Study on Grouper fish mortality phenomenon at the east costal Libyan area of the Mediterranean Sea with reference to bacteriological and parasitological examinations

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Abstract: Grouper fish mortality at the East Cost Libyan area of the Mediterranean Sea is one of the major problems encountered at autumn season starting from October. Sudden environmental changes associated with water pollution were recorded. Adult fish especially the grouper types were affected. This phenomenon was previously recorded in Libya at 1985. The present study was carried out to demonstrate the most prevalent isolates that may lead to this phenomenon. It was found that the gram negative oxidase positive bacterial group (*Pasteurella*, *Vibrio* and *Aeromonas spp*.) were the most isolated bacteria with high incidence refers especially to *Pasteurella piscicida* with an incidence of 64%. Black or metallic colour cysts (Microspordiosis, *Glugea spp*. and *Plistophora spp*.) representing spores or larvae of this parasite were observed on the visceral organs and abdominal cavity. Other parasites included larval stages of *Contracaecum spp*. and *Gonapodasmius epinepheli* (Didymozoid digenes). The clinical and postmortem lesions were mostly characterized by unilateral or bilateral corneal opacity and haemorrhagic spots on the skin with ulcer formation in some cases. Abdominal distension and anal prolaps were also recorded. All the internal organs were congested. The swim bladder was greatly swollen and filled with gas.
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Key words: Grouper fish; Pasteurella, Aeromonas, Vibrio; API; Monogenea, Digenean.

1. Introduction:

The groupers belong to the family Serranidae and the subfamily Epinephelinae. They are widely distributed in the tropical and subtropical regions. The groupers are of great economic value and are the major component of the coastal fisheries in Asia. At least 21 species of groupers are cultured in Asia (Leong, 1994),

A major production constraint in grouper culture is heavy mortality due to various diseases. Aside from the health status of the fish during culture, they are also subjected to considerable stress in the nature as wild type or during collection and transportation for aquacultures. (Erlinda, et al. 1996)

The pathogenic bacteria that have been identified were related to. *Vibrio spp., Aeromonas spp., pasteurella spp., and Streptococcus spp*.

Bacteria are very common in the aquatic environment. The most bacterial disease agents are part of the normal flora of the water. They may cause disease only when the fish are stressed by poor environmental conditions. Due to the development of intensive aquaculture in coastal areas in the United States, Japan, Europe, and the Mediterranean region, conditions favoring the establishment of disease by this highly pathogenic halophilic organism have been created. In Japan, this pathogen has been proven to be detrimental to wild and farmed fishes, and responsible for significant losses. Photobacteriosis causes severe losses in wild and cultured yellowtail juveniles (Kubota et al. 1970; Kusuda and Yamaoka 1972), and mortalities in other fishes, including ayu, red sea bream, black sea bream, oval file fish, and red grouper.

Prior to 1990 there were no any reported cases of photobacteriosis in Europe. Subsequently, epizootics were reported in many areas in Europe, beginning with 1990 outbreak in northwestern Spain in a gilthead sea bream population (Toranzo et al. 1991). Simultaneously, epizootics occurred in France, Turkey, and Greece, affecting mainly sea bass, but in Italy, Malta, and Portugal, affecting populations of gilthead sea bream. The causative organism of fish photobacteriosis was initially isolated from natural populations of white perch and striped bass in 1963 during a massive epizootic in Chesapeake Bay, USA (Snieszko et al. 1964).

.A large numbers of parasites are reported; some cause significant problems for grouper aquaculture. The parasites (protozoans, myxozoans, microsporans, monogeneans, trematodes, crustaceans, nematodes, cestodes, acanthocephalans and hirudineans) include external (skin and gills) and internal parasites. Parasite fauna of healthy and diseased E. malabaricus in Malaysia, Thailand and Philippines was most extensively studied by Leong

and Wong 1990). It has spread to at least twelve other cultured species in Japan including three species of grouper. Benedeniid monogeneans are particularly dangerous in net cage culture systems, where their eggs entangle the net meshing with elongated appendages, which makes re-infection much easier for the parasites (Ogawa, 1996).

