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Monitoring the Cause of Mortality in Some Marine Fishes in Matrouh Governorate, Egypt During the Summer 2008

[1]M.S. Marzouk, [1]M.I. Hanna and [2]Amany M. Kenawy

[1]Department of Fish Disease and Management,
Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
[2]Department of Hydrobiology, National Research Centre, Dokki, Giza, Egypt

Abstract: This study was designed to declare the cause of sudden mortality in different types of marine fishes, namely red grouper, sea bass, sea bream and rabbit, along the sea shore of Matrouh-Saloum in Matrouh Governorate, Egypt during the period from July to October, 2008. The recorded clinical signs in the collected fishes were external hemorrhage, inflamed hemorrhagic vent and abdominal distension in some fishes, while others were apparently normal. P/M examination revealed mottled liver, congestion of the internal organs and also severe inflammation of both gastric and intestinal mucosa with excess amount of mucoid exudates. Histopathological examination revealed variable degrees of hepatocytic vacuolar degeneration, circumscribed areas of melanomacrophage cells aggregations in between the hepatocytes, vasculitis and bile duct hyperplasia. Spleen showed focal depletion in the haemopoietic tissue, increased number of melanomacrophage centers and congestion in the spleenic blood vessels. Brain showed vascular congestion and oedema in the cerebral cortex together with focal gliosis. Bacteriological examination proved the isolation of *Photo. bacterium* sp. (*Pasteurella damsela*) in association with the above mentioned clinical and histopathological changes as a primary cause. *P. damsela* identification was done through phenotypic, culture and biochemical characters. In conclusion: *Pasteurella damsela* could be incriminated as the cause of sudden acute mortality of marine water fishes in Matrouh governorate, Egypt during this period.

Key words:*P. damsela* · Clinical signs · Histopathology · API-20E · Red grouper · Matrouh · Summer

INTRODUCTION

Pasteurellosis is a bacterial septicaemic disease which is also referred to as pseudotuberculosis because, in chronic cases, diseased fish show, whitish tubercles consisting of bacterial accumulations in several internal organs. The halophilic bacterium *Fig.bacterium damselae subsp. piscicida* (formerly *Pasteurella piscicida*) is the etiological agent of pasteurellosis. Due to the change in the taxonomic position of the causative agent, some authors have renamed this septicaemia as Fig.bacteriosis. It should be mentioned that a temperature of 20-25°C seems necessary for the development of outbreaks of pasteurellosis [1]. Today, the natural hosts of the pathogen are a wide variety of marine fishes. Pasteurellosis has had a great economic impact in Japan, where it affects mainly yellowtail cultures and in the Mediterranean area, because of the losses it causes in seabream and sea bass farms. *Pasteurella piscicida*, is one of the most threatening diseases of wild and cultured marine fish and has been reported from many geographical areas including the USA, Japan and the Mediterranean countries. *Fig.hacteriun damsela subsp. piscicida* is the causative agent of the fish pasteurellosis. This disease was first observed in natural populations of white perch (*Morone americanus*) and striped bass (*Morone saxtilis*) in 1963 in Chesapeake Bay, USA [2]. In 1969, the pathogen became economically significant with detrimental fish losses in the Japanese fish culture industry [3]. Until recently, Europe was considered to be free of fish pasteurellosis. However, in 1990 several outbreaks of fish pasteurellosis occurred in cultured

Corresponding Author: Dr. Amany M. Kenawy, Department of Hydrobiology, Veterinary Research Division, National Research Centre, Postal code: 12622, Dokki, Giza, Egypt

