aeromonas spp

#### 2.4.1 Bacterial DNA extraction

Each Aeromonad isolate was grown overnight in 5 ml of BHI broth at28 °C for 24 hrs. Qiagen DNeasy DNA extraction protocol for bacterial cultures adapted from Qiagen DNeasy handbook, 2006 and stored at −20 °C till use. Two specific primers used for *Aeromonads* (SB Aer1 f 5'-CAGAAGAAGCACCGGCTAACTC 3') and (SB Aer1 r 5'-TTACCT TGTTACGACTTCACC 3'), (Taq, Promega, USA) according to Panigrahy *et al*, [38].

#### 2.4.2 PCR amplification

It was performed in a DNA thermal cycler (Eppendorf, Hamburg, Germany). PCR reactions were performed in a final volume of 25 µl containing 20 ng of DNA, 0.1–0.3 µl of each primer, and 1 µl of Hot Star Taq Master Mix containing MgCl2, Hot Star Taq DNA polymerase and deoxynucleotide triphosphate mix (dNTPs). PCR conditions were as follows: 95 °C for 15 min, 30 cycles at 95 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, followed by a final elongation at 72 °C for 7 min. The PCR products (12 µl) were analyzed by electrophoresis on 2.5% agarose gels stained with ethidium bromide using 1 µM Tris-Acetate-EDTA buffer and were visualized by UV transillumination [38].

## 3. Results

### 3.1 Clinical picture

**Plate 1:** Showing the clinical signs of the clinically examined fish species, *O. niloticus*, redness around the mouth, (Fig. 1), detached scales and deep ulcers on skin, (Fig. 2), unilateral exophthalmia, (Fig. 3), abdominal distention and darkness on skin, (Fig. 4), and redness on skin with ulcer formation, (Fig. 5). *C. gariepinus*, redness and ulcer on skin, (Fig. 6). *M. cephalus*, redness on skin, base of fins and at vent, (Fig. 7 and 8**)**.

**Plate 2:** Showing the developed lesions of the clinically examined fish species, *O. niloticus*, enlargement with hemorrhagic patches in liver tissue, (Fig. 1). *C. gariepinus*, congestion and enlargement in all internal organs and musculature, (Fig. 2).

### 3.2 Bacteriological identification

Colonies suspected of *Aeromonas* spp appeared round, convex, shiny and creamy on TSA media. Gram staining of these colonies gives gram-negative coccobacilli to rod-shaped and motile. On RS medium produced yellow colonies after 24 hrs of incubation, non- lactose fermentable on MacConkey agar while on Aeromonas base agar media gives small, dark green colonies with a dark center and on XLD media gives large, round, convex yellow colonies.

Colonies suspected of *Pseudomonas* spp appeared spindle shape on TSA media and after 24 hrs produced a diffusible yellow-greenfluorescence pigment. Gram-negative, a rod-shaped and motile bacterium. Non-lactose fermented colonies on MacConkey agar. On RS media, appeared greenish in color after 24 hrs incubation.

Colonies suspected of *Vibrio fluvialis* was lactose fermentative bacteria on MacConkey agar and produce green colonies on TCBS media. Gram-negative, rod and motile bacterium microscopically. Colonies suspected of *Citrobacter* spp. were variable lactose fermentative bacteria on MacConkey agar and produce yellow colonies with $H_2S$ production on Aeromonas agar media. Gram staining of these colonies gives gram-negative, rod-shaped and motile by making motility test.

Colonies suspected of *Serratia* spp appeared non-lactose fermentative bacteria on MacConkey agar and may

producered colonies on TSA media. *Enterobacter* spp is gram-negative, facultatively anaerobic, rod-shaped microscopically. Ferment lactose during a 48-hours incubation at 35 °Con MacConkey agar.

**3.3 Biochemical characters**

*Aeromonads* gave a positive reaction for oxidase, catalase, and indole test also ferment glucose with the production of acid and gas. They gave negative results toward H2S production, urea hydrolysis, and non-lactose fermentation and produced variable results with V.P, Indole production, gelatin hydrolysis, nitrate reduction and sugar fermentation (Mannitol, Inositol, Sorbitol, and Arabinose).Beside them; there were some different species of isolated bacteria as *Pseudomonas* spp, *Serratia* spp, *Citrobacter* spp, *Vibrio fluialis*, and *Enterobacter* spp. which were identified by anAPI20E system which showed the different result as presented in Table 1.