A wide variety of parasitic organisms have been reported as causing significant problems in grouper aquaculture. In the hatchery and nursery stages, parasitic diseases of groupers are caused predominantly by protozoans, particularly the ciliates. When grouper fry are transferred to grow-out facilities, they are subjected to handling and transport stress. These fish often carry a large variety and high intensity of ciliated protozoans, skin and gill monogeneans and caligid copepods. Dealing also with the major parasites of groupers including infections caused by protozoans, monogeneans, didymozoid digeneans, nematodes, caligid copepods, isopods and leeches.(Kazuya and Erlinda 2004)

The present work aimed to study the predisposing factors and causative agents which lead to high mortality rate in groupers fish, this phenomenon was firstly recorded in Libya at September 1985 in the same region and season.

2. Materials and Methods

- Samples:

a- Fish samples:

30 naturally infected grouper fish of different species were taken. Some of them were caught by special fishermen harvesters and dives from different area of the East costal region (Tobrok and El-Bordy) in Libya. 20 samples were clinically diseased and 10 samples were apparently healthy. They were transferred in a special aquarium for the transfer lab. for different laboratory examinations.

b- Water samples:

6 samples of water were collected from different localities of the suspected area. They were transferred in special sterile bottles to lab. for bacteriological examination.

c- Sediment sample:

4 samples of sediment from the sea bottom at the same locality were collected by special dives. They were also transferred in special sterile bottles for bacteriological examination.

-All fish samples were subjected to clinical and postmortem examination then carried to the transfer lab. for their bacteriological and parasitological examination.

- Isolation of different types of bacteria:

- Grouper fish samples were cultured onto different types of media (Brain heart broth, Brain heart agar, TSA, Aeromonas specific medium and semisolid agar) (Oxoid) with adding 2% of Nacl for prevailing suitable pH and salinity for bacterial growth

- Identification of bacteria:

- Culture character and Microscopic examination
- Oxidase test
- Sensitivity test to O /129 Vibriostatic disc and Novobiocin media for differentiation between Aeomonas ,Pseudomonas and Vibrio
- API test

I- Isolation and identification of different types of bacteria

Bacteriological samples were taken from liver, spleen and kidney of the affected fish. The cultures were examined according to (Koneman et al., 1994). Pure colonies were preserved into semisolid agar for further examinations.

- Biochemical identification of the isolated colonies were done by the API-20 E and API-20NE test strip according to analytical profile index (BioMerieux SA). (Buller, 2004). It was made in bacteriological lab of Poultry and Fish Diseases Department, Faculty of Veterinary Medicine, Al-Fateh University.

II- Parasitological examination: It was done according to Jesus et al (2008) by direct mount technique using skin, gill and intestinal smears, stained by Giemsa stain and examined using penetrating stereoscope. Samples were preserved in 70 % alcohol where thought to have some parasite.

3. Result:

1- Clinical examination of grouper fish

- **All examined cases are related to the following grouper spp.:**

- *Epinephelus aneus- Epinephelus guaza*

- *Epinephelus alexandrines - Epinephelus caninus*

Clinical examination of the apparently healthy and diseased grouper revealed in some cases uni or bilateral corneal opacity with partial or total blindness and exophthalmia. All affected cases revealed distended abdomen with anal prolapse. Some of examined samples showed hemorrhagic spots on the skin and ulcer formation in other cases. Gills were swollen and congested. Most examined cases revealed yellow parasitic cyst in gills with high mucus secretions. Skin depigmentation or discoloration with hemorrhagic patches were also seen. Fig (1,2,3,4,5,6).

