Original Research Paper

Studies on Bacterial Pathogens in Some Marine Fishes in EL-Mansoura, Egypt

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Abstract: A total of 200 marine fish of two different species (100 *Tilapia zillii* and100 *Mugil capito*) were randomly collected from different markets, during different seasons. Fish subjected to clinical, postmortem and bacteriological studies. The common clinical signs were darkness of the skin, hemorrhage in the fins base, eyes and different parts of the body, abdominal distention, congestion in gills and increasing in mucous secretion. The Post Mortem findings showed white serous fluid in the abdominal cavity and sometimes tinged with blood and pale or congested liver, kidney and spleen. The bacteriological examination, revealed that the prevalence of bacterial pathogens among naturally infected marine fishes were *A. hydrophila* (52; 39.39%), *V. alginolyticus* (38; 28.79%), *Ps. fluorescens* (24; 18.18%), *V. cholera*e (10; 7.58%) and *Ps. aeruginosa* (8; 6.06%). The highest prevalence of bacterial infection was during summer (33.33%) followed by spring (24.42%), then autumn (21.97%), while the least prevalence was in the winter season (20.46%). *A. hydrophila* was highly pathogenic to *T. zillii* causing 100% mortality followed by *V. alginolyticus* causing (90%) mortality, then *Ps. fluorescens* (80%), *Ps. aeruginosa* (60%) while *V. cholerae* was non-pathogenic. The results of antibiogram revealed that ciprofloxacin and nalidixic acid were effective against *A. hydrophila*, while ciprofloxacin and rifampicin were effective against *Ps. fluorescens.* Ciprofloxacin and amikacin were effective against *Ps. aeruginosa.* While *V. alginolyticus* was highly sensitive to ciprofloxacin. High prevalence of pathogenic and resistant bacterial strains among marine fishes in Egypt, requires strict control measures from the responsible authorities.

Keywords: Marine, Fish, Bacteria, Season, Antibiotics

Introduction

Aquaculture is an important source of high nutritive value, cheap animal proteins and it becomes an important economic activity in many countries. In this way, the Egyptian Government within the strategy of the food paid special interest to fish meat and within this strategy fish aquaculture and fish industry are one of the main sources of the Egyptian. In the near future, the limitation of aquaculture development in Egypt will be due to water resources especially freshwater. Thus, mariculture is the alternative solution.

Aeromonas, *Vibrio* and *Pseudomonas* species are among the economically important bacterial fish diseases affecting marine fish cultures (Falaise *et al*., 2016).

The infectious bacterial diseases in marine fish affected by the following: (i) Significant economic losses in cultured fish are caused by a relatively small number of pathogenic bacteria; (ii) Several typical of fresh water aquaculture diseases turns as marine culture serious problems; (iii) The disease clinical signs depend on the host species, age; (iv) Absence of any correlation between the external and internal signs; and (v) The lack of the stressful conditions, resulted in increasing the mortality and the severity of the disease in cultured fish than that in wild fish (Toranzo *et al*., 2005).

The use of antibiotics in the aquaculture leads to a variety of public health hazards. The greatest potential risk to public health is thought to be the transfer of resistant organisms through consumption of contaminated fish, the development and spread of antimicrobial resistant bacteria and resistance genes and the dissemination of the resistance genes by horizontal gene transfer (Lukkana *et al*., 2012; Aly, 2013).

The bacterial diseases in marine fish are responsible for important economic losses worldwide. So the present study aimed to isolate and identify the bacterial pathogens in some marine fishes and evaluate the seasonal variation, prevalence in different organs and their antibiogram against the most commonly used antibiotics in maricultures. In addition, an experimental infection was performed to determine the mortality rate caused by different bacterial pathogens.

Material and Methods

Sampling

A total of 200 marine fish of two different marine fish species (100 *Tilapia zillii* and 100 *Mugil capito*) were freshly collected from different markets in EL-Mansoura city, during the four seasons. A total of twenty-five fish of each species were collected in each season. Clinical and postmortem examination were carried out according to (Schaperclaus *et al*., 1992).

Isolation of Suspected Bacteria

Samples from liver, kidney, spleen and gills were cultured on tryptic soy broth, tryptic soy agar (oxoid) supplemented with 2% NaCl, thiosulphate citrate bile salt agar (oxoid), *Aeromonas* base media supplemented with ampicillin and *Pseudomonas* agar base media supplemented with 2% NaCl and 2% glycerin. The plates were incubated at 28°C for 1-2 days.