populations of sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) in different European countries including France [4], Italy [5], Greece [6], Portugal [7], Turkey [8] and Malta and Israel [9]. Pasteurellosis continues to be a serious problem in the intensive culture of different fish species in the Mediterranean Sea and Japan. The taxonomic position of *P. damselae subsp. piscicida* is controversial. Based on morphological and biochemical characterization, the organism was first placed in the genus Pasteurella and given the name '*Pasteurella piscicida'* or 'fish killer' [10]. Although this species is clearly distinguishable from the other species within the genus Pasteurella (i.e. by positive reactions for arginine dihydrolase and the methyl red test, as well as negative reactions for nitrate reduction and growth at 37 and 42°C, the organism remained in this genus. However, it was reported that, based on analysis of rRNA cistrons, *'Pasteurella piscicida*' should be classified in the family Vibrionaceae [11]. This argument was supported by fatty acid methyl ester profiling [12]. More recently, it was found that, based on rRNA sequence and DNA-DNA hybridization data, the pathogen was closely related to *P. damsela* and proposed that it be placed in the genus photo. bacterium sp as a subspecies of *P. damsela* [13]. Pasteurellosis has been identified in several wild fish species, including the following: white perch (*Morone americanus*), striped bass (*Morone saxatilis*), menhaden (*Bsevoortia tyrannus*) and sea mullet (*Mugil cephalus*). Pasteurellosis has also been found in a wide variety of economically important mariculture species, including: yellowtail (*Seriola quinqueradiata*), ayu (*Plecoglossus altivelis*), red sea bream (Pagrus major), gilthead sea bream (*Sparus aurata*), sea bass (*Dicentrachus labrax*), striped jack (*Pseudocaranx dentex*) and hybrid striped bass (*Morone saxatilis* x *M. chrisops*) [14]. It is also recorded in red grouper [15]. The disease has great economic impact both in Japan, where it affects mainly yellowtail cultures and in the Mediterranean area, due to the losses it causes in seabream and seabass farms [16]. Identification of *Pasteurella piscicida,* the causative agent of fish Pasteurellosis, can be performed with the use of classic isolation onto agar plates and identification based on biochemical profiles (use of API 20E) [6].So this study was planned to investigate the role of Fig.bacteium sp. as a suspected cause of acute mortality in some wild marine water fishes, namely red grouper, sea bass, sea bream and Rabbit, along the coastal area of Matrouh governorate, Egypt with application of different laboratory procedures for morphological and biochemical characterization of the isolated bacteria. Also, the recording of both clinical and histopathological changes in moribund marine fishes,is one of the objectives of this study.

MATERIAL AND METHODS

Fish Sampling: After the history of sudden fish mortality in different locations namely Matrouh, Sedi-Elbarani and Saloum in different marine fishes in the period between July to October,2008, specimens from diseases red grouper, common sea bream, Sea bass and rabbit fishes were collected from these areas.Both moribund and apparently normal specimens were killed with an overdose of MS-222 in sea water (65 mg/ml, final concentration; Sigma Chemical Co., St Louis, MO, USA) and immediately processed for bacteriological analysis.

Clinical Investigation and Post Mortem Examination: The moribund fishes were properly examined for any external clinical abnormalities and PM lesions [17].

Bacteriological Examination: Samples collected under strict aseptic precautions from the spleen, liver and kidneys of the dead specimens were seeded on tryptic soy agar and broth (Difco, Detroit, MI, USA) supplemented with 1.5% NaCl (TSAS, TSBS), thiosulfate-citrate-bile salt-sucrose agar (TCBS, Biolife, Milan, Italy. The inoculated media were incubated at 25°C for 2-5 days. The isolate was subjected to taxonomical analyses according to *Bergey's Manual of Determinative Bacteriology* [18]. The API 20E system (BioMérieux, Madrid, Spain) was used to confirm the biochemical characterizations.

Water Analysis: many water samples were collected from different sites along the coastal area of Matrouh governorate from areas with a water depth ranged from 4-5m.Water parameters namely temperature, dissolved oxygen, salinity and pH were measured on spot using temperature and oxygen meter, salinometer and pH meter, respectively.

Histopathological Studies: Tissue specimens from liver, spleen,brain, kidneys and intestine were taken from naturally infected fish. The samples were fixed in 10%

formal saline, processed by conventional method, sectioned at 4 µm and stained with Haematoxylin and Eosin [19,20].

RESULTS

The most observed clinical signs and postmortem changes in naturally infected and sacrified fishes, especiallyred grouper were darkening of the skin with abdominal distension in as well as haemorrhagic inflamed vent (Figs. 1-3). Pale gills and mottled liver were also observed (Figs. 4,5). Congestion of the internal organs and severe inflammation of both gastric and intestinal mucosa with excess amount of mucoid exudates were clear (Figs. 6,7). It is of significance to state that sea bass is the type of fishes collected without any clinical signs. The mortality rate is not properly determined but it is generally law and it is decreased and finally stopped in association with gradual decrease in water temperature.

Table 1: Biophysical and biochemical characteristics of the isolated Fig.bacterium species

Characteristic	Reaction
Gram stain	+
Bipolar staining	Short rods
Cell morphology	-
Motility	
Growth on:	+
Nutrient agar	+
BHI agar	-
MacConkey agar	-
Blood agar	+ with non haemolytic
zone	
TCBS	-
Growth at:	
10°C	-
25°C	+
37°C	-
Growth in:	
0% NaCl	-
0.5% NaCl	+
1.5% NaCl	+
3% NaCl	+
Acid production from:	
Glucose	+
Mannose	+
Galactose	+
Fructose	+
Maltose	-
Sucrose	-

Table 2: Water parameters of different examined water samples

Water parameter	Average value
Water temperature (°C)	24.0±0.5
Water salinity (ppt)	40.0±0.1
Dissolved oxygen (ppm)	8.5±0.1
Water pH	8.3±0.1

The cultured bacterial colonies appeared convex, viscous, regular, opaque to translucent colonies,1-2 mm diameter and were developed within 48 hrs of the incubation when incubated at 25°C (Figs. 8,9). The isolated bacteria were Gram negative,bipolar-staining, non motile bacillus (Figs. 10,11). The biophysical and biochemical characteristics of the isolate are to be seen in Table 1. The measured water parameters were recorded in Table 2.

