Assessment of Some Bacterial Pathogens in Cultured Nile Tilapia in Fayoum Province, Egypt

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Abstract: Fish diseases are the result of interaction between host, pathogen and environmental conditions. The need to better understand the contribution of each element on development of fish diseases has always been a priority to improve the fish aquaculture practices. In this study we evaluated the water parameters as well as bacterial fish pathogens and the possible relationships between them in five different aquaculture locations in Fayoum Governorate, Egypt. Fish specimens were collected and examined for bacterial infections and its histopathological alterations, water samples were analyzed for chemical water parameters as well as levels of heavy metals pollution. The results showed high prevalence of pathogenic bacteria among the five aquaculture sites with positive linear correlation between pollution levels and percentage of infection prevalence with correlation coefficient r (Fe = 0.74, Zn =0.63, Ni = 0.58). Aeromonas hydrophila was the most prevalent species which showed resistance to commonly used antibiotics Oxytetracyclin and Amoxicillin, however less commonly used antimicrobials and antibiotics (trimethoprim–sulfamethoxazole, Ciprofloxacin, Kanamycin, novobiocin, and chloramphenicol) proved to be effective. PCR detection of virulence factors showed the presence of Act, Ast,Alt and Ela virulence genes in the isolated strains.

Key words: Fish diseases • Heavy metals • Water pollution • Aquaculture

INTRODUCTION

Nile tilapia is the most cultured fish in Egypt [1]. Egypt achieved advanced global rank in tilapia production in recent years [2]. With earthen pond semi-intensive culture system being the most implemented culturing system in use, and the use of agricultural drainage in the aquaculture water input makes the quality of water arise to form a real concern in the advancement of fish production in Egypt [3]. Agricultural drainage can be a source of many toxic pollutants that negatively affect the health of cultured fishes. Some heavy metal pollutants as cadmium, lead, manganese, mercury and zinc can be attributed to agricultural activities [4].

Fish diseases has always been perceived as a result of interaction of three main factors; host, pathogen and environment. Having it affecting both pathogen load and host immunity; the environmental conditions has always been viewed as the principal determining factor in the development of disease [5]. However the bacterial load and host immunity and health state play a principal role in the prevention or advancement of the disease.

Bacterial diseases are considered as one of the most important problems facing the aquaculture industry; it has been estimated that 10% of fish loss in aquaculture is due to disease and more than 50% of these losses are due to bacterial agents [6]. For those bacterial diseases, the presence of specific bacterial agents is essential for the infection to happen. Many of fish bacterial pathogenic agents can present in the digestive tract of apparently healthy fish or even naturally commensals in the ambient environment like the Aeromonas species; but their ability to cause a disease outbreak is always dependant on the weakening of the host organism and/or an increase in the organism virulence.
The aim of this work is to assess the levels of heavy metals pollution and bacterial pathogens in Fayoum Governorate’s tilapia cultures as well as assessment of their role in the development of fish diseases as one of the most devastating problems facing aquaculture industry in Egypt.

MATERIAL AND METHODS

Fish Sampling and Bacterial Identification: A total of 250 live Nile tilapia fish (Oreochromis niloticus) specimens were randomly collected from five different private aquaculture farms (50 fish from each farm) from Fayoum Governorate. Samples for bacterial isolation were taken from kidney, liver, and gills and cultured on Tryptic soy broth (TSB), tryptic soy agar medium (TSA), Aeromonas agar media with ampicillin supplement, and pseudomonas agar media with supplement. The inoculated plates were incubated at 25°C for up to 48 h. The purified colonies were phenotypically characterized using commercial API20NE kit (BioMerieux, France) according to the manufacturer’s instructions.

Water Samples: Water samples were collected in a sterile colourless 500 ml screw covered plastic bottles from fish farms under investigation (one sample from each aquaculture site) at the same time of fish collection at depth 20-30 cm below the water surface. Collected samples were stored in ice box containing crushed ice till transferred to the laboratory for physico-chemical analysis.

Histopathological Examination: Specimens for histopathological processing were freshly taken from organs and tissues of infected fishes. They were trimmed and fixed in 10% phosphate buffered formalin, then prepared and stained according to the protocol described by Roberts [7].

