# Isolation and molecular characterization of some bacterial pathogens in El-Serw fish farm, Egypt

**Manal I. El-Barbary[1] and Ahmed M. Hal[2]**
1- Fish Disease Lab, National Institute of Oceanography and Fisheries.
2- Genetics and Genetic Engineering Lab., National Institute of Oceanography and Fisheries.
E-mail address: manal278@yahoo.com

## ABSTRACT

The current study was carried out to isolate and identify some of bacteria infecting fish of El-Serw fish farm during the harvest season from December to March 2014-2015**.** Fish samples (155) were collected from different ponds of farm; 65 Nile tilapia (*Oreochromis niloticus*), 55 catfish *(Clarias gariepinus)* and 35 mullet (*Liza ramada*) were used to isolate bacteria in fish organs (gills, liver and kidney) using different selective media, from the examined fish, bacteria belonging to *Pseudomonas*, *Aeromonas* and *Enterobacteriaceae* were isolated. The group most frequently isolated from fish was *Aeromonas*, the highest number of putative *Aeomonas* isolates were obtained from *O. niloticus* 19 (29%), followed *C. gariepinus* 12 (21.8%) and *L. ramada* 5 (14.2%). Whereas putative *Pseudomonas* sp. showed fewer prevalence 9 (13.8%), 8 (14.5%) and 4 (11.4%) from *O. niloticus*, *C. gariepinus* and *L. ramada*, respectively, while only two colonies were recovered from Yersinia Agar Base. Seven isolates were selected and identified by sequencing of 16S rRNA. The analysis of sequence of 16S rRNA gene using universal primers (RW01 and DG74) resulted in the identification of three bacterial isolates of *Pseudomonas fluorescens*, one of *Pseudomonas putida*, two of *Aeromonas hydrophila* and one of *Klebsiella oxytoca*. The antibiogram test of these isolates revealed their sensitivity to ciprofloxacin, norfloxacin, and gentamycin. This study concluded that the performance 16S rRNA assay has the prospective to create an important contribution to infection management providing rapid of identification the bacterial pathogens in fish.

**Keywords:** *Pseudomonas fluorescens*, *Pseudomonas putida*, *Aeromonas hydrophila*, *Klebsiella oxytoca*, 16S rRNA gene, Antibiotic sensitivity

## INTRODUCTION

Aquaculture is an emerging food producing sector that needs constant research with scientific, technical and innovation developments. Most infectious diseases of fish are opportunistic; this means that the simple presence of the pathogen in the environment of the fish is insufficient to cause a disease outbreak (Yanong and Francis-Floyd, 2002). Water pollution stresses farmed fish and increases susceptibility to infectious diseases, resulting in high mortalities (Sarmento *et al.*, 2004). El-Serw fish farm is located on the southwest shore of Manzala Lake near El-Serw navigation canal, about 200 km northeast Cairo, the conservation of farmed fish from pollution is a major concern and agricultural drainage water is one of the most important sources of water for the El-Serw fish farm (Al-Afify *et al.*, 2014). El-Serw fish farm suffers from poor water quality because water in aquaculture in Egypt is contaminated with the sewage disposal and agrochemical, in addition to exposure to high environmental factors leading to the outbreak of bacterial infection among fish, besides the sewage

wastes discharged from the neighboring villages along Damietta without any treatment into the Damietta branch (Abdo, 2004).

Bacteria considered as a reliable indicator of pollution (Clark and Pagel, 1977) and it plays an important role in global ecosystems which are major factors in controlling water quality and determine the fate of environmental pollution (Atlas and Bartha, 1993).

Among the etiological agents of bacterial fish diseases *Pseudomonas* and *Aeromonas* are considered the most important fish pathogens. These bacteria are responsible for ulcer type diseases including ulcerative syndrome, bacterial hemorrhagic septicemia, tail and fin rot, and ascites (Paniagua *et al.*, 1990). These bacteria have been reported to cause septicemia in *O. niloticus* in Egypt that was more prevalent during winter period (El-Sayyad *et al.*, 2010). Fish species that are affected by *A. hydrophila* include freshwater fish; tilapia, catfish and common carp (Ullal *et al.*, 2008; Abd-El-Rhman, 2009; Jeney, 2009 and Yin *et al.*, 2009) and brackish and marine water fish; Meagre, Grouper, Sea bream and Mullet (El-Barbary, 2010 a,b). Although *A. hydrophila* is commonly considered as a secondary pathogen associated with disease outbreaks, it could also become a primary pathogen, causing outbreaks in fish farms with high mortality rates, resulting in severe economic losses to the aquaculture sector worldwide (Nielsen et al., 2001; Fang et al., 2004). The symptoms of *A. hydrophila* infection include swollen tissues, ascites, necrosis, red sores, ulceration, and hemorrhagic septicemia (Karunasagar *et al.*, 1989; Azad *et al*., 2001).

Also, in Egypt, the genus *Pseudomonas* have been described as causative agents of diseases in fish, where *P. fluorescens, P. angulliseptica, P. aeruginosa* and *P. putida* were identified in various species of fish as etiological agents of *pseudomonas* septicemia (Sakar and Azza, 2008; Eissa *et al.*, 2010; EL-Nagar, 2010).