The microscopical examinations of all tissues of red grouper revealed various pathological changes among different organs. The histopathological examination of the liver of many cases demonstrated variable degrees of the hepatocytes vacuolar degeneration where the hepatocytes were found swollen, vacuolated with pyknosis of their nuclei and /or associated with small areas of necrosis (Fig. 12). Congestion of hepatic blood vessels accompanied with hyperplasia in the wall of hepatic blood (vasculitis) were observed (Fig. 13). Several cases showed presences of large numbers of circumscribed areas of aggeregated melanomacrophage cells associated with infiltration of chronic inflammatory cells in between the hepocytes (Fig. 14). Bile duct hyperplasia were also seen (Fig. 15). The pancreas exhibited slight degenerative changes in the pancreatic acinar cells with some peri-gl andular mononuclear cell infiltrations.

Examination of the spleen revealed increased numbers of melanomacrophage centers associated with congestion and hyperplasia in the wall of spleenic blood vessels. Focal lymphoid depletion was also detected (Figs. 16,17).

The histopathological changes in the brain revealed congestion in the submeningeal and cerebral vessels, pale and shrunken neurons and necrotic glial cells associated with focal gliosis (Fig. 18) and oedema in the cerebral cortex (Fig. 19).

The Histopathological changes in the intestine exhibited mucinus degeneration and desquamation in the epithelial lining the intestinal villi accompanied with mononuclear cell infiltrations in the lamina propria (Fig. 20).