Sensitivity Test (Antibiogram): The antimicrobial susceptibility of the A. hydrophila isolates was determined by the disc diffusion method on Mueller Hinton Agar as described by the Clinical and Laboratory Standards Institutes [8]. The antibiotic discs used in this study were purchased from (Sigma, USA). The following antibiotics were tested: amoxicillin, oxytetracycline, trimethoprim–sulfamethoxazole, Ciprofloxacin, Kanamycin, novobiocin, and chloramphenicol.

Detection of Virulence Genes: Aeromonas isolates from different localities were subjected to PCR assays to detect the presence of six virulence genes; cytotoxic heat-labile enterotoxin harb (Act), cytotoxic heat-stable enterotoxins (Ast), Lipase (Lip), elastase (Ela), cytotoxic heat-labile (Alt) and flagella A and flagella B (Fla) using the same primers sequences and PCR conditions described by Sen and Rodgers [9].

Statistical Analysis: Bivariate Pearson Correlation Statistical analysis is performed using PS AW V. 18. The analysis is carried on between the prevalence levels and heavy metal parameters in different aquaculture location.

RESULTS

Bacterial Isolation and Identification: Bacterial isolation from sampled fishes showed high prevalence of bacterial infection among the five aquaculture sites, Aeromonas species were the highest prevalent bacterial isolates in all fish farms with 18.8% for A. hydrophila and 13.2% infection with A. sobria. Other isolated bacteria where Pseudomonas fluorensce, P. putida, Flavobacterium sp. and Staphylococcus aureus with prevalence ratio of 10%, 14%, 8.4% and 2.4% respectively, Table (1).

The clinical examination of infected fishes, revealed many external pathological lesions including: irregular haemorrhages distributed over many parts of the body, on the ventral aspect of the body, around the vent, on the mouth region, operculum and at the base of the fins especially pectoral, dorsal and anal fins. Some fishes showed eye lesions including opacity and exophthalmia, skin lesions were apparent in many fishes including scale detachment and darkening of the skin.

Necropsy findings of the dissected diseased fishes showed reddish serous fluid in the abdominal cavity. Examination of the hepatopancreas showed alternation between prominent paleness in some fishes and severe congestion, haemorrhages with irregular patches of paleness and necrotic nodules throughout the surface in others. Spleen showed great variation ranging from normal to highly enlarged and congested in examined fishes. Posterior kidney was congested and slightly enlarged. The intestine was inflamed and congested, some cases showed serous haemorrhagic fluid in the abdominal cavity.
Table 1: Total prevalence of bacterial infections in examined fishes divided per causative agent bacteria

<table>
<thead>
<tr>
<th></th>
<th>A. hydrophila</th>
<th>A. sobria</th>
<th>P. fluorescene</th>
<th>P. putida</th>
<th>F. columnare</th>
<th>Staph. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of exam fish</td>
<td>No. of inf fish</td>
<td>% No. inf</td>
<td>% No. inf</td>
<td>% No. inf</td>
<td>% No. inf</td>
</tr>
<tr>
<td>Aquaculture 1</td>
<td>50</td>
<td>30</td>
<td>34</td>
<td>17</td>
<td>22</td>
<td>11</td>
</tr>
<tr>
<td>Aquaculture 2</td>
<td>50</td>
<td>29</td>
<td>26</td>
<td>13</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>Aquaculture 3</td>
<td>50</td>
<td>41</td>
<td>12</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aquaculture 4</td>
<td>50</td>
<td>50</td>
<td>8</td>
<td>4</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Aquaculture 5</td>
<td>50</td>
<td>38</td>
<td>14</td>
<td>7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>250</td>
<td>188</td>
<td>18.8</td>
<td>47</td>
<td>13.2</td>
<td>33</td>
</tr>
</tbody>
</table>

Percentage was calculated according to the total number of sampled fish.

Fig. 1: Infected *Oreochromis niloticus* showing unilateral exophthalmia and eye opacity.

Fig. 2: Infected *O. niloticus* showing marked abdominal distension, darkening of the body, haemorrhagic spots with skin erosions.

Fig. 3: Infected *O. niloticus* showing severe inflammation with haemorrhagic spots distributed all over the body surface.

Fig. 4: Dissection of Infected *O. niloticus* showing severe inflammation and congestion of intestine and haemorrhage in abdominal cavity.

**Histopathological Picture of Infected Fishes:** Many degenerative changes were observed in tissues of sampled fishes. The hepatopancreas showed severe congestion in the hepatic blood vessels accompanied with severe degenerative and necrotic changes in the hepatic cells. Posterior kidney showed prominent necrotic degenerations in both glomerular and interstitial tissues. The latter were infiltrated most probably by oedematous fluid in which many necrotic cells were distributed (plate 1,a).