Molecular techniques have provided faster results and high-resolution insights into the structure and diversity of bacterial communities (Kim *et al.*, 2007; Wu *et al.*, 2010). Bacteria can be identified by direct sequencing of the PCR-amplified bacterial 16S rRNA followed by comparing this sequence with known sequences stored in GenBank (Clarridge, 2004). Thus, it has been widely used to measure the genetic diversity of biological samples (Baker *et al.*, 2001). This approach has played a key role in identifying thousands of novel prokaryotic microorganisms and in improving our understanding of the level of microbial complexity. 16S rRNA contains variable and highly conserved regions, PCR primers targeting the conserved regions of rRNA amplify variable sequences of the rRNA gene (Relman, 1999; Buller, 2004). A universal PCR with primers RW01 and DG74 demonstrated to be a sensitive screening method for detection of bacterial pathogens (Greisen *et al.*, 1994). 16S rRNA gene is an important tool when used alongside biochemical tests to identify microbes in the diagnostic laboratory (Buller, 2004). Phylogenetic analysis of 16S rRNA allows identification of so far unknown organisms or bacterial isolates and provides appreciated data of the biodiversity from the respective ecosystem (Olsen *et al*., 1986; Ludwig *et al*., 1998). Microorganisms can be grouped together via similarities in their genes, which reflect their evolutionary relationships (Woese, 1987).

The aim of this study was isolation and identification of some of bacteria infecting fish of El-Serw fish farm during the harvest season using 16S rRNA assay to provide accurate detection of bacterial pathogens.

## MATERIALS AND METHODS

**Fish:**

A number of 155 apparently healthy and naturally infected fishes of different species; 65 Nile tilapia (*O. niloticus*), 55 catfish *(Clarias gariepinus)* and 35 mullet (*Liza ramada*) were collected from different ponds in El-Serw fish farm along the harvest season (December to March 2014-2015). These species are very important fish economically because they have high market value and have been successfully cultivated by fish farmers. These fishes were subjected to full clinical examination and recorded any abnormal signs.

**Culture and Incubation:**

Swabs from each organ (liver, kidney and gills) were inoculated into trypticase soy broth (TSB; Oxoid) and incubated at 37°C for 24h. A loopful of the obtained broth culture was streaked on the selective media Thiosulfate-citrate- bile salt- Sucrose (TCBS) agar (Vibrio selective media), Aeromonas Medium Base (Oxoid, Ltd.), Pseudomonas Agar Base (LabM, UK) and Yersinia Agar Base (LabM, UK), followed by incubation at 27±1°C/ 24hr to isolate non *Enterobacteriaceae* bacteria. While, Yersinia selective agar media was used to isolate and detected *Enterobacteriaceae* bacteria such as *Yersinia* sp. Morphologically similar and dominant bacterial colonies were selected and streaked onto nutrient agar plates for 24 h at 27±1 °C in order to obtain pure cultures. Pure colonies were transferred to nutrient agar slants and stored at 4 °C for further identification.

**Identification of bacteria isolates:**

All purified isolates were identified by studying colony growth characteristics. Smears were prepared from the colonies and stained with Gram's stain and examined microscopically to demonstrate of morphology, arrangement and staining reaction of microorganism, also the motility of each isolate was tested. The bacteria isolates were identified according to schemes of biochemical reactions provided in Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994). After that, the selective isolates were tested for universal 16S rRNA gene with complementary tests.

**Bacterial genomic DNA purification protocol:**

**DNA isolation from cultured bacteria:**

The pure isolates were incubated overnight in tryptic soy broth at 27±1 °C, in order to isolate the DNA. Bacterial genomic DNA was extracted using Gene JET Genomic DNA Purification Kit (Thermo Scientific) according to the manufacturer's instruction. The eluted DNA was used as a template for PCR detection of universal 16S rRNA gene. Universal bacterial primers DG74 5′-AGGAGGTGATCCAACCGCA-3′ and RW01 5′-AACTGGAGGAAGGTGGGGAT-3′ were used for detection of 16S rRNA (Greisen *et al*., 1994). The locations of primers were 1521-1540 and 1170-1189, respectively.

**PCR and 16S rRNA gene sequencing**:

To identify bacteria, universal primers specific for 16S rRNA gene of eubacteria were used**.** PCR reactions were performed using 12.5 µl of DreamTaq Green PCR Master Mix (2x) (Thermo Scientific #K1082) with 2 µl of Template DNA and water along with nuclease-free to reach the final volume of 25 µl at room temperature. The amplification was performed using a thermal cycler (Biometra) with the following parameters: initial denaturation at 95°C for 5 min, followed by 40 cycles of amplification (denaturation at 95°C for 30s, primer annealing at 58°C for 30s, extension at 72°C for 30s) and a final extension step of 72°C for 10 min. After amplification, 10 µl of the PCR sample was loaded on a 1.5% agarose gel in

Trisacetate-EDTA containing ethidium bromide (0.5µg/ml). The PCR product was purified with a QIAquick PCR purification kit and directly sequenced with a 3500/3500xL Genetic Analyzer (Applied Biosystems, at Faculty of Agriculture Research Park, Faculty Agriculture, Cairo University.

**Phylogenetic analysis:**

The 16S rRNA sequence of bacteria were analyzed and compared to all bacteria rRNA sequences by BLASTN (http://www.ncbi.nlm.nih.gov/blast) to find related species. The 16S rRNA genes of 27 related species selected from the GenBank database and their accession numbers are shown in the phylogenetic tree. Phylogenetic tree was constructed using MEGA6 (Tamura *et al.*, 2013), performed using the Neighbor-Joining method (Saitou and Nei, 1987).

**Antibiotic susceptibility testing:**

The susceptibility was determined by the agar diffusion method using 6 mm diameter commercial discs (Oxoid Limited, Basing stoke, Hampshire, England) included the following antibiotics, (µg $disc^{-1}$): streptomycin 10 (S), cephazolin 30 (CZ), erythromycin 15 (E), fusidic acid 10 (FA), gentamycin 10 (GN), oxytetracyclin 30 (OTC), ciprofloxacin 5 (CIP) and sulfamethoxazole 25 (SXT), nalidixic acid 30 (NA), norfloxacin 30 (NOR). Antibiotic sensitivity was tested on Mueller-Hinton agar plates, which were inoculated with 0.1 ml of an 18-h old culture of the every test bacteria in glucose-supplemented (1%) nutrient broth. Antibiotic discs were placed on the solid medium plates and incubated at 27±1 $^{o}$C for 24 h. Zones of inhibition formed around the discs were measured and antibiotic sensitivity was assayed from length of the diameter of the zones (in mm). Zone diameters were interpreted as sensitive, intermediate and resistant according to CLSI (2010).