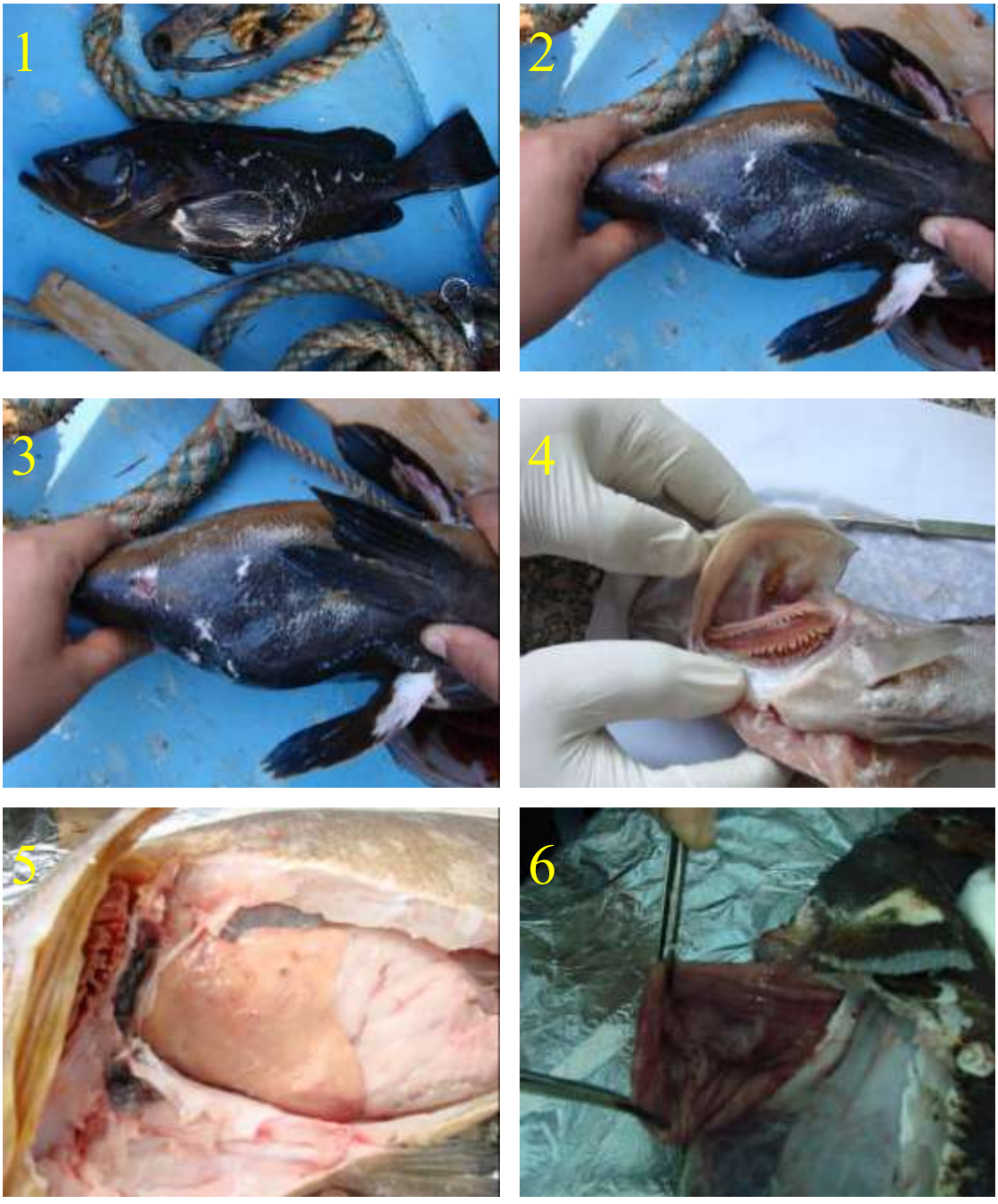

Fig. 1: A grouper catched from the coastal area of Matrouh.
Fig. 2: Red grouper with darkening of the body colour and abdominal distension.
Fig. 3: Red grouper with haemorrhagic inflamed vent.
Fig. 4: Grouper fish with pale anaemic gills.
Fig. 5: Grouper fish with mottled enlarged liver.
Fig. 6: Red grouper with severe inflammation of the gastric mucosa.