The columnar epithelium of the intestinal villi and crypts undergone hyperplasia and showed increased secretory activity with vacuolated clear cytoplasm and were infiltrated by inflammatory cells (Plate 1,b).

The spleen showed clusters of melanomacrophage centres consisting of polyhedral large cells (melanomacrophage cells) appeared brownish or dark brown in colour. These were increased both in number and extension in the splenic parenchyma especially in areas showing features of necrosis and degeneration (Plate 1,c). Depletion of lymphocytic elements, and the presence of necrotic and fragmented cells, vascular changes and oedema were also recorded (Plate 1,d).
Plate 1: a: Posterior kidney of *O. niloticus* showed necrosis in both the glomerular and interstitial tissue. Infiltration of oedematous fluid in-between the interstitial haemopoietic renal tissue (H&E, X400).

b: Intestine of *O. niloticus* showed hyperplasia in the columnar epithelium of the intestinal villi and crypts, increased secretory activity with vacuolated clear cytoplasm and infiltrated by inflammatory cells (H&E, X400).

c: Spleen of *O. niloticus* showed increase in the number and extension of melanomacrophage centers in the splenic parynychyma near by areas showing features of necrosis and degeneration (H&E, X400).

d: Spleen of *O. niloticus* showed depletion of lymphocytic elements, presence of necrotic and fragmented cells, vascular changes and oedema (H&E, X400).

Table 2: Heavy metals levels in 5 sampled fish farm locations in Fayoum Governorate

<table>
<thead>
<tr>
<th>Farm</th>
<th>Farm 2</th>
<th>Farm 3</th>
<th>Farm 4</th>
<th>Farm 5</th>
<th>Permissible limits*</th>
<th>Correlation Coefficient to Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>0.41</td>
<td>0.78</td>
<td>2.1</td>
<td>1.8</td>
<td>0.53</td>
<td>1000</td>
</tr>
<tr>
<td>Cu</td>
<td>0.011</td>
<td>0.03</td>
<td>0.02</td>
<td>0.015</td>
<td>0.026</td>
<td>70</td>
</tr>
<tr>
<td>Cd</td>
<td>0.08</td>
<td>--</td>
<td>0.15</td>
<td>0.10</td>
<td>0.12</td>
<td>0.72</td>
</tr>
<tr>
<td>Co</td>
<td>0.28</td>
<td>0.37</td>
<td>0.41</td>
<td>0.32</td>
<td>0.38</td>
<td>--</td>
</tr>
<tr>
<td>Ni</td>
<td>0.41</td>
<td>0.5</td>
<td>0.75</td>
<td>0.58</td>
<td>0.6</td>
<td>8.2</td>
</tr>
<tr>
<td>Pb</td>
<td>0.36</td>
<td>0.24</td>
<td>0.40</td>
<td>0.32</td>
<td>0.39</td>
<td>2.5</td>
</tr>
<tr>
<td>Zn</td>
<td>0.18</td>
<td>0.0</td>
<td>0.31</td>
<td>0.25</td>
<td>0.062</td>
<td>81</td>
</tr>
<tr>
<td>As</td>
<td>27.7</td>
<td>21.4</td>
<td>--</td>
<td>--</td>
<td>36</td>
<td>--</td>
</tr>
</tbody>
</table>

*Permissible limits are adapted from [10].

** All values are expressed in Part Per Million (PPM)

**Heavy Metals Pollution:** Heavy metals levels in five different aquacultures in Fayoum Governorate have been evaluated for Iron (Fe), Copper (Cu), Cadmium (Cd), Cobalt (Co), Nickel (Ni), Lead, Zinc (Zn) and Arsenic (As). All of measured heavy metals were within the permissible limits recommended by US Environmental Protection Agency for fresh water aquaculture Continuous exposure Concentration [10]. Aquaculture site 1 was the lowest in most of pollutants levels while the Aquaculture site 3 showed the highest levels of heavy metal pollutions in most of measured parameters (Table2).

The Pearson product-moment correlation coefficient R was calculated for each measured pollutant and bacterial prevalence rates in corresponding aquaculture.