**3.4 Prevalence of bacterial infection among naturally infected fishes**

Total prevalence of bacterial infection was (55.3%); among the species*; O. niloticus* showed high incidence 62.5%, followed by *C. gariepinus* 45% and *M. cephalus* 32.5%, (Table 2 and Fig.1). The seasonal prevalence of infected *O. niloticus* was 33.3, 66.7, 91.7 and 58. 3% in winter, spring, summer, and autumn, respectively, while in *C. gariepinus* was 0, 55, 65 and 60 % in winter, spring, summer, and autumn, respectively. In addition; in *M. cephalus* was 0, 40, 60 and 30% in winter, spring, summer and autumn, respectively so summer season revealed the highest prevalence in the examined fishes, (Table 3 and Fig. 2)**.** Based on phenotypic and biochemical characterizations using API20E; the isolated bacteria identified as *Aeromonas* spp. (*A. hydrophila, A. veronii, A. caviae,* and *A. sobria*). *Pseudomonas* species (*Ps. fluorescence, Ps. aeroginosa,* and *Ps. putida*), *Citrobacter* spp (*C. koseri, C. freundii*, and *C. braakii*), *Serratia* spp (*S. odorifera*, *S. liquefaciens*, and *S. marcescens*), *Enterobacter* spp (*E. sakazakii* and *E. cloacae*) and *Vibrio fluialis.* The most prevalent bacterial isolates were *Aeromonads* (12 isolates, 48%), among them; *A. hydrophila* was the predominant species (5 isolates, 20%), and followed by *A. veronii* (3 isolates, 12%), *A. sobria* (3 isolates, 12%) and *A. caviae* (1 isolates, 4%). *O. niloticus* was the most infected fish species (14 isolates) followed by *C. gariepinus* (9 isolates) and *M. cephalus* (2 isolates). Almost all the bacterial detected were isolated from kidneys and liver samples, (Table 4).

**3.5 Molecular identification of aeromonas spp.**

Molecular characterization with conventional PCR to confirm biochemically identified 12 *Aeromonads* isolates using the16S r DNA region retrieved band at 900 bp (lanes 1-12), (Fig. 3).

**4. Discussion**

Bacterial diseases considered the most serious disease problem among freshwater fishes **[35]**.Isolation of 25 different species of bacterial pathogens from three freshwater fishes during the current study adds more evidence for the wide geographical distribution of bacterial diseases. The isolated bacteria identified as Aeromonas spp. (*A. hydrophila, A. veronii, A. caviae* and *A. sobria*).Pseudomonas species (*Ps. fluorescence, Ps. aeroginosa,* and *Ps. putida*), *Citrobacter* spp. (*C. koseri, C. freundii* and *C. braakii*), *Serratia* spp. (S. *odorifera*, S. *liquefaciens,* and *S. marcescens*), *Enterobacter* spp. (*E. sakazakii* and *E. cloacae*) and *Vibrio fluialis.* Concerning the clinical picture of naturally infected fishes which observed in this study nearly similar with that obtained by Austin and Austin, Abd El- Aziz, Hussein, Rasmia Hanafy, Toranzo *et al*, Cagatay and Sen and Basma *et al*. [9, 1, 27, 41, 49, 17, 13] who isolated different bacterial species from some freshwater fishes showing signs of septicemia. Consistent with the current findings, hemorrhagic septicemia was also reported in fish (Milkfish, *Chanos chanos*) due to *Aeromonas* infection, especially when fish are under stress due to the opportunistic pathogen (such as *Aeromonas* spp) causes infection only when the host resistance has been lowered by environmental stress factors, such as high organic load, overcrowding, and sub-lethal oxygen levels **[46, 20].**