## RESULTS

**The clinical and postmortem findings for naturally infected fish species**:

The clinical examination of the naturally infected fish species *(O. niloticus, C. gariepinus* and *L. ramada* ) showed one or more of the following signs; hemorrhages in different areas of the body (fin base, mouth and anal region) some fish showed corneal opacity, exophthalmia, swelling of the abdomen, separated scales with ulcers of the skin and erosion of fins. In addition, gills were congested and in other cases were pale and swollen while liver, spleen, kidney and gall bladder showed congestion and enlargement.

**Bacteriological examination:**

After aseptic isolation from liver, kidney and gills of the suspected fish species and culturing on general and specific media, staining procedures and some biochemical tests it was clearly that the isolated species of bacteria from the studied fish were categorized into Gram negative bacteria. Some of the biochemical characters of the presumptive *Aeromonas* and *Pseudomonas* species were showed in Table 1. All isolates are Gram-negative rods, oxidase negative and motility. A according to schemes of biochemical reactions provided in Bergey's Manual of Systematic Bacteriology for identification, the obtained results indicate that *Aeromonas* sp. formed glucose, manitol, catalase and maltose and produced indol. While *Pseudomonas* species does not produce indol and formed glucose. *Pseudomonas* species showed variation in gelatin hydrolysis.

Table 1: Some biochemical characterization of some *Aeromonas* and *Pseudomonas* species isolated from fish.

| Characteristics | Isolates | |
|---|---|---|
| | ***Aeromonas* sp.** | ***Pseudomonas* sp.** |
| **Gram stain** | - | - |
| **Cell shape** | Short rods | Short rods |
| **motility** | + | + |
| **Colony color** | dark green | yellow-green |
| **Oxidase test** | + | + |
| **Indol production** | + | - |
| **Methyl red** | - | + |
| **Vogus-proskour** | + | - |
| **Gelatin hydrolysis** | + | V |
| **Utilizationof Glucose** | + | - |
| **Mannitol** | + | + |
| **Maltose** | + | + |
| **Catalase** | + | + |

+ Positive - Negative V Variable

## Prevalence of the isolated bacteria:

A total of 59 bacterial colonies were recovered from the 155 examined fish. Selective isolation and preliminary identification reflected that 36 resembled *Aeromonas* sp., 21 resembled *Pseudomonas* sp. and 2 resembled *Klebsilla* sp. (Table 2).

*A. hydrophila* strains were isolated from *O. niloticus C. gariepinus* and *L. ramada* with a prevalence of 29 %, 21.8 % and 14.2%, respectively. While *Pseudomonas* sp. isolated from the different organs with a prevalence of 13.8%, 14.5 % and 11.4% from *O. niloticus, C. gariepinus* and *L. ramada*, respectively. On the other hand, *Klebsiella oxytoca* was isolated only from gills of *O. niloticus* with a prevalence of 3%.

Table 2: Prevalence and distribution of bacteria among the examined fish.

| **Number of Fish species** | Name and number (%) of isolates | | | | | | | **Total of isolates (%)** | |
|---|---|---|---|---|---|---|---|---|---|
| | *Aeromonas* sp. | | *Pseudomonas* sp. | | *Klebsiella* sp. | | *Vibrio* sp. | | |
| | N | % | N | % | N | % | N | N | % |
| *O. niloticus (n=65)* | 19 | 29 | 9 | 13.8 | 2 | 3 | 0 | 30 | 46.1 |
| *C. gariepinus* (n=55) | 12 | 21.8 | 8 | 14.5 | - | - | | 20 | 36.3 |
| *L. ramada* (n=35) | 5 | 14.2 | 4 | 11.4 | - | - | 0 | 9 | 25.7 |

N= number of isolates; %= Percentage of isolates calculated to the number of examined fish

## Analysis of 16S rRNA gene:

Seven isolates were selected to identify by 16S rRNA (1-7) and sequenced by 3500 Genetic Analyzer (Applied Biosystems) and were compared to those available in the GenBank database (Table 3 and Figs. 1). DG74 and RW01 primers were used to amplify of 16S rRNA of bacterial isolates, with amplicon size ~370 bp (Fig. 1).

Compared with GenBank database, the nucleotide sequences of 16S rRNA gene could detect the isolates of bacteria in the level species, according to levels of homology of nucleotide ranging from 98–100%.

Table 3: 16S rRNA based identification of isolates.

| No. of isolate | Isolates | Fish species |
|---|---|---|
| 1 | *A. hydrophila* | *O. niloticus* |
| 2 | *A. hydrophila* | *C. gariepinus* |
| 3 | *P. fluorescens* | *O. niloticus* |
| 4 | *P. fluorescens* | *C. gariepinus* |
| 5 | *P. fluorescens* | *L. ramada* |
| 6 | *P. putida* | *L. ramada* |
| 7 | *Klebsiella oxytoca* | *O. niloticus* |

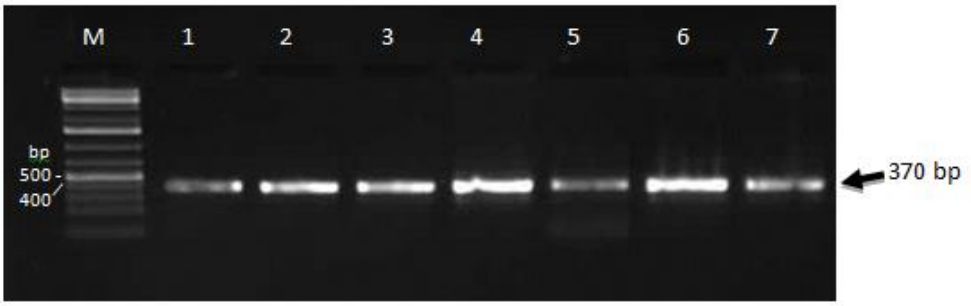


Fig. 1: Agarose (1.5%) gel electrophoresis analysis of the PCR products from 16S rRNA gene of bacteria with universal primers (DG74 and RW01). Lane 1-7: Bacterial isolates.