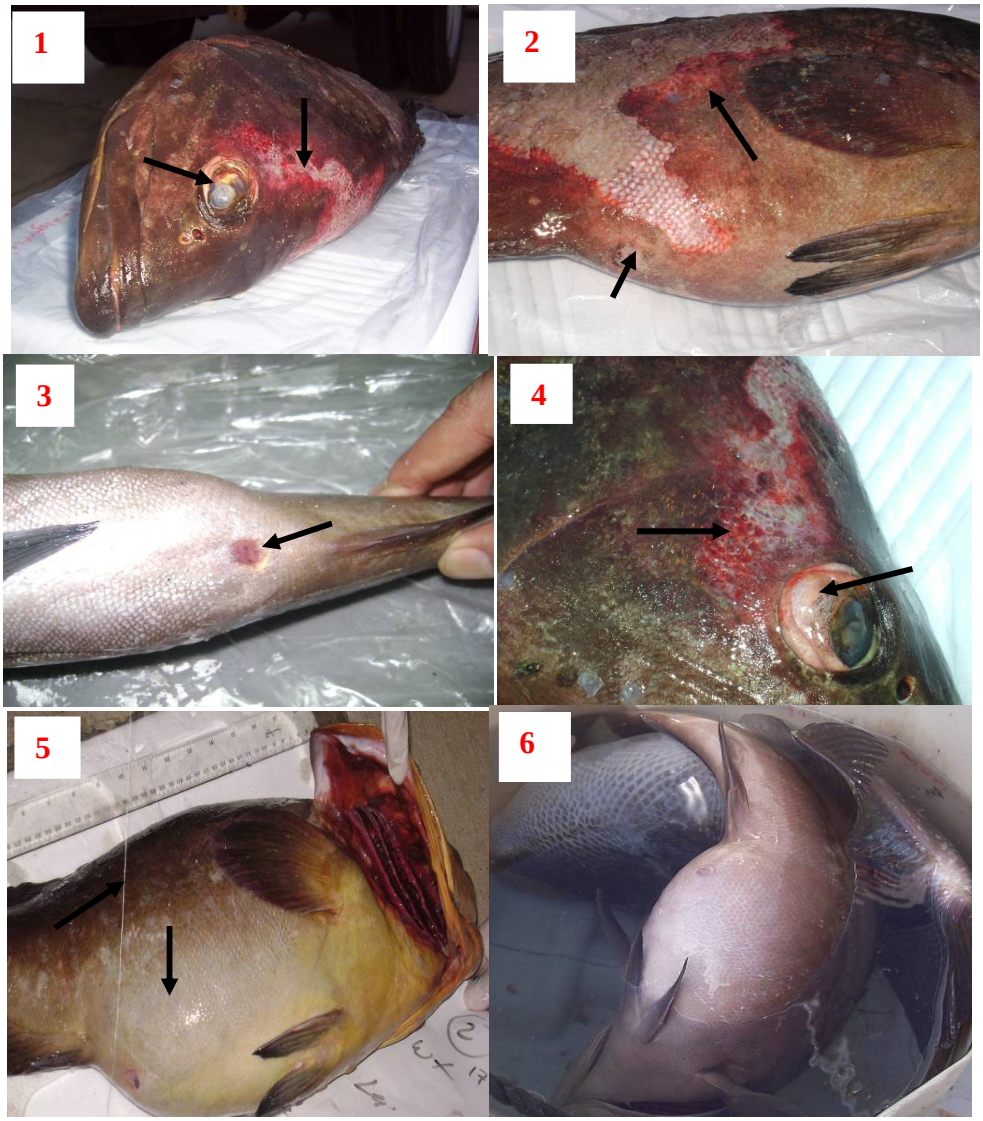

Fig. (1): Grouper fish showing hemorrhagic patches on the head region with corneal opacity
Fig. (2): Grouper fish showing abdominal dropsy and anal protrusion with skin hemorrhage in different body areas
Fig (3): Grouper fish showing abdominal distension and anal inflammation
Fig (4): Grouper fish showing hemorrhagic spots with exophthalmia
Fig. (5): Grouper fish showing hyper distension of abdomen due to hyper filling of swim bladder with air.
Fig. (6): Grouper fish showing floating position at one side and abdominal distension

2- Postmortem examination

Post mortem examination of most cases revealed empty stomach and intestine, parasitic cyst on the digestive tract and abdominal cavity which may be the primary cause or predisposing cause for bacterial infection. All visceral organs are swollen and congested with ulcerative lesions in some cases. The liver was pale in some cases. Adhesion between the swim bladder and the visceral wall with yellowish abdominal fluid tinged with blood (purulent like exudates or discharge). White nodules were present in some examined cases affecting liver, spleen and kidney. Presence of black or metallic colure cyst in which may represent the larvae of parasites filling all abdominal cavity (heavy infestation). Fig. (1,2,3,4).

