Streptococcosis in tilapia: Clinico-pathological picture of experimentally infected tilapia

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Abstract: Six groups of Tilapia fish were inoculated intraperitoneally using isolates of *S. pluranimalium, S. dysgalactiae, E. fecalis, E. gallinarum, L. garvieae, and S. anginosus* isolated from natural infection in different localities in Egypt, with infective dose $3x10^7$ CFU ml^{-1}. The inoculated fish were observed for 21 days for any clinical abnormalities and careful postmortem examination. The infected fish showed loss of appetite, slight enlargement of the abdomen; except for which inoculated by *S. pluranimalium*, most of the fish showed slight redness at the base of the dorsal fin. No mortalities were recorded in fish groups inoculated with *S. pluranimalium*, *S. anginosus*, *E. fecalis*, and *E. gallinarum*, while mortalities reached 100% in the other fish groups. Specimens were taken from liver, kidney, spleen, and brain for histopathological examination revealed inflammatory cells infiltration, degenerative changes, different stages of necrosis, and marked fatty changes specially in liver and kidney, renal and splenic thrombosis specially in fish group inoculated with *S. anginosus*, congested blood vessels was noticed in all organs specimens examined, meningitis especially of the meninges of the telecephalon characterized by thickening with congested blood vessels and inflammatory cells infiltration, marked brain edema was observed in all groups mostly as perivascular and pericellular edema was noticed and necrotic and degenerative changes were observed in the neurons.

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I- Introduction

Streptococcosis is a septicemic disease caused by different *Streptococcus* species and other closely related genera including *Lactococcus* and *Enterococcus*. Stress is usually one of the predisposing factors resulting in streptococcosis outbreaks such as poor environmental condition; rising in the environmental temperature, harvesting, bad handling, transportation, and poor water quality (Francis-Floyd and Yanong, 2013).

The interest in studying streptococcosis in fish has been increased due to the dramatic economic losses results from its outbreaks which is estimated hundred million dollars annually or exceeding (Haghighi, 2010). Streptococcosis attacking both freshwater and marine fishes (Kusudaand Komatsu, 1978, Kitao, 1993, and Austin and Austin, 1999) causing high mortality rate which may reach more than 50% within 3 to 7 days in acute cases, or few mortalities over a long period of time "several weeks" in the chronic cases (Francis-Floyd and Yanong, 2013).

Mortalities may reach 100 % within 2-4 days, if the bacterial concentration in aquaculture is high (Ferguson, *et al.*, 1994), accordingly most of the Asian countries considering it as a devastating disease of high economic importance (Pasnik *et al.*, 2005).

Streptococcosis in fish has a wide host range and can affect different fish species including rainbow trout, seabream, tilapia, yellowtail, catfish species, killifish, menhaden species, mullet, and silver pompfret, sturgeon, sea bass, eel, sea trout, striped bass, mullet, salmon, bottle nose dolphin and tilapia (Inglis *et al.*, 1993, Evans *et al.*, 2005, and Abdullah, 2013). It's also known as "Pop-eye disease", and this is due to the accumulation of mucopurulant exudates around the eye (Romalde and Toranzo, 1999). The clinical signs differ according to the fish species, and the chronicity of the disease as it sometimes doesn't associated with any apparent clinical signs, and in other cases it causes: erratic swimming (as it affects the nervous system), lethargy, loss of buoyancy, darkening of the skin, uni-lateral or bilateral exophthalmia (pop-eye); corneal opacity, hemorrhage inside or around eye, the gill plate, base of the fins, vent/ anus, or on the body, ascites (accumulation of yellow or reddish yellow fluids in the abdominal cavity / dropsy/ bloating), yellow exudates covers the

peritoneum and in the cranial cavity, hemorrhage in muscle and intestine, anal prolaps, pale, yellow, or mottled red liver, congested kidney and spleen, splenomegaly, sometimes empty stomach and intestine, and intestinal hemorrhage may be observed (Ferguson *et al.*,1994, El-Bouhy, 2002, Zeid, 2004, Torky *et al.*,2006, Haghighi *et al.*,2010, Abdullah *et al.*,2013 and Francis-Floyd and Yanong, 2013).