The seven bacterial species belonged to three families *Pseudomonadaceae*, *Aeromonadaceae* and *Enterobacteriaceae*. The closer relationship among sequences of 16S rRNA was grouped to each other in the phylogenetic tree, where phylotypes were distributed in the branches (Fig. 2). The cluster of the genera *Pseudomonas* harbored four bacterial isolates; three bacterial isolates were clustered to, the type strain of *Pseudomonas fluorescens* and showed 99% of homology with *Pseudomonas fluorescens* in GenBank (accession numbers KX527632, KX817232 and KF770987) however, one of bacterial isolate was grouped with *Pseudomonas putida* and was also closely related to *Pseudomonas putida* (GenBank accession no. KX817235). On the other hand, phylogenetic lineages in the branches of the genera of *Aeromonas* had two bacterial isolates which they were *Aeromonas hydrophila* and had an average nucleotide identity percentage, 98.5%, with *Aeromonas hydrophila* (GenBank acc. nos. KJ781365, KU711812 and KU872254). The last bacterial isolate was clustered to *Klebsiella oxytoca* strains, showed 100% of similarity with *K. oxytoca* in GenBank (acc nos. KX911722 and HQ219863) and *Klebsiella* sp. (acc. no. KX500307). All of bacterial isolates were identified using 16S rRNA and were confirmed by biochemical test.

**Antibiotic sensitivity:**

The sensitivity of bacterial strains that have been isolated from different fish species was included in Table (4). Results showed that the antibiotics resistance was common in all isolates except *K. oxytoca.* However, all bacterial isolates were found to be sensitive to at least 2 antibiotics (NOR, CIP and GN) and showed various resistance for OTC, S and STX.