The characteristic colonies of *Aeromonas* spp appeared round, convex, shiny and creamy on TSA media. Gram-negative, coccobacilli to rod-shaped and motile, gives yellow colonies due to maltose fermentation on RS Media, non- lactose fermentable on MacConkey agar while on *Aeromonas* base agar media gives small, dark green colonies with dark center and on XLD media gives large, round, convex yellow colonies, these findings similar with that recorded by Hazen *et al*. [25] who stated that RS Media was 94% efficient for isolation of Aeromonas spp. while Pseudomonas spp. appeared spindle shape on TSA media with a diffusible yellow-green fluorescence pigment. Gram-negative, rod shape and motile. Non-lactose fermented colonies on MacConkey agar. On R S media appeared greenish in color after 24 hrs incubation. These results like that obtained by Cruickshank *et al.*, Austin and Austin and Masbouba [23, 9, 32]. Colonies suspected of *Vibrio fluvialis* was lactose fermentative bacteria on MacConkey agar and produce green colonies on TCBS media. Gram-negative, motile, rod bacterium. These observations nearly similar with that recorded by Cruickshank *et al*. and Austin and Austin [23, 9]. Colonies suspected of *Citrobacter* spp were variable lactose fermentative bacteria on MacConkey agar and produce yellow colonies with $H_2S$ production on Aeromonas agar media. Gram-negative, rod-shaped, motile. These findings were like those recorded by Cruickshank *et al.*, Sato *et al.*, Austin and Austin and Mohamed [23, 42, 9, 33]. *Serratia* spp appeared non-lactose fermentative bacteria on MacConkey agar and may produce red or yellow colonies on TSA media. These results were like those recorded by Cruickshank *et al.*, Baya *et al.*,and Austin and Stobie [23, 14, 11]. *Enterobacter* spp is gram-negative, facultatively anaerobic, rod-shaped. Ferment lactose during a 48-hours incubation at 35-37 °C on MacConkey agar. These observations like that obtained by Cruickshank *et al* and Sato *et al*, [23, 42].

The API-20E microbiological kits are effectively used to identify Enterobacteriaceae, according to the results of API 20 E test, several 12 strains were suspected to be Aeromonas spp., these results nearly similar with that recorded by Lund *et al*., and Cantas *et al*., [30, 18] who reported that some of the biochemical reactions of the standardized API20E test, showing positive reaction and others gave negative results but variation in another one. Beside Aeromonas spp.; there were some different species of isolated bacteria as *Pseudomonas* spp., *Serratia* spp., *Citrobacter* spp., *Vibrio fluvialis*, and *Enterobacter* spp which were identified by API20E system which showed different results in some reactions. Nearly similar results were observed by several authors Cruickshank *et al*., Sato *et al.*, Baya *et al.*, Austin and Austin, Mohamed 2000, Lund *et al*., Masbouba and Cantas *et al.* [23, 42, 14, 9, 33, 30,

[32, 18]. However, the API20E patterns cannot be related to fish host and geographical location.

The prevalence of bacterial pathogens has been well documented in several cultured and wild freshwater fish species associated with septicemia [7]. In this work, the total prevalence of bacterial infection was (55.3%), these findings were higher than that recorded by Rasmia hanafy [41] who found the total prevalence of bacterial infection was 32.5% and these findings were lower than that recorded by Ali [5] who found the total prevalence of bacterial infection was76.5%**.** This difference may be attributed to the site of sample collection, the number of examined fish, the size of fish and environmental conditions**.** Among the species*; O. niloticus* showed high incidence 62.5%, followed by *C. gariepinus* 45% and *M. cephalus* 32.5%. These observations like that recorded by Mohamed, Stock and Wiedemann and Hussein [33, 45, 27]. These results may be due to *O. niloticus*is considered the main cultured fish species and genetically highly suitable to the infection while *C. gariepinus* and *M. cephalus* may be more immunologically protected from that infection and may be due to the system of rearing and environmental conditions**,** Mohamed [33]. Regarding the seasonal prevalence of infected fish species, summer season revealed the highest prevalence among examined fishes. These observations nearly similar with that recorded by Mohamed, Stock and Wiedemann, Hussein and Rasmia hanafy [33,45,27, 41],this may be due that in summer; high temperature, low dissolved oxygen and the subsequent other alteration in water parameters that induce stressors on fish compromise the immune response make the fish more susceptible to bacterial infection**.**The most prevalent bacterial isolates were *Aeromonads* (12 isolates, 48%), among them; *A. hydrophila* was the predominant species (5 isolates, 20%), and followed by *A. veronii* (3 isolates, 12%), *A. sobria* (3 isolates, 12%) and *A. caviae* (1 isolates, 4%). These findings nearly like that recorded by Mohamed, Stock and Wiedemann, Hussein, Rasmia hanafy, Basma *et al*. and Ahmed and Shoreit [33, 45,27, 41, 13, 3]. Almost all the bacterial detected were isolated from kidneys and liver samples. These findings were similar with obtained by Thompson *et al.*, Toranzo *et al.* and Kusdarwati *et al.* [47, 49, 29]. These findings were due to these organs has haemolytic function so highly susceptible to infection**.**

Molecular characterization using conventional PCR to confirm the biochemically identified *Aeromonad* species using 16S r DNA region retrieved specific band at 900 bp. These results agreed with that of Panigrahy *et al.* [38] who described PCR amplification of the 16S rDNA coding region of *Aeromonads* isolates which all eight isolates had bands at the 900 bp DNA fragment level. This method has shown discriminatory properties in the identification up to the species level and in the typing of the bacteria.