The most common species results in streptococcosis in fish are *S. milleri* (*S. anginosus)* (Whiley, 1990), *Streptococcus difficilis* (Austin and Austin, 1999), *S. agalactiae* (Brian,2001) *S. dysgalactiae* (Austin and Austin, 2007), *S. parauberis*, and *Streptococcus iniae* (*S. shiloi*) (Francis-Floyd and Yanong, 2013),.There are closely related genera to *Streptococcus* causing signs like of streptococcosis as (Austin and Austin, 1999), *Lactococcus garvieae*) *Enterococcus seriolicida* (Eldar *et al.*,1996), *L. piscium*, *Vagococcus salmoninarum* (Francis-Floyd and Yanong, 2013), and *Enterococcus faecalis* (Torky *et al.*,2006).

This study evaluates the pathogenicity of different pathogens causing streptococcosis in experimentally infected Tilapia fish and following up the disease progress through recording the clinical signs observed and the histopathological findings of the specimens collected from organs of freshly dead and moribund fish.

II- Material and methods

Seventy apparently healthy Tilapia fish weighting 50 ± 5 grams were supplied from a private fish farm at El-Tal El-Kebir. They were divided into 7 groups, 10 fish per each, one served as control and the other six groups used for experimental inoculation. The fish were transferred in boxes supplied with aeriators to the "Fish Disease Department" in Animal Health Research Institute in Dokki. Fish were maintained in glass aquaria supplemented with dechlorinated water with temperature 25± 2 ° c using thermostatic heaters and aerators. Fish were fed 3% of their body weight / day commercial fish diet throughout of the experiments duration (El-Sayed, 2006).

Six bacterial isolates were isolated from naturally infected tilapia fish from different locations in Egypt (*Streptococcus pluranimalium* (*S. pluranimalium), Streptococcus dysgalactiae (S. dysgalactiae), Enterococcus fecalis (E. fecalis), Enterococcus gallinarum (E. gallinarum), Lactococcus garvieae (L. garvieae), and Streptococcus anginosus (S. anginosus).*

They were cultivated on brain heart infusion agar supplemented with 5% sheep blood agar then incubated at 25 ºC for 24 - 48 hour (Torky *et al.*,2006). A bacterial suspension was prepared from each isolate with turbidity matching with McFarland standard number 1 (which is equivalent to 3 x 10^{8} CFU ml^{-1}), and then 10 fold serial dilution was done for each isolate to reach a concentration 3 x 10^{7} CFU ml^{-1}, 0.2 ml of the bacterial suspension was inoculated intraperitoneally in the corresponding fish group (Austin and Austin, 1999, Moustafa *et al.*, 2010 and Abuseliana *et al.*, 2011). The inoculated fish groups were observed for 21 days and any clinical signs were recorded. At the end of the experimental period, all fish still alive were sacrificed and careful postmortem examination was carried out. Tissue specimens were collected from liver, kidney, spleen, and brain from both moribund and freshly died, or sacrificed fish with pitting technique for histopathological examination. The specimens were fixed in 10% neutral buffered formalin for 24 hours, then washed using tap water, and serial dilutions of ethyl alcohol were used for dehydration. The specimens were routinely cleared in xylene, embedded in paraffin. Paraffin blocks were sectioned at 4 microns thickness by microtome. The obtained tissue sections were stained by haematoxylin and eosin (H&E) stain according to (Roberts, 2001).

III- Results

1- Clinical signs and postmortem lesions

All fish groups were observed for 21 days. No abnormal signs appeared in the control fish group. While, the inoculated fish groups showed variable clinical abnormalities including; loss of appetite in the first 5 days post inoculation, then slight enlargement of the abdomen in all fish groups except for the group inoculated by *S. pluranimalium*. Most fish groups showed slight redness at the base of the dorsal fin. Concerning mortalities; no mortalities were recorded in all fish groups inoculated by *S. pluranimalium*, *S. anginosus*, *E. fecalis*, and *E. gallinarum*, while mortalities reached 100% in the other fish groups; in which 100% of fish inoculated with *S. dysgalactiae* in the 4th day post inoculation. While 16 days post inoculation of fish group inoculated by *L. garvieae* .

Regarding the postmortem (PM) examination of moribund, freshly died, and sacrificed fish revealed dark pigmentation of the skin, ophthalmic abnormalities including eye hemorrhage and opacity, some fish exhibited congested or pale gills, distended abdomen, congested base of the fins. Some fish groups showed pale friable liver while livers of others were congested, renal enlargement and congestion. In addition, some fish groups showed clear or hemorrhagic ascites, and few showed congested dark spleen (Figure1). The observed PM lesions in all fish groups are summarized in (table1).