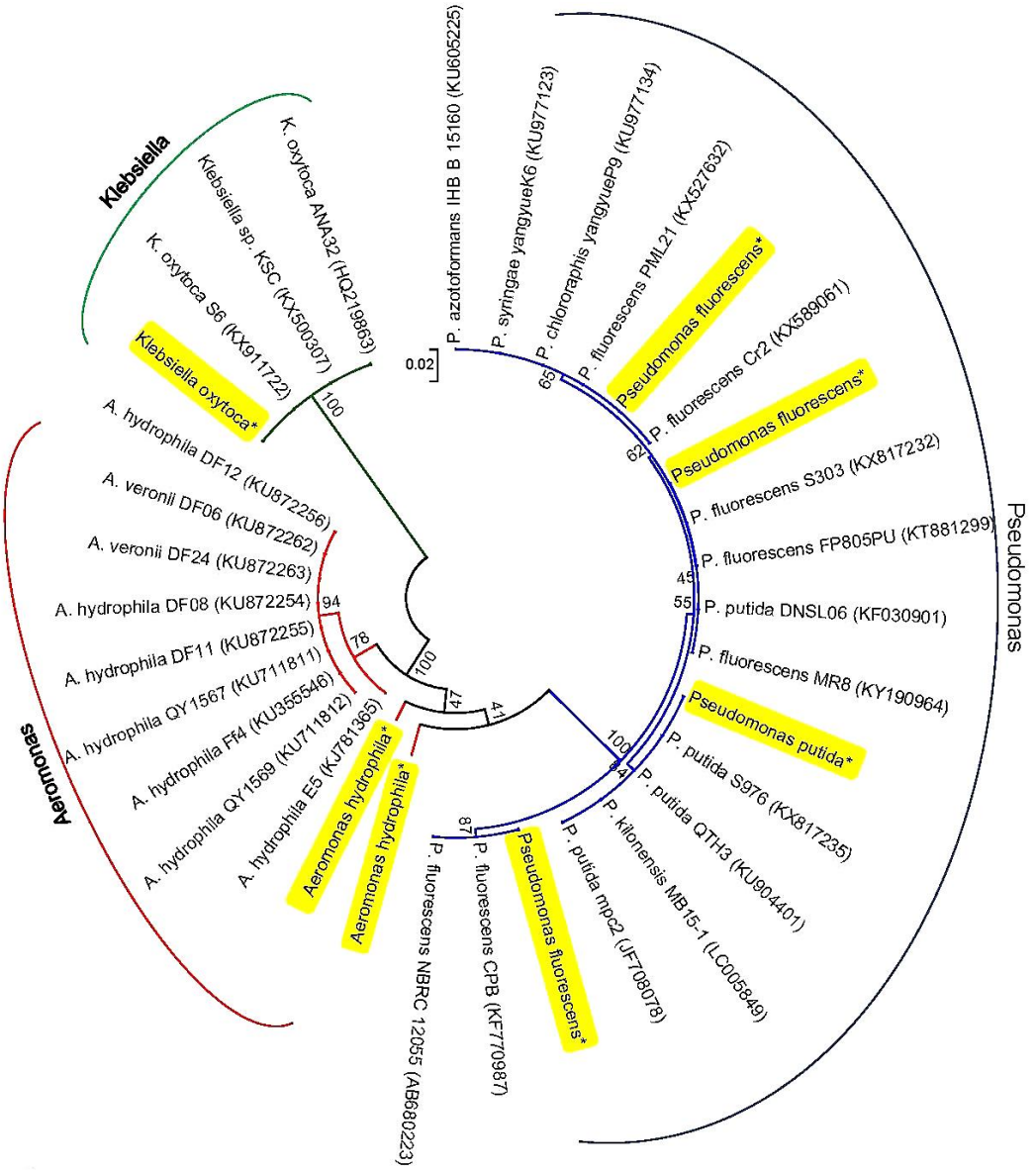


Fig. 2: The phylogenetic relationships, based on 16S rRNA gene sequences, were constructed by the neighbor-joining method. Numerals at nodes indicate bootstrap percentages derived from 1,000 replications. The bacterial isolates from this study designated with an asterisk and highlight. Accession numbers of 16S rRNA genes are shown in brackets.

Table 4: Antibiotic sensitivities of identified isolates.

| Isolates | **Antibiotic tested** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **NOR** | **NA** | **CIP** | **CZ** | **E** | **FA** | **GN** | **OTC** | **S** | **STX** |
| ***A. hydrophila* 1** | S | R | S | R | R | R | S | R | R | I |
| ***A. hydrophila* 2** | S | I | S | R | I | I | S | S | R | S |
| ***P. fluorescens* 3** | S | R | S | I | R | R | S | S | S | R |
| ***P. fluorescens* 4** | S | R | S | S | R | R | I | R | S | I |
| ***P. fluorescens* 5** | S | R | I | R | R | R | S | I | I | I |
| ***P. putida* 6** | S | R | S | R | R | R | I | I | S | I |
| ***K. oxytoca* 7** | S | I | S | I | I | R | S | S | S | S |

NOR= Norfloxacin, NA= Nalidixic Acid, CIP = Cirprofloxacin, CZ = Cephazolin, E= Erythromycin, FA= Fusidic acid, GN= Gentamycin, OTC= Oxytetracyclin, S= Streptomycin, SXT= Sulfamethoxazole. R= Resistant, S = Sensitive, I = Intermediate.

## DISCUSSION

The results in the Table (1) showed that, all *Pseudomonas* and *Aeromonas* species are characterized by Gram-negative motile rods with cytochrome oxidase and those findings agree with Altinok *et al.* (2006); El-Barbary (2010a,b); Nathan *et al.* (2011) whose reported that *Pseudomonas* species are Gram negative, oxidase positive, rod shaped bacteria that exist in the environment. *P. fluorescens* showed variation in some phenotypic tests such as gelatin hydrolysis where it has ability to hydrolyze gelatin in contrast to *P. putida* (Krieg and Holt, 1984).

Data in Table (2) showed that *A. hydrophila* was the most prevalent in the studied fish. This might be attributed to the harvest season, winter season, where Moustafa *et al.* (2010) reported the highest prevalence of *A. hydrophila* in winter season. Similarly, Pathak *et al.* (1988) recorded that the highest isolation rates of *A. hydrophila* occurred during late winter followed by a gradual decline in intensity during the summer and monsoon seasons. Popovic *et al.* (2000) mentioned that there was clear seasonality in the prevalence of *A. hydrophila* where there were no isolates recovered in the summer months. This could be attributed to the suppressed immunity to cultured fish caused by cold weather and low water temperature, which most warm water fish couldn't tolerate especially Tilapia spp. rendering fishes more vulnerable to different pathogens (Abdel-Hadi *et al.*, 2008). In this study, the clinical and postmortem findings for naturally infected fishes showed hemorrhages, detachment of scales and ulcer on the skin, abdominal distention and fin rot. Similar lesions as hemorrhages over all the fish body especially at the base of fins, tail and fins rot, detachment of scales and skin ulceration and abdominal distention of naturally infected fishes by some of *Aeromonas* sp. and *Pseudomonas* sp. were recorded by Okaeme (1989); Karunasagar *et al.* (2001); Eissa *et al.* (2010); Hanna *et al.* (2014).

*Klebsiella oxytoca* was isolated only from gills of *O. niloticus* with a prevalence of 3%, it is an indicator for sewage pollution, where Rajasekaran (2008) reported that *Enterobacteraceae*, as *Klebsiella oxytoca*, are considered as an indicator for sewage pollution and has been reported as opportunistic pathogen in fish. Pathogenic bacteria can be detected or diagnosed by using a variety of test methods, include growth on selective media followed by biochemical test, in addition to nucleic acid detection by polymerase chain reaction (PCR). The present study used PCR technique targeting 16S rRNA to detect bacterial isolates from infected fish through universal primers (RW01 and DG74) which has been reported as a sensitive screening method for detecting bacterial communities (Greisen *et al.*, 1994; Teng *et al.*, 2004).

The analysis of 16S rRNA gene gave a quick and accurate identification of bacteria (Genmoto *et al.*, 1996; Kim and Jeong, 2001; Tringe and Hugenholtz, 2008; Tang *et al.*, 2011). Many sets of primers have been designed to amplify different regions of 16S rDNA and have been shown to have different sensitivities and specificities (Greisen *et al.*, 1994). Moreover, phylogenetic analysis placed the bacterial isolates in three genera *Pseudomonas*, *Aeromonas* and *Klebsiella* based on homology, where bacteria with < 98.7% of similarity of 16S rRNA gene sequence considered to be different species (Schleifer, 2009). It is worth mentioning that, weak evidence of identification of *A. hydrophila*, in this study, was due to amplification of short sequence of 16S rRNA gene. This observation was faced Jenkins *et al.* (2012) through analysis of large sequence of 16S rRNA gene that improved species discrimination than short sequence. The results support the sensitivity of 16S rRNA