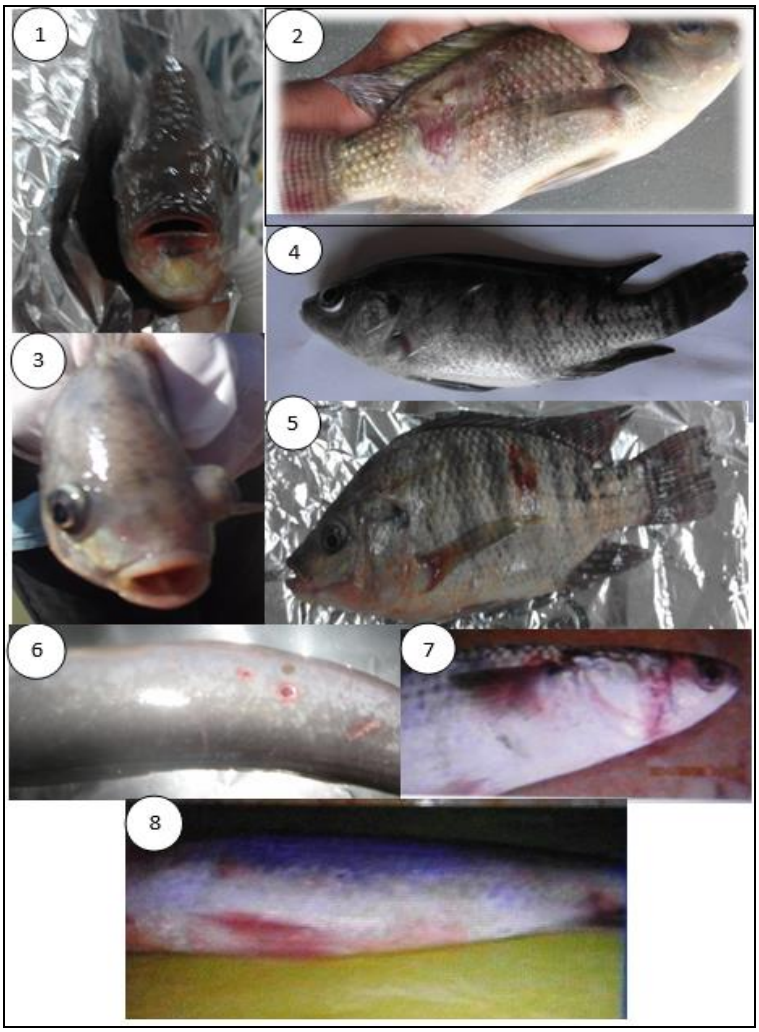


**Plate 1:** Showing the clinical signs of the clinically examined fish species, *O. niloticus*, Fig. 1: Redness around the mouth Fig. 2: Detached scales and deep ulcers on skin, Fig. 3: Unilateral exophthalmia, Fig. 4: Abdominal distention and darkness on skin and Fig. 5: Redness on skin with ulcer formation. *C. gariepinus*, Fig. 6: Redness and ulcer on skin. *M. cephalus*, Fig. 7 and 8: Redness on skin, base of fins and at vent.

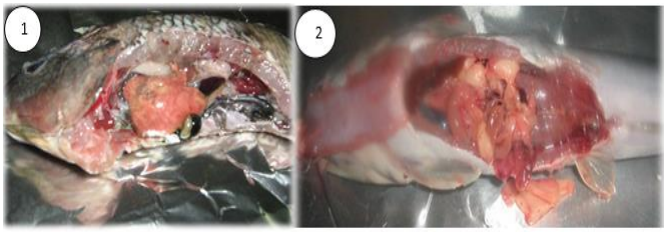


**Plate 2:** Showing the developed lesions of the clinically examined fish species, *O. niloticus*, Fig. 1: Enlargement with hemorrhagic patches in liver tissue. *C. gariepinus*, Fig. 2: Congestion and enlargement in all internal organs and musculature.