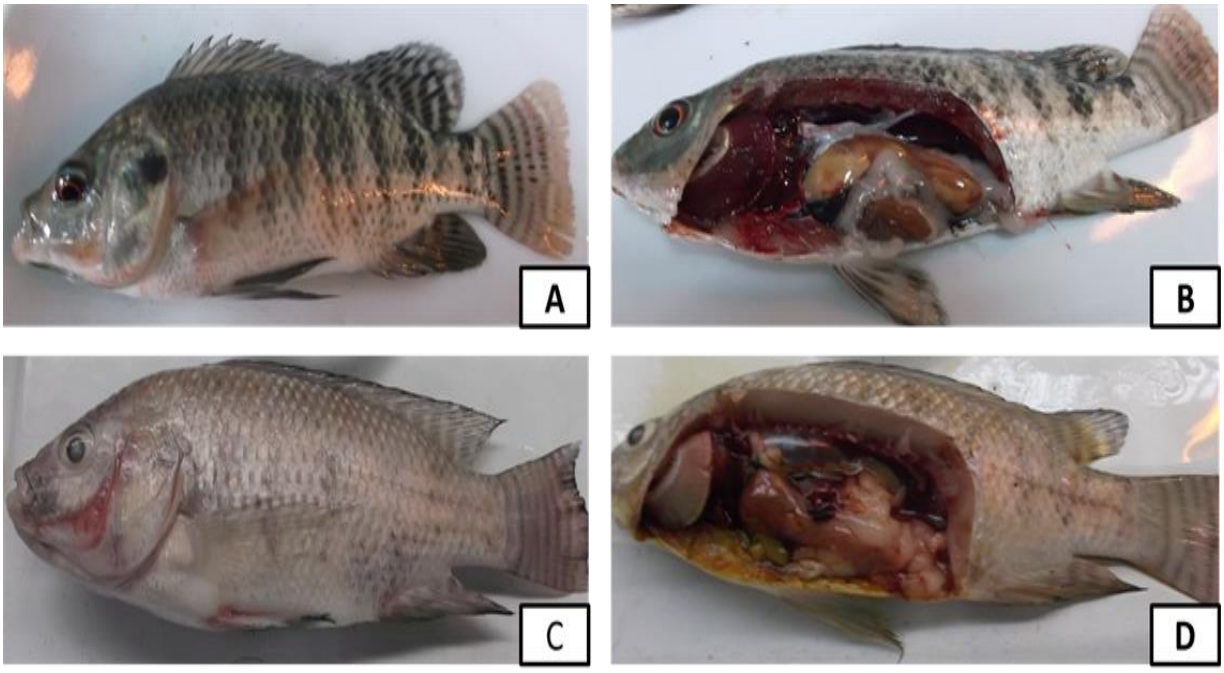


Figure 1: Freshly died tilapia fish inoculated with *S. dysgalactiae* showing externally **A**: eye hemorrhage, dark skin discolouration in the form of transverse strips, slightly distended abdomen, while internally **B:** congested gills, enlarged and congested kidney, enlarged friable mottled liver, dark gall bladder, intestinal congestion, and congested heart. **C:** freshly died tilapia fish inoculated with *L. garvieae* showing externally pale skin with detached scales, eye opacity, abdominal distension, while internally **D:** friable pale liver, hemorrhagic ascites, pale gills, and enlarged congested kidney.

2-Histopathological examination results

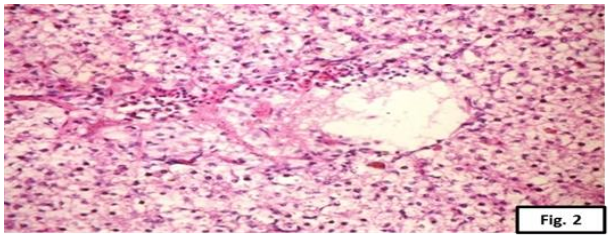
Specimens were taken from liver, kidney, spleen and brain of different groups of fish inoculated with different strains revealed histological alterations that were more or less the same in all of the inoculated groups but of variable degrees of intensity.