Identification of the Isolated Bacteria

Bacterial isolates were stained with Gram's stain to examine their morphology. The isolates then biochemically identified according to (Bergey's, 2005). The analytical profile index of API20 E system was used to the final confirmation of the bacterial isolates identification (Buller, 2004).

Antibiotic Sensitivity

The sensitivity of the isolated bacterial strains to antibiotics was performed using the disc diffusion method on Muller's Hinton agar medium against the oxytetracycline, ampicillin, amoxicillin, lincomycin, ciprofloxacin, colistin sulfate, nalidixic acid, amikacin, rifampicin and erythromycin (Schaperclaus *et al*., 1992).

Experimental Infection

A total of 70 live and apparently healthy *T. zillii* fish with an average body weight 20±5 g, were obtained from EL Abbassa fish farm, EL sharkia, Egypt. They were used for experimental infection with *A. hydrophila*, *Ps. fluorescens*, *Ps. aeruginosa*, *V. alginolyticus* and *V. cholerae* which isolated from naturally infected marine fish. The Fish were maintained in glass aquaria supplied with well aerated dechlorinated tap water with the addition of salt to be acclimated. All experimental fish were fed with a commercial ration at a rate of 5% body weight per day.

Fish were divided into seven groups (10 fish of each) and a bacterial suspension were prepared for I/P injection (Austin and Austin, 1999). Five groups were I/P injected with a bacterial suspension prepared separately from each species (*Ps. aeruginosa, A. hydrophila*, *V. alginolyticus*, *Ps. fluorescence* and *V. cholera*e at a dose of 0.2 mL of (3×10^7 CFU). The sixth group was intra-peritoneal injected with 0.2 ml of saline containing *V. cholera*e (2.5×10^8 CFU ml^{-1}) (Austin and Austin, 2007) the last group was left as control and injected I/P with 0.2 ml sterile saline (Table 5). Fish observed daily for 1-2 weeks and the clinical signs and mortalities were recorded. Freshly dead fish were examined for postmortem gross lesions. Pathogenic bacteria re-isolated from dead and scarified fish.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences version 20.0 (SPSS inc. Chicago, USA). Pearson correlation coefficient was used to determine the association between the prevalence of bacterial pathogens during the four seasons. A *p*-value of 0.05 or less was considered as statistically significant.

Results

Clinical and Postmortem Examination

The naturally infected fish showed the darkness of the external body surface, an opacity of eye, increased mucous secretion, exophthalmia and some fish showed hemorrhages in the eye and large irregular hemorrhagic areas into many parts of the body, at the base of fins, on gills cover, at the anal region and anal fin. There was abdominal distention observed in some fish (Fig. 1). The Postmortem examination showed white serous fluid in the abdominal cavity, some tinged with blood. The liver appeared pale anemic, friable with some hemorrhagic patches and pinpoint hemorrhagic on liver surface. Kidney was congested and slightly enlarged. The intestine of some fish was hemorrhagic, inflamed with severe congestion. The spleen was enlarged and congested and in some cases appeared pale and in other cases appeared normal. In some fish hemorrhagic areas appeared in the abdominal wall and on the peritoneum (Fig. 2).

Fig. 1: Diseased *M. capito* with diffused hemorrhage on body surface and on anal and ventral fine

Fig. 2: Diseased *M. capito* showed hemorrhages in peritoneum

Bacteriological Examination

The bacteriological examination showed the isolation of 132 Gram-negative isolates which identified morphologically, biochemically and confirmed by API20 into *A. hydrophila, Ps. fluorescens, Ps. aeruginosa, V. alginolyticus* and *V. cholerae* (Table 1). A total of (22; 14.29%) isolates were oxidase-negative and (132; 85.71%) were oxidase-positive isolates. Among the oxidase-positive isolates, *A. hydrophila* represented (52; 39.39%), followed by *V. alginolyticus* (38; 28.79%), *Ps. fluorescens* (24; 18.18%), *V. cholerae* (10; 7.58%) *and Ps. aeruginosa* (8; 6.06%) (Table 2).

The results of bacterial isolates distribution in different organs and tissues of the examined marine fish are depicted in Table 3. The highest rate of *A. hydrophila* was in liver (44.23%), while the minimal prevalence recorded in gills (7.69%). *Ps. fluorescens* was isolated mainly from kidney (50%), followed by liver (25%) and spleen (8.33%). *Ps. aeruginosa* was isolated equally from liver and kidney (37.5%), followed by spleen and gills (12.5%). *V. alginolyticus* was isolated mainly from liver (36.84%) and at a lower rate from gills (7.89%). The highest prevalence of *V. cholerae* was in the kidney (40%), while the least prevalence was in gills (10%).