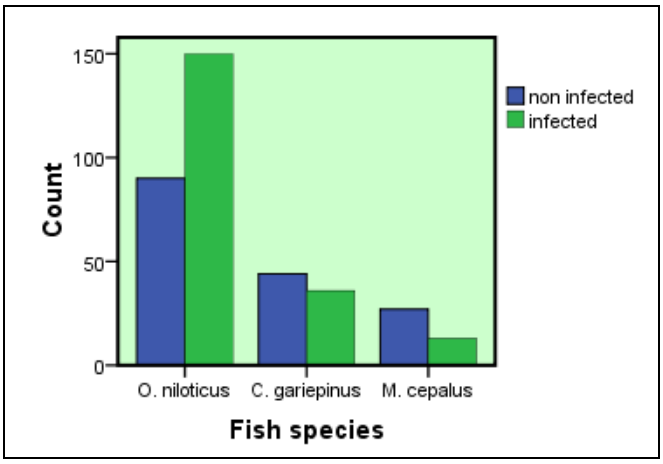


**Fig 1:** Showing total prevalence of bacterial infection among the examined fish species

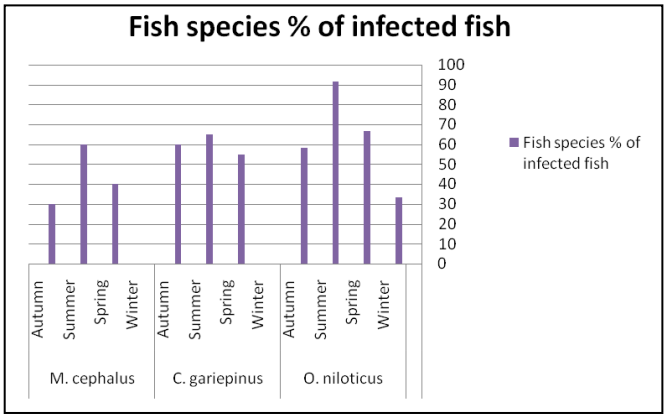


**Fig 2:** Seasonal prevalence of bacterial infection among the examined fish species:

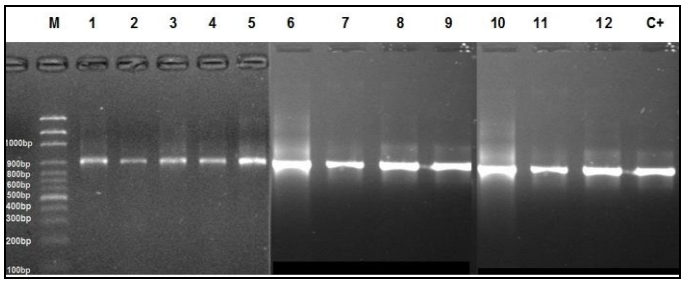


**Fig 3:** Ethidium bromide stained 2 % agarose gel of PCR products showed Aeromonas +ve samples of 900 bp PCR products from bacterial culture (lanes 1-12). M represents a 1000 bp DNA ladder as a size standard.

**Table 1:** Biochemical characters of different bacterial isolates obtained from naturally infected fishes using API 20E system.

| | | ***Aeromonas spp*** | ***Pseudomonas spp*** | ***Vibrio fluvialis*** | ***Citrobacter spp*** | ***Enterobacter spp*** | ***Serratia spp*** |
|---|---|---|---|---|---|---|---|
| Gram stain | | -ve | -ve | -ve | -ve | -ve | -ve |
| Motility test | | Motile | Motile | Motile | Motile | Motile | Motile |
| **API 20E reactions: -** | | | | | | | |
| ONPG | β-galactosidase | + | - | + | + | + | + |
| ADH | Arginine dihydrolase | + | + | + | + | _ | + |
| LDC | Lysine decarboxylase | + | - | + | - | + | + |
| ODC | Ornithine decarboxylase | + | - | + | + | + | + |
| CIT | Citrate utilisation | + | + | + | + | - | + |
| $H_2S$ | $H_2S$ production | - | - | - | + | - | - |
| URE | Urea hydrolysis | - | - | - | - | - | V |
| TDA | Tryptophan deamination | + | + | + | + | + | + |
| IND | Indol production | V | - | V | + | - | - |
| VP | Acetoin production | V | - | V | - | - | V |
| GEL | Gelatin hydrolysis | + | - | + | - | + | + |
| GLU | Glucose fermentation | + | + | + | + | + | + |
| MAN | Mannitol | V | V | V | V | V | V |
| INO | Inositol | V | V | V | V | V | V |
| SOR | Sorbitol | V | V | V | V | V | V |
| RHA | Rhamnose | V | V | V | V | V | V |
| SAC | Sucrose | V | V | V | V | V | V |
| MEL | Melibiose | V | V | V | V | V | V |
| AMY | Amygdalin | V | V | V | V | V | V |
| ARA | Arabinose | V | V | V | V | V | V |
| Oxidase | Cytochrome oxidase | + | + | + | - | - | - |