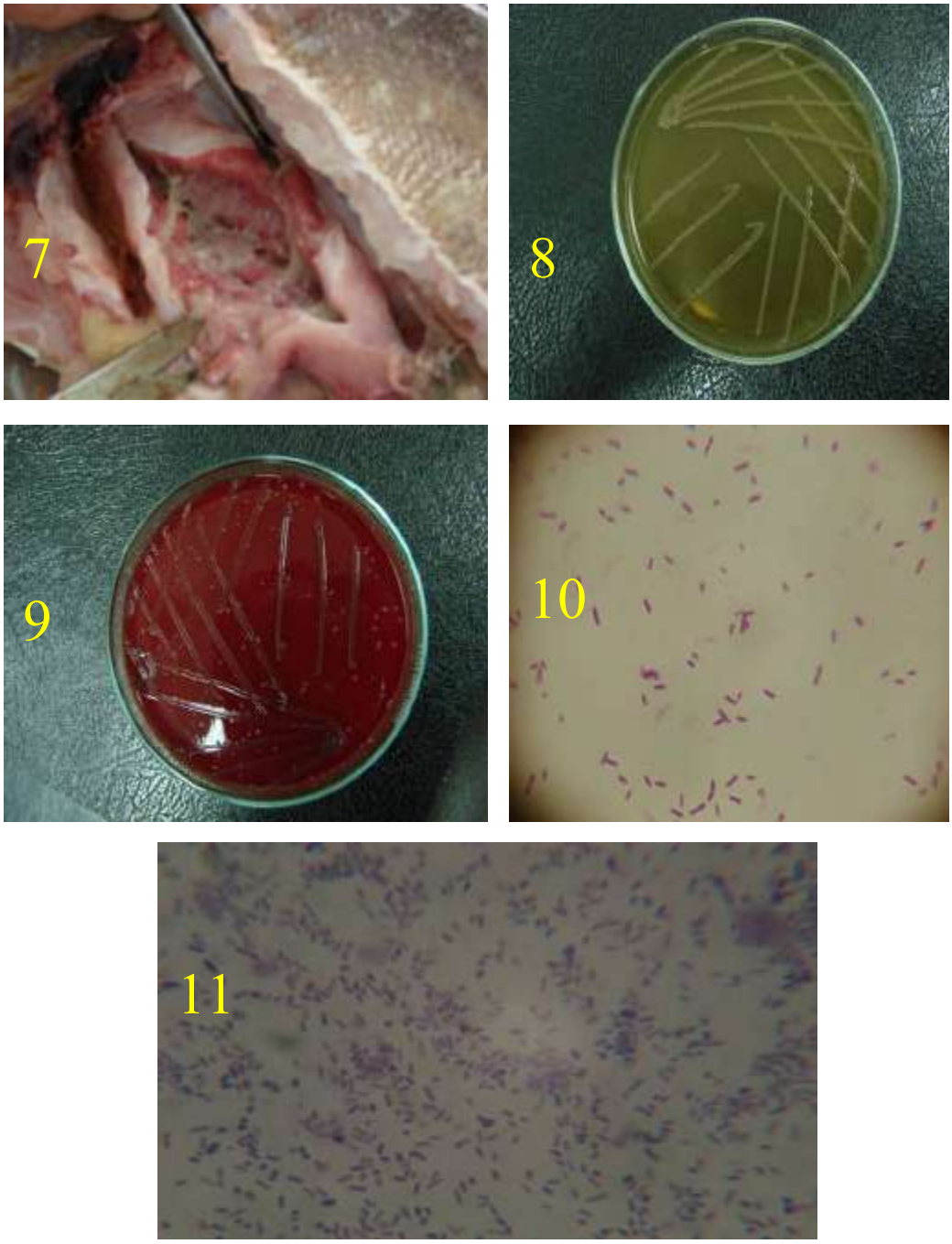

Fig. 7: Severe inflammation of the gastric mucosa with excess amount of mucoid exudates in the examined fish.
Fig. 8: Grayish white viscous colonies of Photobacteria with Ի2mm diameter on Brain Heart Infusion Agar supplemented with 2% Nacl.
Fig. 9: Non-haemolytic colonies of Photobacteria on sheep blood agar..
Fig. 10: Bacterial smear stained with Gram`s stain showing Gram negative , non motile bacillus.
Fig. 11: Bacterial smear stained with Giemsa stain showing bipolarity staining.