Seasonal Variation

The seasonal prevalence of bacterial pathogens among the naturally infected marine fish was the highest during summer (33.33%), followed by the spring (24.24%), then autumn (21.97%), in contrast, the minimal prevalence was during winter (20.46%) (Table 4). The overall prevalence of different bacterial pathogens during summer was statistically significant compared to other seasons (p-values <0.05). *A. hydrophila* highest prevalence was during summer season 18 (15.4%), while *Ps. fluorescens* not reported in summer. *Ps. aeruginosa* was isolated only during spring and summer. *V. alginolyticus* highest prevalence was also during summer. *V. cholerae* reported during different seasons but not during winter.

Antibiotic Sensitivity

The results of antibiotic sensitivity revealed that ciprofloxacin and nalidixic acid were more effective against *A. hydrophila*, while ciprofloxacin and rifampicin more effective against *Ps. fluorescens.* ciprofloxacin and amikacin were effective against *Ps. aeruginosa. V. alginolyticus* was highly sensitive to ciprofloxacin. On the other hand, the bacterial isolates showed resistance against ampicillin, amoxicillin, erythromycin and oxytetracycline.

Table 1: The biochemical and morphological characters of isolated bacteria from examined marine fish

		A. hydrophila	*Ps. fluorescens*	*Ps. aeruginosa*	*V. alginolyticus*	*V. Cholerae*
1.	Gram stain	-	-	-	-	-
1.	Shape	Short rod.	Short rod	Short rod	vebrionic bacilli	vebrionic bacilli
2.	Motility	+	+	+	+	+
3.	Cytochrome Oxidase. (Ox)	+	+	+	+	+
4.	Catalase Test	+	+	+	+	+
5.	B-Galactosidase Production (OPNG)	+	-	-	-	+
6.	Arginine Dihydrolase production (ADH)	-	+	+	-	-
7.	Lysine Decarboxylase production (LDC)	-	+	-	+	-
8.	Ornithine Decarboxylase production (ODC)	+	+	-	+	+
9.	Citrate utilization (CIT)	-	+	+	-	-
10.	H_2S production (H_2S)	-	-	-	-	-
11.	Urease Production (URE)	-	-	+	-	-
12.	Tryptophan Deaminase Production (TDA)	-	-	-	-	-
13.	Indole production (IND)	+	-	-	+	+
14.	Acetoin production (VP)	-	-	+	-	+
15.	Gelatinase production (GEL)	+	-	+	+	+
16.	Acid from glucose	+	-	-	+	v
17.	Acid from manitole	+	-	-	+	+
18.	Acid from inositol	+	-	-	-	-
19.	Acid from sorbitol	+	-	-	-	-
20.	Acid from rhaminos	-	-	-	-	-
21.	Acid from sacrose	+	-	-	+	+
22.	Acid from melobiose	-	-	-	-	-
23.	Acid from amylase	+	-	-	-	-

Table 2: Prevalence of Gram-negative, oxidase positive bacterial isolates in examined marine fish

A. hydrophila	*Ps. fluorescens*	*Ps. aeruginosa*	*V. alginolyticus*	*V. cholerae*
52 (39.39 %)	24 (18.18 %)	8 (6.06 %)	38 (28.79 %)	10 (7.58 %)

Table 3: Incidence of Gram-negative bacterial species from examined tissues and organs of marine fish

	A. hydrophila		*Ps. fluorescens*		*Ps. aeruginosa*		*V. alginolyticus*		*V. Cholerae*		Total	
Organ	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Liver	23	44.230	6	25.00	3	37.50	14	36.84	2	20.00	48	36.36
Kidney	19	36.540	12	50.00	3	37.50	12	31.58	4	40.00	50	37.84
Spleen	6	11.540	2	8.33	1	12.50	9	23.68	3	30.00	21	15.91
Gills	4	7.690	4	16.67	1	12.50	3	7.89	1	10.00	13	9.85
Total	52	39.390	24	18.18	8	6.06	38	28.79	10	7.58	132	100.00

Table 4: Seasonal prevalence of Gram-negative bacterial species in examined marine fish