**Table 2:** Total prevalence of bacterial infection isolated from diseased fish species:

| Fish species | Parameters | Examined fishes | | Total |
|---|---|---|---|---|
| | | Non-infected | Infected | |
| *O. niloticus* | Count | 90 | 150 | 240 |
| | % within fish species | 37.5% | 62.5% | 100.0% |
| | % within infected | 55.9% | 75.4% | 66.7% |
| | % of Total | 25.0% | 41.7% | 66.7% |
| *C. gariepinus* | Count | 44 | 36 | 80 |
| | % within fish species | 55.0% | 45.0% | 100.0% |
| | % within infected | 27.3% | 18.1% | 22.2% |
| | % of Total | 12.2% | 10.0% | 22.2% |
| *M. cephalus* | Count | 27 | 13 | 40 |
| | % within fish species | 67.5% | 32.5% | 100.0% |
| | % within infected | 16.8% | 6.5% | 11.1% |
| | % of Total | 7.5% | 3.6% | 11.1% |
| Total | Count | 161 | 199 | 360 |
| | % within fish species | 44.7% | 55.3% | 100.0% |
| | % within infected | 100.0% | 100.0% | 100.0% |
| | % of Total | 44.7% | 55.3% | 100.0% |

**Table 3:** Seasonal prevalence of bacterial infections isolated from diseased fish species.

| Fish species | Season | No. of examined fish | No. of infected fish | % of infected fish |
|---|---|---|---|---|
| *O. niloticus* | Winter | 60 | 20 | 33.3 |
| | Spring | 60 | 40 | 66.7 |
| | Summer | 60 | 55 | 91.7 |
| | Autumn | 60 | 35 | 58.3 |
| *C. gariepinus* | Winter | 20 | 0 | 0 |
| | Spring | 20 | 11 | 55 |
| | Summer | 20 | 13 | 65 |
| | Autumn | 20 | 12 | 60 |
| *M. cephalus* | Winter | 10 | 0 | 0 |
| | Spring | 10 | 4 | 40 |
| | Summer | 10 | 6 | 60 |
| | Autumn | 10 | 3 | 30 |

**Table 4:** Bacterial isolates, number of isolates, % of isolation in relation to the total number of isolates and site of isolation in each fish species

| Fish species | Bacterial isolates | Number of isolates | % | Sites of isolation |
|---|---|---|---|---|
| *O. niloticus* | *A. hydrophila* | 2 | 8 | Kidneys, Liver, Skin lesions |
| | *A. sobria* | 1 | 4 | Kidneys |
| | *A. veronii* | 3 | 12 | Gills, Kidneys, Spleen |
| | *Ps. putida* | 1 | 4 | Kidneys |
| | *Vibrio fluvialis* | 2 | 8 | Liver, Intestine |
| | *S. liquefaciens* | 1 | 4 | Kidneys |
| | *S. marcescens* | 1 | 4 | Liver |
| | *C. freundii* | 1 | 4 | Liver |
| | *C. braakii* | 1 | 4 | Kidneys |
| | *E. cloacae* | 1 | 4 | Intestine |
| *C. gariepinus* | *A. hydrophila* | 2 | 8 | Kidneys, Skin lesions |
| | *A. sobria* | 1 | 4 | Kidneys |
| | *A. caviae* | 1 | 4 | Spleen |
| | *Ps. fluorescens* | 1 | 4 | Kidneys |
| | *Ps. aeruginosa* | 1 | 4 | Liver |
| | *S. odorifera* | 1 | 4 | Liver |
| | *C. koseri* | 1 | 4 | Kidneys |
| | *E. sakazakii* | 1 | 4 | Intestine |
| *M. cephalus* | *A. hydrophila* | 1 | 4 | Skin lesions |
| | *A. sobria* | 1 | 4 | Kidneys |
| Total | | 25 | | |

## 5. Conclusions

From the previous findings, it can be concluded that *Aeromonads* were the most prevalent bacterial species, were widely distributed in warm water aquaculture, and causing great economic losses. Additionally, *O. niloticus* was the highly susceptible fish species. Further studies related to the sequencing of the PCR product were done (unpublished data).

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