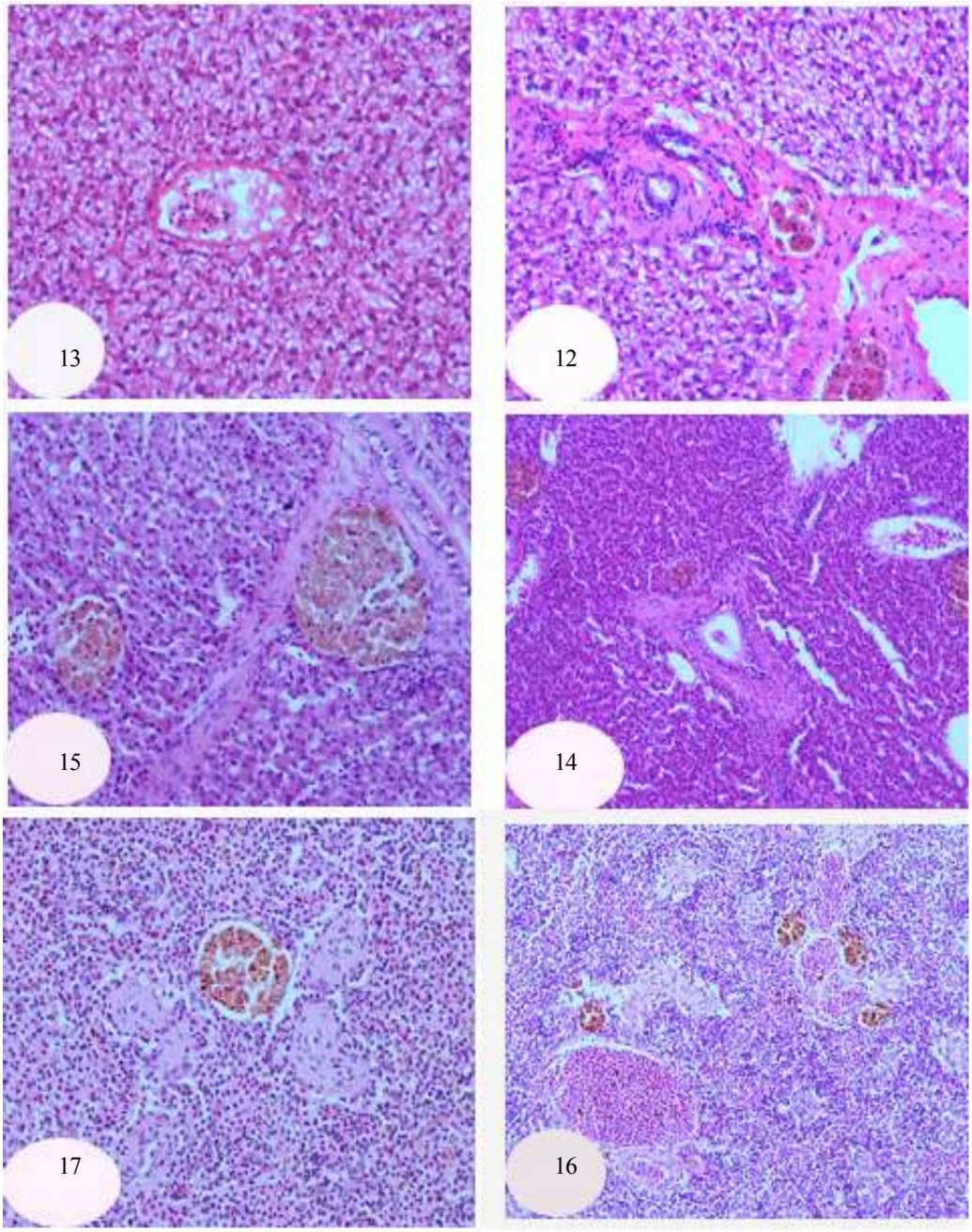

Fig. 12: Liver showing vacuolar degeneration in the hepatocytes, pyknosis of their nuclei and small areas of necrosis H&E stain, X 250.

Fig. 13: Liver showing congestion of hepatic blood and hyperplasia in the wall of hepatic blood (vasculitis) H&E stain, X 250..

Fig. 14: Liver showing circumscribed areas of aggeregated melanomacrophage cells and infiltration of chronic inflammatory cells in between the hepocytes H&E stain, X 250.

Fig. 15: Liver showing Bile duct hyperplasia H&E stain, X100.

Fig. 16: Spleen showing increase in the numbers of melanomacrophage centers H&E stain, X 250.

Fig. 17: Spleen showing congestion and hyperplasia in the wall of spleenic blood vessels. Focal lymphoid depletion H&E stain, X 100.