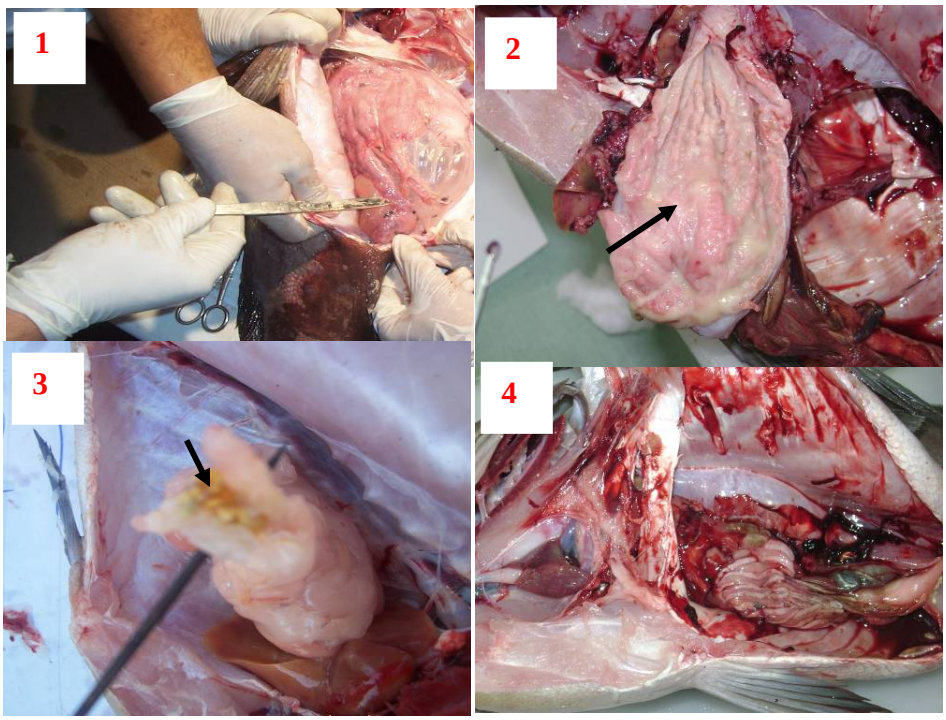

Fig (1): Grouper fish PM examination showing heavy infestation of abdominal cavity with parasitic cysts
Fig (2): Grouper fish showing the stomach free from food
Fig (3): Grouper fish showing pus like material with yellowish white nodules in the internal organs
Fig (4): internal sever hemorrhage affecting all internal organs

3- Bacteriological examination

Table (1): The total number of examined samples (Fish, Water and Sediment)

Examined samples		Total No	
Fish	Apparently healthy	10	30
	diseased	20	
Water		6	10
Sediment		4	
Total		**40**	

As shown in table (1) the total No of examined samples were 40. 30 samples resembled the apparently healthy and diseased fish samples and 10 samples resembled by 6 water samples from different sea bottom areas, they were collected in a sterile bottles for bacteriological examination and 4 samples were taken from the sediment of the sand bottom of the sea. All samples were bacteriologically examined. The result of these samples examination was shown in table (2).

Table(2): Incidence of positive and negative samples for bacterial isolation

Samples	Bacterial isolation					
	Positive isolation		Negative isolation		Total	
	No	%	No	%	No	%
Diseased fish	15	75	5	25	20	100
Apparently healthy	5	50	5	50	10	100
Water samples	5	83	1	17	6	100
Sediment samples	3	75	1	25	4	100

-Regarding table (2), out of 30 fish samples; 20 samples were positive for bacterial. The incidence of bacterial isolation in diseased fish was (75 %), while in apparently healthy fish it was (50%). The positive samples of water for bacteriological findings

represented by (83%), while for sediment samples it was (75 %).

Table(3): The incidence of isolated bacteria obtained from different samples (Fish, water and sediment)

Bacterial isolates	Fish		Water		Sediment		Total	
	No	%	No	%	No	%	No	%
Pasteurella piscicida	13	65	3	60	2	67	18	64
Vibrio anguillarum	3	15	1	20	1	33	5	18
Aeromonas salmonicida	2	10	1	20	-	-	3	11
Staphylococcus spp.	1	5	-	-	-	-	1	3.5
Streptococcus spp.	1	5	-	-	-	-	1	3.5
Total	**20**	**100**	**5**	**100**	**3**	**100**	**28**	**100**

From table (3) it was noticed that *Pasteurella piscicida* were isolated with higher incidence (64%). They were isolated from fish, water and sediment samples. Other microorganisms like *Vibrio anguillarum* were isolated with an incidence of (18%), while *Aeromonas sal*monicida were isolated with an incidence of (11%). On the other hand *Staphylococcus spp*. And *Streptococcus spp*. were isolated with very low incidence (3.5%) for each.