Bacterial isolates	Winter	Spring	Summer*	Autumn	Total
A. hydrophila	14 (26.9%)	12 (23.1%)	18 (34.6%)	8 (15.4%)	52 (39.39%)
Ps. fluorescens	8 (33. 3%)	8 (33. 3%)	0	8 (33. 3%)	24 (18.18%)
Ps. aeruginosa	0	4 (50 %)	4 (50 %)	0	8 (6.06%)
V. alginolyticus	5 (13. 2%)	7 (18.4%)	16 (42.2%)	10 (26.3%)	38 (28.79%)
V. cholerae	0	1 (10 %)	6 (60 %)	3 (30 %)	10 (7.59%)
Total	27 (20.45%)	32 (24.24%)	44 (33.33%)	29 (21.97%)	132 (100%)

* Statistically significant (p-values<0.05)

Experimental Infection

The clinical signs of the disease appeared after 24 hrs of fish injection with the pathogenic isolates (*A. hydrophila, Ps. fluorescens, Ps. aeruginosa and V. alginolyticus*) and the signs included inflammatory changes at site of injection, hemorrhages all over the body, bilateral distention of abdomen, congested liver, kidney and spleen. The pathogenic isolates were re-isolated from all freshly dead and scarified infected fish. The mortality rates caused by *A. hydrophila, Ps. fluorescens, Ps. aeruginosa, V. alginolyticus* was 100%, 80%, 60% and 90% respectively. While the I/P infected fish groups with two different concentrations of *V. cholerae* showed neither clinical signs nor mortalities (Table 5).

Table 5: Mortality rates of the experimentally infected fish

Group*	Dose per fish	Dead fish during 7 days after Injection of pathogenic bacteria							No. of dead fish	Mortality rate %
		1	2	3	4	5	6	7		
1	*A. hydrophila* 0.2 mL of 3×10^7 CFU	3	3	3	1	-	-	-	10	100%
2	*Ps. fluorescens* 0.2 mL of 3×10^7 CFU	2	2	1	2	1	-	-	8	80%
3	Ps. *aeruginosa* 0.2 mL of 3×10^7 CFU	0	2	2	2	-	-	-	6	60%
4	*V. alginolyticus* 0.2 mL of 3×10^7 CFU	2	3	3	1	-	-	-	9	90%
5	*V. cholerae* 0.2 mL of 3×10^7 CFU	-	-	-	-	-	-	-	-	0%
6	*V. cholerae* 0.2 mL of 2.5×10^8 CFU	-	-	-	-	-	-	-	-	0%
7	Control group 0.2 mL of sterile saline	-	-	-	-	-	-	-	-	0%

* (10 fish each)

Discussion

Marine fish are susceptible to a wide variety of bacterial pathogens. In the present study, naturally infected marine fish (*M. capito and T. zilii*) were clinically examined and found to have darkness of the external body surface, opacity of eye, exophthalmia and some fish showed hemorrhages in eye, scales detachment and large irregular hemorrhagic areas into many parts of the body, at base of fins, anal fin and caudal peduncle. Postmortem findings of most of the examined fish pointed out that liver appeared to be pale anemic, friable with some hemorrhagic patches on its surface and in some cases appear congested. Kidney and spleen were congested and slightly enlarged and in some cases pale. In some fish appeared hemorrhagic areas in the abdominal wall. Our clinical and Postmortem findings were in accordance with what was mentioned by Toranzo *et al*. (2005).

The most prevalent bacterial pathogens affecting fish worldwide are *Aeromonas* and *Pseudomonas* (Kayansamruaj *et al*., 2017) in addition to genus *Vibrio* especially *V. alginolyticus, V. cholerae (non-O1), V. vulnificus (Biotype 2), V. anguillarum* (Kannapiran *et al*., 2009). In the present study, bacteriological examination identified *A. hydrophila, Ps. fluorescens, Ps. aeruginosa, V. alginolyticus and V. cholerae.* Among the bacterial isolates *A. hydrophila* is the most prevalent *(*39.39%), followed by *V. alginolyticus (*28.79%) and *Ps. fluorescens* (18.18%) and these results were in accordance with Zorrilla *et al*. (2003) who isolated the bacterial pathogens as follow: *Vibrio* (69.90%), *Pseudomonas spp*. and *Aeromonas spp*., in which *V. alginolyticus* was the most identified species (21.35%) and with Ullmann *et al*. (2005) who demonstrated that among the bacterial pathogens *A. hydrophila* had the highest prevalence (67%), followed by *A. caviae (*26.1%) and *A. sobria* (6.0%). Also (Abd-El-Malek, 2017) who isolated *ps. fluorescens* from naturally infected Mugil species with a prevalence rate of 21.33%. Thomas *et al*. (2014) isolated 12 (15%) *Ps. aeruginosa* out of 80 *Pseudomonas spp.* from marine fish. Outbreaks of *A. hydrophila* were usually associated with environmental changes and other Stresses such as, sudden change in temperature, overcrowding, poor nutrition and other infections that made stress on fish and increase its susceptibility to the infection (Hayes, 2000).