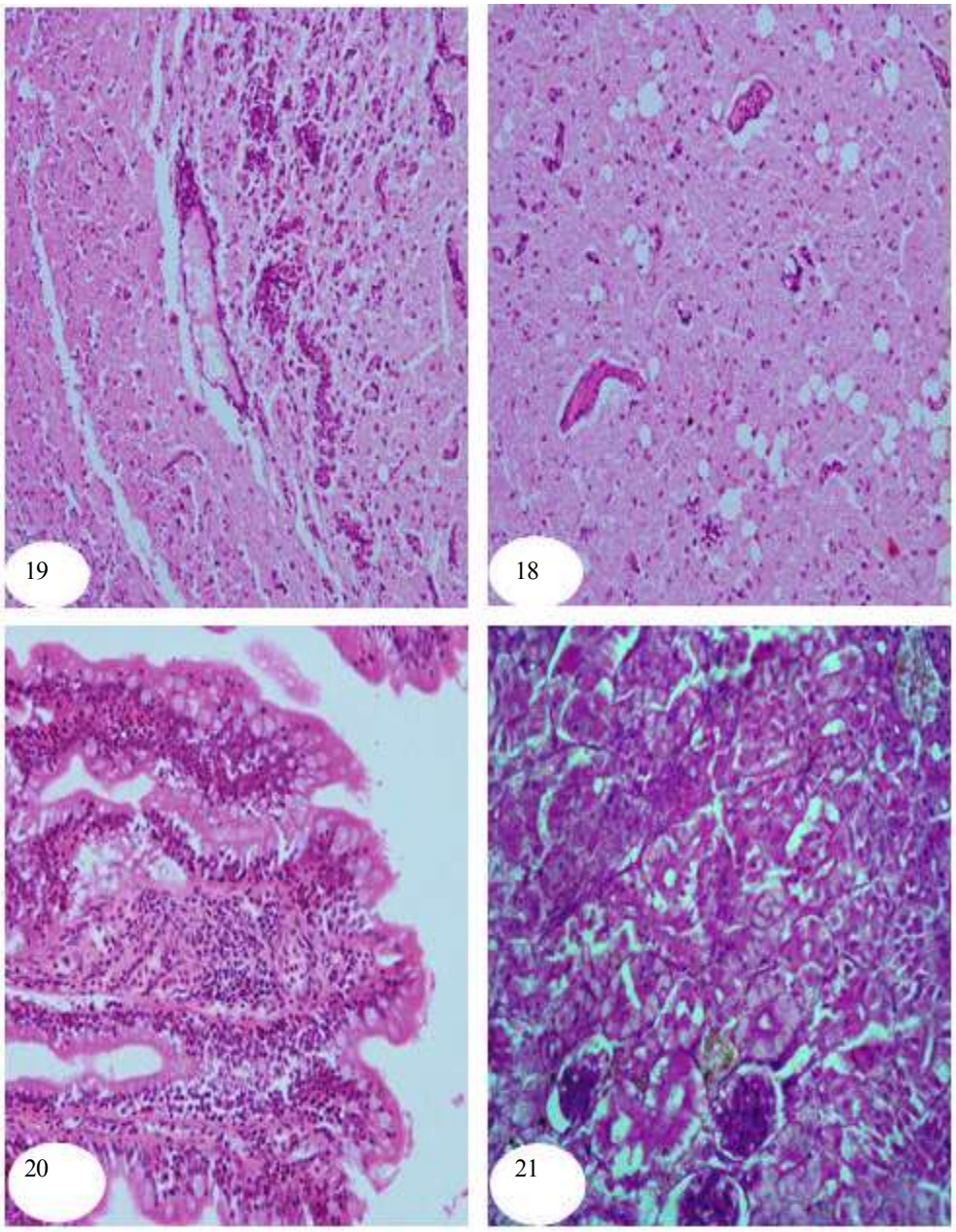

Fig. 18: Brain showing congestion in the submeningeal and cerebral vessels, Pale and shrunken neurons and necrotic glial cells associated with Focal gliosis H&E stain, X 400.
Fig. 19: Brain showing oedema in the cerebral corteX H&E stain, X 400.
Fig. 20: Intestine showing mucinus degeneration and desquamation in the epithelial lining the intestinal villi accompanied with mononuclear cell infiltrations in the lamina propria H&E stain, X 400.
Fig. 21: Kidney showing vacuolar degeneration in the tubular epithelium, focal necrosis in the interstitial tissues, aggregation of melanomacrophage cells in between the interstitial tissues, Periglomerular and peritubular oedema. H&E stain, X 400.

Examination of the kidneys revealed vacuolar degeneration in the tubular epithelium associated with focal necrosis in the interstitial tissues, periglomerular and peritubular oedema. Aggregation of melanomacrophage cells in between the interstitial tissues were also seen (Fig.,21).

DISCUSSION

Pasteurellosis is a bacterial septicaemia also referred to as pseudotuberculosis which continues to be a serious problem in the intensive culture of different fish species in the Mediterranean Sea and Japan. The observed clinical signs and postmortem changes in the moribund fish revealed darkening of fish skin with abdominal distension, hemorrhagic inflamed vent,pale gills and mottled liver in addition to congestion of the internal organs with severe inflammation of both gastric and intestinal mucosa and excess amount of mucoid exudates.The mortality rate was low. Although these findings are non-diagnostic to specific illness,but they are more or less similar to those recorded by Robohom[21] and Hawke [22]. Also, it was stated that the gross external signs of pasteurellosis are usually inconspicuous and there are generally no surface lesions[1,23]. Only some affected fish may exhibit a darkening of body color and/or slight hemorrhagic areas in the head and gills. The external clinical symptoms of the *P. piscicida* infection are darkening of the skin, abdominal distention, hemorrhagic areas in the head and gills and, in some cases, anaemia in acute form [14,24]. In chronic cases, white nodules are present in several internal organs. The mortality in the cultured species ranges from low, in sub-clinical forms, which occur at low temperatures, to as high as 90%, in some cases [25]. Usually, the loss is around 10%. In wild populations, extreme cases of estimated 50% mortality have been reported (first isolation in wild population, in Chesapeake Bay, USA) [2]. Mass mortality of mullet has been observed along the coast of some Mediterranean countries including the Campania region of Southern Italy in August 2006 [26].

The phenotypic, biophysical and biochemical criteria of the isolated bacteria1) are resemble to those recorded for *P. piscicida* (Fig.bacterium species) [2, 10, 13, 20, 21, 27, 28]. In agreement with Kent [28] although *P. piscicida* is not actually included in the API 20E code index, this system is valuable for a rapid identification of presumptive *P. piscicida,* because all the strains analyzed had a similar pattern (no. 2 005 004), with no false-positive or -negative reactions being detected [29].

It seems that the recorded water temperature (24± 0.5°C) was a suitable environmental temperature for Fig.babcterium species bacteria.In this aspect,it was mentioned that a temperature of 20-25C seems necessary for pseudotuberclosis outbreak [1]. *Ph. damselae* subsp. *piscicida* (formerly *P. piscicida*), the causative agent of pasteurellosis/ pseudotuberculosis, is a serious condition of both farmed and wild-fish populations [30]. Substantial mortalities, i.e. 40-50% of the stock, have occurred during summer months and it is thought that infection takes place in seawater at 25°C [30].