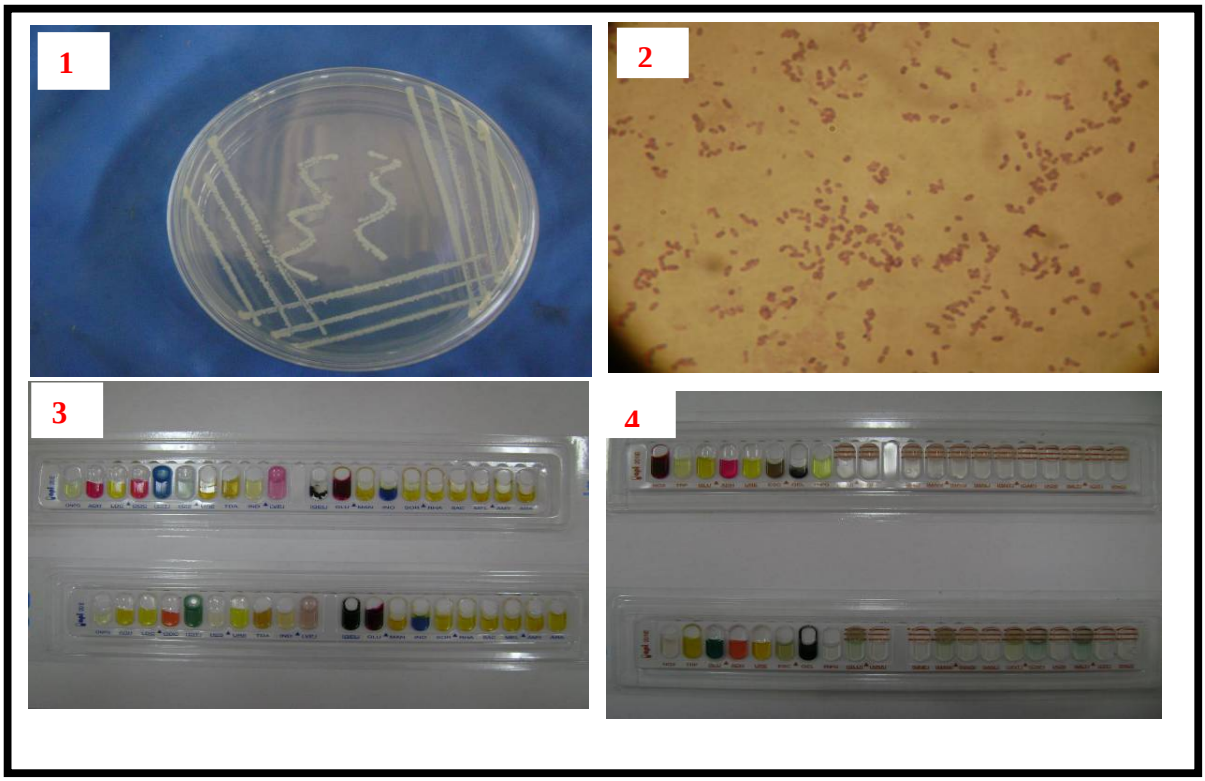

Fig.(1): White small rounded and convex colonies growing onto TSA media representing *Pasteurella piscicida* as pure colonies
Fig. (2) *Pasteurella piscicida* under oil immersion with X 100 showing bipolarity Gram –ve bacilli
Fig. (3): API Identification system of type 20E
Fig (4): API Identification system of type 20NE

4- Parasitological examination

Diagnosis after careful examination and identification using penetrating stereoscope and high power magnification through all samples. We found that only two of samples were positive and containing the whole parasite (worms, adults or parasitic stages). Other samples contained larval cestodes and scolex with bothridia and four armed proboscides or tentacles, encysted on the viscera and in the body cavity had been

confirmed as Metacestodes of the orders Trhypanothyucha and Eutetrarbynchus species, other parasites were larval nematodes where confirmed as contracaecum species. There was another parasite found but not confirmed due to its larval stages need to be complete its life cycle in living fish which was not possible. Black or metallic colour cysts (Microspordiosis, *Glugea spp*. and *Plistophora spp*.) representing spores or larvae of this parasite were seen on the visceral organs and abdominal cavity. Other parasites included larval stages of *Contracaecum spp*. and ***G****onapodasmius epinepheli* (*Didymozoid digenes*) affecting gills.