Regarding, the seasonal prevalence of *A. hydrophila* the result highlighted that *A. hydrophila* highest prevalence was recorded in summer (34.6%), followed by winter season (26.9%), spring (23.1%) and autumn (15.4%). *Aeromonas* dynamics exhibited a seasonal prevalence in natural seawater. *Aeromonads* decreased very rapidly in sea water during the cold period from the end November to April (Maalej *et al*., 2003). Our findings are agreed with Nam and Joh (2007) who demonstrated that *A. hydrophila* was dominant in August and the density of *Aeromonas* isolates was ten times higher in November than in August because *Aeromonas* was both psychrophilic as well as mesophilic strains.

On the other hand *Ps. fluorescens* prevalence was equal during winter, autumn and spring. While not recorded in the summer season. *Ps. fluorescens* propagate and exerts infection at low temperature (Eissa *et al*., 2013). These results were in accordance with (Blanco *et al*., 2002) who illustrated that the proteinase activity of *Pseudomonas spp*. increase in low temperature. Wang *et al*. (2017) reported that *Pseudomonas* were isolated mainly in cold months. while these results disagreed with (Miguéis *et al*., 2016) who reported a lower prevalence of *Pseudomonas* in winter than in summer. In contrast, our results disagreed with Thomas *et al*. (2014) who isolated *Pseudomonas spp.* with a significant difference among the four seasons, 43.33% in spring, 24.44% in summer, 21.11% in autumn and 17.77% in winter. While, the highest prevalence of *Ps.aeruginosa* was recorded in spring (12.5%), followed by summer (9.09%). But not reported in winter and autumn.

The highest prevalence of *V. alginolyticus* was recorded in summer (42.2%), then autumn (26.3%), followed by spring (18.4%) and winter (13. 2%). The obtained results of seasonal prevalence of *V. alginolyticus* were higher than what reported by Di *et al*. (2017) who isolated *V. alginolyticus* at a prevalence of 8.57% in summer, 5.30% in autumn, 2.04% in spring and 0.81% in winter. This was in a disagreement with (Wang *et al*., 2017) who reported that V. alginolyticus were not associated with a particular season.

Concerning to the rate of bacterial isolates recovery from various organs, our investigation demonstrated that the prevalence of total bacterial isolates was (37.84%) in the kidney so it is the most predominant site for isolation of bacterial pathogens that causing septicemia as it is considered as one of the main hematopoietic organs of fish. Followed by the liver (36.36%), then spleen (15.91%) and finally gills (9.85%) and this result nearly agreed with (Mahmoud *et al*., 2016) who concluded that the bacterial infections affect hematopoietic system mainly liver, kidney and spleen.

Antibiogram sensitivity results cleared that ciprofloxacin and nalidixic acid were highly effective against *A. hydrophila* and these results agreed with (Grande *et al*., 2018).While ciprofloxacin and rifampicin were more effective against Pseudomonas spp. and these results similar to that detected by (Lee and Wendy, 2017). On the other hand, *V. alginolyticus* was highly sensitive to ciprofloxacin and our results supported with that obtained by (Schmidt *et al*., 2017).

Conclusion

It was concluded that the highest prevalence of bacterial pathogens causing infection in marine fish was for *A. hydrophila* followed by *V. alginolyticus*, and *Ps. fluorescens*. While the minimal prevalence was for *Ps. aeruginosa* and *V. cholerae*. The higher rate of infection was in summer, while the minimal incidence was in winter. Ciprofloxacin was considered the drug of choice for treatment and prevention of bacterial infection in marine fish either alone or combined with other antibiotics.

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Author's Contributions

Elsayed, M.E. and A.M. Essawy: Designed the research plan, organized the study and critical review.

I.I. Shabana and M.E. Abou El-Atta: Participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

N.I. EL-Banna: Participated in all experiments and contributed to the writing of the manuscript.

Ethics

The authors confirmed that this manuscript is an original work and do not contain any conflict of interest.

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