The microscopical examination of the liver of several infected cases demonstrated variable degrees of the hepatocytes vacuolar degeneration associated with small areas of necrosis. These findings are more or less similar to those of Hawke *et al*. [31] who observed extensive necrosis and karyorrhexis in the hepatic tissues of naturally *P.damselae*-infected striped bass. Also, the obtained findings are supported by those of Reyad and Salah [32]. They mentioned that the liver displayed vacuolar degeneration of most hepatic cells with pyknosis of their nuclei,congestion accompanied with vasculitis where there are severe hyperplasia in the wall of hepatic blood vessels. In our opinion, these vasculitis which were observed may correspond to bacterial artirities infection. Moreover, Bile duct hyperplasia and presence of large numbers of circumscribed areas of aggeregated melanomacrophage cells associated with infiltration of chronic inflammatory cells in between the hepocyte which were as a chronic granulomatous reaction to bacterial infection [32]. Most of these findings are similar to a great extent to those of Foyle *et al*. [33] who stated that the histopathological examination of naturally infected fish revealed non specific sings of septicaemia migration and extravasation of neutrophils and macrophages in the liver and spleen.

The pancreas exhibited slight degenerative changes in the pancreatic acinar cells with some peri-gl andular mononuclear cell infiltrations. These findings were parallel with that described by Reyad and Salah[32]in that the pancreas exhibited peri-gl andular edema and the pancreatic acinar cells showed more eosinophilic granular cytoplasm with some peri-gl andular mononuclear cell infiltrations in the experimentally infected Nile tilapia with the Ph3 isolate of *Fig.bacterium*, during the acute stage.

Examination of the spleen revealed an increase in the number of melanomacrophage centers, congestion, hyperplasia in the wall of spleenic blood vessels and focal lymphoid depletion. The changes in the spleen were previously described by Foyle *et al*. [33] who found an early necrosis in the splenic ellipsoids in naturally

infected fish with *pasteurella piscicida*. Also [32] could detect degeneration and necrosis of melanomacrophage center with focal lymphoid depletion in the spleen of experimentally infected Nile tilapia with the Ph3 isolate of *Fig.bacterium*, during the acute stage.

Examination of the kidneys revealed vacuolar degeneration in the tubular epithelium, focal necrosis in the interstitial tissues, periglomerular and peritubular oedema. Aggregation of melanomacrophage cells in between the interstitial tissues. These findings were parallel with that described by Foyle *et al*. [33] who stated that there was depletion and some necrosis of renal haemopoietic tissue in naturally infected fish and in experimentally infected fish which was pointed out by Reyad and Salah [32] they found that The renal epithelium showed vacuolation and focal depletion in the hematopoietic tissues. Internally, differences can be seen between the acute and the chronic form of Fig.bacteriosis. In the acute stage there are few pathological changes: these consist of multifocal necrosis in liver, spleen and kidney and bacterial accumulations inside and outside macrophages and in the interstitial spaces. The chronic lesions, in addition to the white nodules are characterized by the presence of necrotized macrophages in the internal organs with intact bacterial cells [14].In wild fish populations,few pathological changes can be noted in fish with acute Fig.bacteriosis where as a chronic infection characterized by military lesions in the kidney and spleen [21,35].

Regarding the histopathological changes in the brain, congestion in the submeningeal and cerebral vessels and oedema in the cerebral cortex were noticed. Pale and shrunken neurons and necrotic glial cells associated with Focal gliosis. These fingings accentuates the findings of by Ahmed [34] in hybrid striped bass infected with *Fig.bacterium damselae subspecies piscida*.

Concerning the histopathological changes in the intestine,mucinos degeneration and desquamation in the epithelial lining the intestinal villi accompanied with mononuclear cell infiltrations in the lamina propria were clear. The same results were also detected by Reyad and Salah [32].

Bacteria were not obvious in any of the organs examined these result was disagree with Tung *et al*. [23], Hawke *et al*. [31], Nelson *et al*.[36], Noya *et al*. [37] and Beatriz *et al*.[38] as they recorded that the liver, kidney and spleen show a multifocal necrosis and the presence of bacterial accumulations, free and within phagocytes, in the capillaries and in the interstitial spaces. In our opinion, based on the above information no lesions were considered severe enough to provide a suitable explanation for the cause of death, but may be attributed to bacterial toxins of pasteurella. These results are in consistent with several previous report of Beatriz *et al*. [38] as they found that The extracellular products (ECP) secreted by a variety of bacterial fish pathogens are important virulence factors since they can contribute to the development of the disease in terms of bacterial nutrition or as aggressins enabling the bacteria to counteract the host's defense mechanisms. The ECP of *Pasteurella piscicida* strains were shown to be lethal for different fish species including gilthead seabream, seabass, turbot and rainbow trout.

In conclusion *P.damsela* was isolated in pure form from the different marine fishes namely grouper,sea bass,sea bream and rabbit fishes in Matrouh governorate, Egypt during the mortality attack, the matter of which indicated its important role in this mortality either alone or in combination with other organisms or even some environmental pollutants. It is a matter of interest to diagnose this pathogenic bacteria in Egyptian coastal areas at Mediterranean-sea therefore further investigations are required.

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