approach could be affected by the size of the 16S rRNA gene fragment amplified. However, the specific media and biochemical test, confirmed the identification with 16S rRNA. The current and previous studies agreed with detection of different variable regions of the 16S rRNA sequences could be a useful method to identify or use different techniques to confirm the identification of bacterial strains. The results of antimicrobial tests in the current study revealed that all tested strains were sensitive or intermediate to ciprofloxacin and norfloxacin and gentamycin, while other antibiotics that have been tested cannot be used to treat fish infected with these bacteria. Some of these results of antibiogram sensitivity are in agreement with those of Eissa *et al*. (2010) who reported that *P. anguilliseptica*, *P. putida* and *P. aureginosa* revealed that almost all of them were sensitive to Gentamicin, Erythromycin Novobiocin and Sulfa-trimethoprime. Also, Altinok *et al.* (2006) reported that *P. putida* has intrinsically high resistance to Chloramphenicol, Ampicillin, Erythromycin, Tetracycline, Rifampicin, Naladixic acid and Streptomycin. In another study, Darak and Barde (2015) reported that *P. fluorescens* was very sensitive to Kanamycin, Nalidixic acid, Gentamicin, Neomycin and less sensitive to Amikacin and Tetracycline, and Chlorophenicol. While El-Barbary (2010a), found that the isolates of *A. hydrophila* were sensitive to Oxytetracyclin, and showed varying resistance to Cephazolin, Erythromycin, Gentamicin and Sylfamethoxazole.

## CONCLUSIONS

The results of this study concluded that, some of fish pathogenic bacteria are related to different taxonomic groups, have been isolated from different fish species, and identified by 16S rRNA gene. The analysis of sequence of 16S rRNA gene using universal primers (RW01 and DG74) succeeded in detecting four different bacterial isolates namely *P. fluorescens, P. putida, A. hydrophila* and *Klebsiella oxytoca* and showed that the performance of 16S rRNA assay has the potential to create an important contribution to infection management by identification the bacterial pathogens in fish. In addition to, the antibiogram test showed that ciprofloxacin, norfloxacin and gentamycin could be used to treat fish infected with these strains.

## REFERENCES

Abdel-Hadi, Y.M.; El-Bouhy, Z.M. and Diab, A.S. (2008). Prevalence of some bacterial agents affecting the gills of some cultured fishes in Egypt. Proceeding of the 30th Malaysian Symposium on Microbiology, August 16-19, 2008, Kuantan, Malaysia.

Abd-El-Rhman, A.M. (2009). Antagonism of *Aeromonas hydrophila* by propolis and its effect on the performance of Nile tilapia, *Oreochromis niloticus*. Fish Shellfish Immunol., 27:454-9.

Abdo, M.H. (2004). Seasonal variations of some heavy metals in macrophytes and water of Damietta branch, River Nile, Egypt. Egypt J Aquat Biol Fish, 8:195–211

Al-Afify, F.D.; Osman, M.E. and Elnady, M. (2014). Ecological Studies on El-Serw Fish Farm, Egypt: Drainage Water Quality, Sediment Analysis and Heavy Metals Pollution in Nile Delta. Farms Paperback– LAP LAMBERT Academic Publishing (2014-06-16). 200 pp.

Altinok, I.; Kayis, S. and Capkin, E. (2006). *Pseudomonas putida* infection in rainbow trout. Aquaculture, 261: 850-855.

Atlas, R.M. and Bartha, R. (1993). Microbial ecology: Fundamentals and Applications, 3rd Edition, Redwood City, Benjamin.

Azad, I.S.; Rajendran, K.V.; Rajan, J.J.S.; Vijayan, K.K. and Santiago, T.C. (2001). Virulence and histopathology of *Aeromonas hydrophila* (Sah 93) in experimentally infected tilapia, *Oreochromis mossambicus* (L.). J. Aquac. Trop., 6: 265- 75.

Baker, G.C.; Gaffar, S.; Cowan, D.A. and Suharto, A.R. (2001). Bacterial community analysis of Indonesian hot springs. FEMS Microbiol. Lett., 200: 103-109.

Buller, N.B. (2004). Bacteria from Fish and Other Aquatic Animals. A Practical Identification Manual. Cabi Publishing, UK.

Clark, I.A. and Pagel, J.E. (1977). Pollution Indicator Bacteria associated with Municipal Raw and Drinking Water Supplies. Can. J. Microbiol., 28: 465-470.

Clarridge, J.E., III (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev., 17: 840–862.

CLSI, (2010). Performance Standards for Antimicrobial Susceptibility Testing, 20th Informational Supplement. Clinical and Laboratory Standards Institute, M100-S20 & M100-S-20-U.

Darak, O. and Barde, R.D. (2015). *Pseudomonas fluorescens* associated with bacteria disease in *Catla catla* in Marathwada region Maharashtra. Int J Adv Biotechnol Res., 6: 189-195

Eissa, N.M.E.; Abou El-Ghiet, E.N.; Shaheen, A. and Abbass, A. (2010). Characterization of *Pseudomonas* Species Isolated from Tilapia "*Oreochromis niloticus*" in Qaroun and Wadi-El-Rayan Lakes, Egypt. Glob. Vet., 5: 116-121.

EL-Barbary, M.I. (2010a). Some clinical microbiological and molecular characteristics of *Aeromonas hydrophila* isolated from various naturally infected fishes. Aquac Int., 18:943–954.

EL-Barbary, M.I. (2010b). Pathogenic characteristics and molecular identification of *Aeromonas hydrophila* isolated from some naturally infected cultured fishes. Egyptian Journal of Aquatic Research, 36(2): 345-356.

El-Nagar, R.M.A. (2010). Bacteriological studies on *pseudomonas* microorganisms in cultured. MSc. thesis, Fac. Vet. Med., Zag. University.