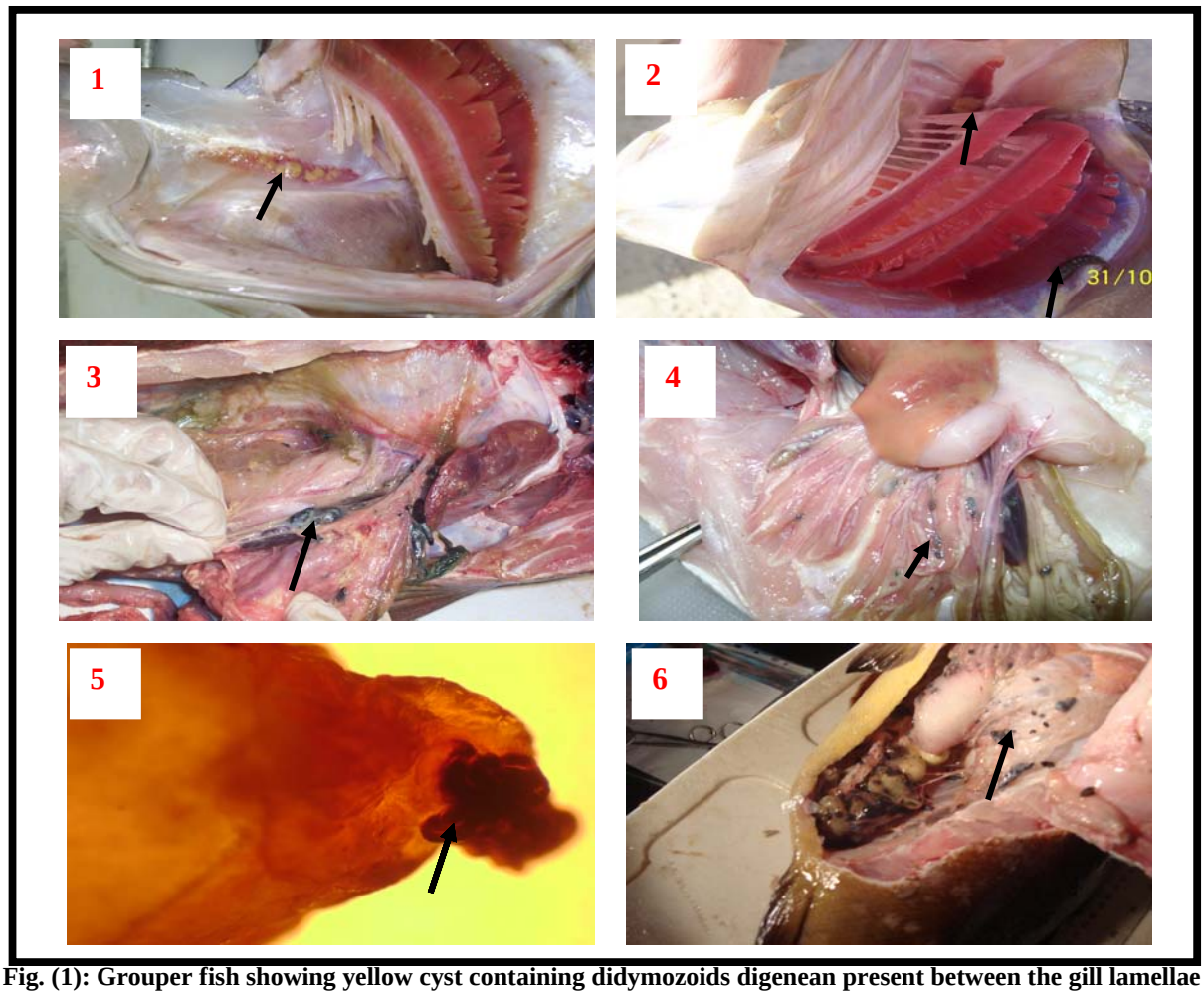

Fig. (1): Grouper fish showing yellow cyst containing didymozoids digenean present between the gill lamellae
Fig. (2): yellow cyst and small black colour larvae present in the gills
Fig. (3): small black cyst (spores of Plistophora spp.) covering the whole visceral organs (heavy infestation). It may take metallic or silver colour
Fig. (4): small black cysts (spores of Plistophora spp.) present between the pyloric caeca
Fig. (5): the whole larva of Plistophora spp under microscope
Fig (6): black cyst of different sizes and shapes filling the abdominal viscera in and around

4. Discussion

Like all animals, marine fish are subjected to numerous diseases, especially bacterial one, in which bacteria play the main role in producing the disease. Diseases are intensified by climatic changes that reflect negatively on the aquatic environment which is a good media for numerous pathogens, and triggered by human interference that lead not only to increase the virulence of these pathogens but at the same time also to the appearance and emergences of new pathogens not present before. Major problems were encountered in grouper fish with heavy mortality due to diseases. Diseases of cultured groupers may be caused by infectious disease agents such as viruses, bacteria, fungi and parasites. Non-infectious disease agents such as nutritional

imbalances and environmental factors may also lead to disease.

The objective of this work is to provide information on diseases observed among the major species of groupers in the East costal area of the Mediterranean sea. It includes bacteriological and parasitological examination with gross clinical signs and postmortem changes. Also to demonstrate grouper fish mortality phenomena at this area. Clinical examination of the apparently healthy and diseased grouper fish revealed that all fish were swimming or floating on water surface with opened operculum and distended air sac or swim bladder, swimming at one side with their abdomen upwardly and rotate around their self as they have nervous manifestation and in some cases sleepy motion.

During catching they showed struggle movement or easily to be catch. In some cases uni or bilateral corneal opacity and all affected fish revealed partial or total blindness with exophthalmia. All the examined cases revealed distended abdomen with anal prolapse. Some of the examined samples showed hemorrhagic spots on the skin with ulcer formation in some cases. Gills were swollen and congested. Most examined cases revealed yellow parasitic cyst in gills with high mucus secretions. Skin depigmentation or discoloration with hemorrhagic spots were also seen.