The Iron levels showed to be the highest correlated with the incidence levels of the bacterial diseases isolated from examined farms with correlation coefficient R: 0.74. Followed by the Zinc levels R: 0.63 and Nickel: 0.59. While the copper, cadmium, cobalt and lead showed no or insignificant correlation with the infection levels in different aquaculture locations.
Sensitivity Testing (Antibiogram): The conduction of sensitivity test on most prevalent isolated bacterial species *A. hydrophila* has showed the strong susceptibility to ciprofloxacin antibiotic showing the largest zone of inhibition in all tested bacteria. Other antibiotics showed moderate action on the bacterial isolate with moderate zone of inhibition were trimethoprim–sulfamethoxazole, kanamycin, novobiocin, and chloramphenicol. Bacteria showed high resistance to the antibiotics oxytetracycline and amoxicillin with no recorded zone of inhibition.

Detection of Virulence Genes: Virulence genes play an important role in pathogenesis of bacterial pathogens. The results in this study showed the distribution of the six virulence variety in the distribution of virulence factors in the *A. hydrophila* isolates. The PCR results revealed the presence of an amplification product at 232bp for Act gene, 331bp for the Ast gene. Ela was detected at 513 and Alt gene was detected at 442 bp band, while both Fla and Lip genes were not detected in the examined samples.

**DISCUSSION**

In this study, the possible link of heavy metals pollution and bacterial diseases in fish in Fayoum Governorate has been evaluated, water samples from different localities have been collected and examined for chemical and physical parameters as well as heavy metals levels. Fish specimens were examined for bacterial infections and histopathological alterations. The prevalence of bacterial diseases showed high levels of infection by different bacterial fish pathogens which dictates the presence of high load of different pathogenic bacteria as well as weakening of host immunity due to sub-lethal pollution levels as well as stress caused by culturing conditions [11].

The most prevalent bacterial species found to be *Aeromonas hydrophila* 18.8, followed by *A. sobria* 13.2%, *P. fluorescens* 10%, *P. putida* 14%, *Flavobacterium* Sp. 8.4% and *Staphylococcus* Sp. 2.4%. showing that the natural commensals Aeromonas and Pseudomonas bacteria can turn into pathogenic bacteria when fish suffer stressed conditions[12].

The infected fishes showed many gross lesions including haemorrhages both on external surface and internally in the abdominal cavity, eye opacity and exophthalmia as well as scale detachment and darkening of the skin. Internal organs suffered from alternate conditions of severe paleness or sever congestion. These findings were attributed to bacterial infection and was consistent with clinical picture observed in septicaemic bacterial diseases[13].

Histopathological picture of infected fishes showed severe damage in different organs and tissues caused by bacterial infections, where the hepatopancreas, posterior kidney and gills suffered congestion in some areas and necrotic degenerations in other regions, that picture was consistent with histopathological alterations in septicaemic bacterial diseases [14, 15].

The heavy metal pollution levels were not higher than the permissible levels that are recommended by USA Environmental protection Agency [10] however the levels of heavy metals differed drastically among different examined sites where site one and two were much lower in pollution levels than the other 3 locations that can be attributed to the geographical location of the sites where site one and two where using the irrigation source at the beginning of the water canal before passing on agricultural fields, while the other locations where at the end of the canal after passing on agricultural fields and after collection of agricultural wastes.

Although the pollution levels weren’t above the recommended levels, chronic exposure to pollutions has been proved to have a hazardous effect on fish health [16]. The correlation between some heavy metals pollution and the rate of infection with bacterial strains has been estimated and found to be positive correlation with iron, zinc and nickel levels even in low levels. While copper, lead, cadmium and cobalt showed no effect on fish diseases rate at these levels. Such relation has been found consistent with findings of other studies [17].

Antibiogram of most prevalent strains showed development of resistance against most commonly used antibiotic in aquacultures oxytetracycline. However less commonly used antibiotics showed good activity against isolated strains. Such antibiotic resistance profile was consistent with other studies [18].

The PCR detection of virulence determinants confirmed the presence of four virulence genes Act gene, Ast gene, Ela and Alt gene in the isolated strains, while both Fla and Lip genes were not detected in the examined samples.

**CONCLUSION**

The presence of heavy metals pollution in aquatic environment is linked to occurrence of many bacterial diseases that usually have drastic effect on the fish health and productivity. The extensive use of antibiotics to
control such diseases at the long run loses its value as bacteria gains resistance against the commonly used antibacterial. Which highlight the importance of usage of clean water source and controlling the pollution levels in aquaculture environment.

REFERENCES