El-Sayyad, H.I.; Zaki, V.H.; El-Shebly, A.M. and El-Badry, D.A. (2010). Studies on the effects of bacterial diseases on skin and gill structure of *Clarias gariepinus* in Dakahlia Provinence, Egypt. Annal. Biol. Res., 4 (1): 106-118.

Fang, H.M.; Ge, R. and Sin, Y.M. (2004). Cloning, characterisation and expression of *Aeromonas hydrophila* major adhesin. Fish Shellfish Immunol., 16:645-58.

Genmoto, K.; Nishizawa, T.; Nakai, T. and Muroga, K. (1996). 16S rRNA targeted RT-PCR for the detection of *Vibrio penaeicida*, the pathogen of cultured kuruma prawn *Penaeus japonicus*. Dis Aquat Organ., 24: 185-189

Greisen, K.; Loeffelholz, M.; Purohit, A. and Leong, D. (1994). PCR primers and probes for the 16S ribosomal RNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J. Clin. Microbiol., 32(2):335-351.

Hanna, M.I.; El-Hady, M.A.; Ahmed, H.A.; Elmeadawy, S.A. and Kenwy, A.M. (2014). A contribution on *Pseudomonas aeruginosa* infection in African Catfish (*Clarias gariepinus*). Res J Pharm Biol Chem Sci., 5: 575-588.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. (1994). Bergey's Manual of Determinative Bacteriology, 9th edn. Baltimore: Williams & Wilkins

Jeney, Z., Rácz, T., Thompson, K.D.;Poobalane, S.;Ardó, L.; Adams, A. et al. (2009). Differences in the antibody response and survival of genetically different varieties of common carp (*Cyprinuscarpio* L.) vaccinated with a commercial *Aeromonas salmonicida* /*A. hydrophila* vaccine and challenged with *A. hydrophila*. Fish Physiol.Biochem., 35: 677-82.

Jenkins, C., Ling, C.L., Ciesielczuk, H.L., Lockwood, J., Hopkins, S., Mc Hugh, T. D., Gillespie, S.H., and Kibbler, C.C. (2012). Detection and identification of bacteria in clinical samples by 16S rRNA gene sequencing: comparison of two different approaches in clinical practice. J Med Microbiol., 61: 483–488.

Karunasagar, I.; Rosalind, G.M.; Karunasagar, I. and Gopal Rao, K. (1989). *Aeromonas hydrophila* septicaemia of Indian major carps in some commercial fish farms of West Godavari District, Andhra Pradesh. Curr. Sci., 58:1044-5.

Kim, D.H.; Brunt, J. and Austin, B. (2007). Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). J Appl Microbiol., 102: 1654- 1664.

Kim, M.S. and Jeong, H.D. (2001). Development of 16S rRNA targeted PCR methods for the detection and differentiation of *Vibrio* v*ulnificus* in marine environments. Aquacul., 193: 199–211.

Krieg, N.R. and Holt, J.G. (1984). Bergey's Manual of Systematic Bacteriology, vol. 1.Williams and Wilkins, Baltimore, MD, p. 964

Ludwig, W.; Strunk, O.; Klugbauer, S.; Klugbauer, N.; Weizenegger, M.; Neumaier, J.; Bachleitner, M. and Schleifer, K.H. (1998). Bacterial phylogeny based on comparative sequence analysis. Electrophoresis, 19: 554-568

Moustafa, M.; Laila, A.M.; Mahmoud, M. A.; Soliman, W.S. and El-gendy, M. Y. (2010). Bacterial Infections Affecting Marine Fishes in Egypt. Journal of American Science, (6): 603 – 612.

Nathan, P.; Rathinam, X.; Kasi, M.; Rahman, Z.A. and Subrama-niam, S. (2011). A pilot study on the isolation and biochemical characterization of *Pseudomonas* from chemical intensive rice ecosystem. Afr J Biotechnol., 10: 12653-12656.

Nielsen, M.E.; Hoi, L.; Schmidt, A.S.; Qian, D.; Shimata, T.; Shen, J.Y. and Larsen, J.L. (2001). Is *Aeromonas hydrophila* the dominant motile *Aeromonas* species that cause disease outbreaks in aquaculture production in the Zhejiang Province of China?. Dis. Aquat. Organ., 22: 23–29.

Okaeme, A.N. (1989). Bacteria associated with mortality in tilapias, *Heterobranchus bidorsalis*, and *Clarias lazera* in indoor hatcheries and outdoor ponds. J Aquacult Trop., 4: 143-146.

Olsen, G.J.; Lane, E.J.; Giovannoni, S.J.; Pace, N.R. and Stahl, D.A. (1986). Microbial ecology and evolution: a ribosomal RNA approach. Annual Review of Microbiology, 40: 331-355.

Paniagua, C.; Rivero, O.; Anguita, J. and Naharro, G. (1990). Pathogenicity Factors and Virulence for Rainbow Trout (*Salmo gairdneri*) of Motile *Aeromonas* spp. Isolated from a River. J Clin Microbiol., 28(2):350-355.

Pathak, S.P.; Bhattacherjee, J. W.; Kalra, N. and Chandra, S. (1988). Seasonal distribution of *A. hydrophila* in river water and isolation from river fish. J. Appl. Bacteriol., 65:347-52.

Popovic, T. N.; Teskeredzic, E.; Perovic, I. S. & Rakovac, R. C. (2000): *A. hydrophila* isolated from wild fresh water fish in Croatia.Vet. Rsearch communic., 24: 371-377.

Rajasekaran, P. (2008). *Enterobacteriaceae* group of organisms in sewage-fed fishes. Advanced Biotech, 8, 12-14

Relman, D. A. (1999). The search for unrecognized pathogens. Science. 284:1308–1310.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol., 4:406-425.