Data from other investigations revealed nearly the same clinical signs and postmortem lesions .Balebona *et al*. (1998), Evans *et al*. (2000), Varvarigos (2001), Ping *et al*. (2004), Golomazou *et al*. (2006) and Mladineo *et al*. (2006), reported that the affected fish presented several external signs of infection, such as exophthalmia, dark skin pigmentation, erratic swimming, corneal opacity, mucus-covered and pale gills, eroded fins, hemorrhagic gills, and necrotic and epidermic ulcers. The most predominant internal symptoms were the presence of a fatty hemorrhagic liver with petechial hemorrhage and necrotic areas, splenomegaly, enteritis, pale liver, and abdominal swelling with ascitic liquid. Aforementioned lesions are nearly similar to that obtained in our work..

The results in table (1) and (2) indicated that the incidence of bacterial isolation in diseased fish samples was 75% and in apparently healthy one it was 50%, while it was represented in both water and sediment samples by 83 and 75% respectively. From table (3) it was clear that *pasteurella piscicida* were isolated with higher incidence (64 %). They were isolated from fish, water and sediment samples. Other microorganisms like *Vibrio anguillarum* were isolated with an incidence of (18 %), while *Aeromonas salmonicida* were isolated with an incidence of (11 %). On the other hand *Staphylococcus spp*. and *Streptococcus spp* were isolated with very low incidence (3.5 %) for each and this nearly similar to that obtained by Mladineo *et al*. (2006). The previous result indicated that *P. piscicida* were the most prevalent bacteria isolated from grouper fish and were represented in all examined samples (fish, water and sediment) and this indicated that it may be the most important cause of grouper fish mortality in addition to other factors like bad environmental conditions with the heavy infestation with external and internal parasites which were isolated from the same affected cases. *Pasteurella piscicida* causes the same symptoms and PM lesions in all marine fish similar to discussed by Esther et al (2007). Also *Vibrio, Aeromonas* and *Strept spp*. causing to some extent the same disease in marine fish which is called hemorrhagic septicemia.

Also from the previous investigations done by Varvarigos (1997) who suggested that the most economically important bacterial pathogens causing systemic disease in marine fishes were *V. anguillarum* and *Pasteurella piscicida*. Author added that Gram- positive cocci including *Streptococcus spp* and Staph. aureas are also implicated causing septicemia to all fish species mainly during late spring and early summer when sea water temperatures are high. Balebona et al. (1998) conducted a bacteriological survey in three marine fish spp. like gilt-head sea bream, *Sparus aurata* L. and grouper one in southwestern Spain. The authors declared that the main pathogenic microorganisms isolated from diseased fishes were *Vibrio spp* (67.8%), Pseudomonas spp (13.5%), *P. piscicida* (6.7%), Cytophaga or Flexibacter-like bacteria (4.8%), *Aeromonas spp* (0.5%), and Gram positive bacteria. (6.7%). Authors noticed that the highest percentages of isolates corresponded to *Vibrio spp* and *Pseudomonas spp.* The strains of *P. piscicida* and Flexibacter-like bacteria caused epizootics with highest degree of mortalities. On the other hand Dumontet *et al*. (2000) recorded that Vibrios and Aeromonads were the most prevailing bacterial agents detected in sea water and sediment samples collected from the southern coast of Italy during early and late summer. Aeromonads were detected in (62%) of the samples while (42%) were positive for *Vibrio spp*. and these results are disagree with our results where Pasteurella were the main bacterial isolates from water and sediment samples collected from the affected areas.

Hartono et al. (2000) found that diseases caused by bacteria may cause heavy mortality in both wild and cultured grouper fish .Several gram-negative and rod shape bacteria have been identified from internal organs i.e. kidney, spleen and liver. Many of them show sub-clinic infections. The pathogenic bacteria that have been identified were

Vibrio spp., Aeromonas spp., Pasteurella spp., and *Streptococcus spp*. The main suspected factor that may lead to the same clinical signs and postmortem lesions with histopathological findings was related to *Pasteurela piscicida* which cause high mortality and morbidity rate as mentioned also by Zorrilla *et al*. (2003) who conducted a bacteriological study of 25 outbreaks affecting gilthead sea bream, *Sparus aurata* L. in southwestern Spain. The highest number of outbreaks recorded during winter. Most of the isolates (93.19%) were Gram negative. The most frequently isolated bacterial agents were vibrios (69.90%), pseudomonads (9.7%), *P. piscicida* (8.73%), cytophaga (3.88%) and aeromonads (0.97%). *V. alginolyticus* was the *Vibrio* spp most frequently isolated (21.35%). Authors noticed that outbreaks of the highest mortalities were caused by *P. piscicida*. Other studies were done by Alicia *et al*., (2005), they suggested that the most threatening bacterial diseases occurring in marine cultured fishes worldwide are vibriosis, photobacteriosis, furunculosis, flexibacteriosis, pseudomonads septicemia and streptococcosis. Esther et al (2007) demonstrated that the isolates were subjected to taxonomical analyses according to Bergey's Manual of Determinative Bacteriology. The API 20E system (BioMérieux, Madrid, Spain) was used to confirm the biochemical characterizations. The bacterial species identified as *Pasteurella. damselae subspp. damselae* were isolated from the two outbreaks affecting redbanded seabream and from two outbreaks affecting white seabream *P. damselae subspp. Piscicida*, while George and Pantelis (2009) demonstrated that bacterial outbreaks causing serious problems have been mainly induced by *Photobacterium damsella subspp. piscicida, Vibrio alginolyticus, V. harveyi, Tenacibaculum maritimum* and *Flavobacterium spp*. The methods for identification of these bacteria were similar to the methods of isolation and identification performed in our work which demonstrated that the most predominant bacteria isolated from affected grouper fish, water samples and sediment were *P. piscicida* which in our research affecting only grouper fish causing this mortality phenomenon and the symptoms were clear in this type of fish only where this may be refer to the environmental stress factors and high susceptibility of this fish to this microorganism. It was also refer to the sever infestation with parasites that made it more susceptible to bacterial infection, so we focus discussion more on bacterial infection in our research than parasitic infestation.

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