Sakar, S.F.M. and Azza, M.M.A. (2008). Contribution on *Pseudomona* septicemia caused by *Ps. anguilliseptica* in cultured *Oreochromis niloticus*. Fish Diseases Dept., Central lab for Aquaculture Research, Abbassa, Agriculture Research Center, Egypt, 1177-1197.

Sarmento, A.; Guilhermino, L. and Afonso, A. (2004). Mercury chloride effects on the function and cellular integrity of sea bass (*Dicentrarchus labrax*) head kidney macrophages. Fish Shellfish Immunol., 17(5):489- 98.

Schleifer, K.H. (2009). Classification of Bacteria and Archaea: Past, present and future. Syst. Appl. Microbiol., 32; 533-542.

Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A. and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol., 30: 2725-2729.

Tang, J.; Zheng, A.; Bromfield, E.S.P.; Zhu, J.; Li, S.; Wang, S.; Deng, Q., and Li, P. (2011). 16S rRNA gene sequence analysis of halophilic and halotolerant bacteria isolated from a hypersaline pond in Sichuan, China. Ann Microbiol., 61:375–381.

Teng, L.; Hsueh,P.; Huang, Y. and Tsai, J. (2004). Identification of *Bacteroides thetaiotaomicron* on the Basis of an Unexpected Specific Amplicon of Universal 16S Ribosomal DNA PCR. J. Clin. Microbiol., 42: 1727-1730.

Tringe, S.G., and Hugenholtz, P. (2008). A renaissance for the pioneering 16S rRNA gene. Curr Opin Microbiol., 11:442–6.

Ullal, A.J.; Litaker, R.W. and Noga, E.J. (2008). Antimicrobial peptides derived from hemoglobin are expressed in epithelium of channel catfish (*Ictalurus punctatus*, Rafinesque). Dev. Comp.Immunol., 32:1301-12.

Woese, C.R. (1987). Bacterial evolution. Microbiol. Rev., 51: 221-71.

Wu, S.; Gao, T.; Zheng, Y.; Wang, W.; Cheng, Y., et al. (2010) Microbial diversity of intestinal contents and mucus in yellow catfish (*Pelteobagrus fulvidraco*). Aquacul., 303: 1-7.

Yanong, R.P.E. and Floyd, R.F. (2002). Streptococcal infections of fish. Florida Cooperative Extension Service. IFAS, University of Florida, pp. 1-5. Circular, FA0 57.

Yin, G.; Ardó, L.; Thompson, K.D.; Adams, A.; Jeney, Z. and Jeney, G. (2009). Chinese herbs (*Astragalus radix* and *Ganoder malucidum*) enhance immune response of carp, *Cyprinus carpio*, and protection against *Aeromonas hydrophila*. Fish Shellfish Immunol., 26:140-5.

## ARABIC SUMMARY

### العزل والتوصيف الجزيئي لمسببات الامراض البكتيريه في مزرعه السرو السمكيه، مصر

**منال إبراهيم البربرى[1]،أحمد محمد حال[2]**
1- معمل أمراض الأسماك، المعهد القومى لعلوم البحار والمصايد، مصر
2- معمل الوراثه والهندسه الوراثيه ، المعهد القومى لعلوم البحار والمصايد، مصر

تم تقييم الحاله البكتريولوجية لأسماك مزرعه السرو خلال فتره حصاد الأسماك (ديسمبر – مارس 2014-2015). تم تجميع 155 عينه من أسماك البلطي النيلي والقرموط وسمك الطوبار وتم فحص خياشيم وكلي وكبد هذه الأسماك بكتيريولوجيا بإستخدام بيئات بكتيريه متخصصه وتم عزل بكتيريا أجناس الايروموناس والسيدوموناس والكليبسيلا .وكانت عزلات الايروموناس هي الأكثر إنتشارا في أعضاء الأسماك المدروسه حيث سجلت نسب 19%, 21.8% و14.2% في أسماك البلطي والقرموط والطوبار من مجموع العزلات الكلي علي التوالي. بينما كان تواجد عزلات السيدوموناس في الأسماك السابقه 13.8% و 14.5% و11.4% علي التوالي. تم عزل عدد 2 مستعمره فقط علي البيئه المتخصصه لميكروب اليرسينيا ولم يتم عزل أي مستعمرات بكتيريه علي البيئه المتخصصه لميكروب الفيبريو من أي من الأنواع السمكيه. ولتأكيد التعريف بالطرق الحديثه تم تعريف هذه العزلات لمستوي الأنواع بإستخدام تحليل تتابع جين 16S rRNA لضمان دقه التعريف. وتم إختيار 7 عزلات من الأجناس المعزوله وتم تحديد 3 عزلات من ميكروب السيدوموناس فلورسينس وعزله من نوع سيدوموناس بيوتيدا وعزلتين من نوع الايروموناس هيدروفيلا وهي ميكروبات سالبه لجرام وممرضه للأسماك وتسبب خسائر إقتصاديه كبيره .وعزله من نوع كليبسيلا اوكسيتوكا وهي من ميكروبات القولون ودليل علي تلوث المياه المستخدمه في الإستزراع السمكي بالصرف الصحي وتبين هذه الدراسة ان تحليل 16S rRNA يمكنه تقديم مساهمة هامة في السيطره علي العدوى البكتيريه عن طريق تحديد مسببات الأمراض البكتيرية في الأسماك. كما تم عمل إختبار لدراسه مدى حساسيه الأنواع البكتيريه المعزوله للمضادات الحيويه وأظهر الإختبار حساسيه كبيره للعزلات لكل من المضاد الحيوي نورفلوكساسين وسيبروفلوكساسين وجينتاميسين .