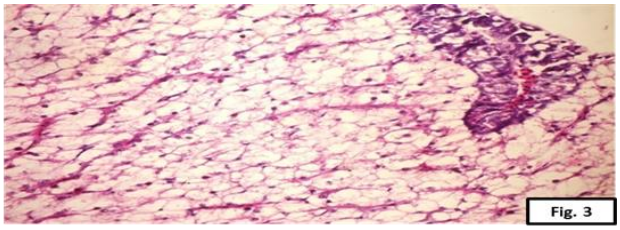
Regarding the examined liver tissue specimens of various groups revealed; congestion of central veins, hepatic sinusoids of fish group inoculated with *S. anginosus* and hepatic blood vessels, focal areas of hemorrhage of variable sizes as well as disarrangement of hepatocytes and thrombosis of portal blood vessel (Figure 2) all were noticed specially in groups inoculated with *E. fecalis* and *S. dysgalactiae*. The hepatocytes showed various degrees of necrobiotic changes as granular and vacuolar degeneration together with hepatocellular necrosis ranged from single cell necrosis, scattered groups of necrotic cells to massive necrosis of hepatocytes (*S. dysgalactiae*) (Figure3 and 4). The later necrotic cells appeared with pyknotic or karyorrhectic or karyolytic nuclei or without nuclei. Marked fatty change was observed in liver of fish inoculated with *S. dysgalactiae* (Figure5). Furthermore, focal areas of mononuclear inflammatory cells infiltration were noticed in hepatic tissue of fish inoculated with (*E. gallinarum*, *E. fecalis*, *S. dysgalactiae*). The former infiltrates were observed in liver infected with *E. fecalis* and *S. dysgalactiae* as replacing multiple focal areas of necrotic and completely destructed hepatocytes.

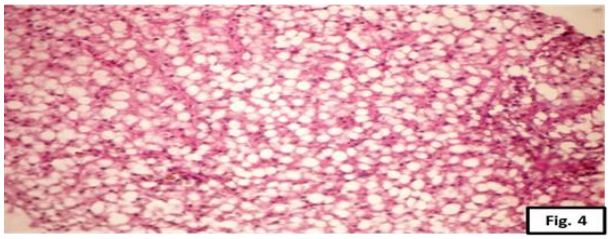
In addition, the hepatopancreas showed vacuolation of the acinar cells and scattered necrotic cells that appeared with pyknotic nuclei or without nuclei. While, the hepatopancears of fish infected with (*E. fecalis*, *S. anginosus*, *L. garvieae*) showed completely disorganized and destructed acini (Figure 6) as well as inflammatory cells infiltration among the acinar cells. Moderate inflammatory cells infiltration was observed in the portal triads of fish infected with *S. anginosus* (Figure 7). Increase the melanomacrophage center as groups of cells carrying pigment was an evident observation in all fish groups.

Table (1) showing PM lesions results of challenged fish

Strain	Gross Lesion					Internal Organs				
	Gills	Eye	Skin	Fins	Abdomen	Liver	Kidney	Spleen	Abdominal Cavity	Brain
S. pluranimalium	Pale white	Dark eye	Erosion and ulcers	-	-	Pale enlarged liver	Congested	Congested	_	_
S. anginosus	Congested	_	_	Redness at the base of dorsal fin	Slightly distended	Very pale friable	Congested black enlarged	Severely congested	Enlarged congested stomach, hemorrhagic ascites	Congested
E. faecalis	Congested	Dark eyes	Dark strips in the form of strips	-	Slightly distended	Friable enlarged pale, fatty liver	Congested enlarged	Severely congested enlarged	Mild hemorrhagic ascites	Slightly congested
E. gallinarum	_	Eye hemorrhage	Dark spots on skin, lower jaw abscess (30% of the fish group)	-	Distended	Enlarged friable, fatty liver (marble like)	Slightly congested	Congested enlarged	_	_
S. dysgalactiae	_	Eye hemorrhage (only 20% of the fish group)	Pale skin (20% of the fish group) Dark skin discoloration (30% of the fish group)	Hemorrhage at the base of: pectoral, and dorsal fins (20% of the fish group)	Distended (100% of the fish group)	Congested (8/10) Mottled (2/10)	Congested, enlarged	Congested	Ascites	Slightly congested (1/10)
L. garvieae	Very pale (80% of the fish group) – unilateral ulcerated operculum (only 10% of the fish group)	Congested sclera (30% of the fish group) – opacity (unilateral in only 10% of the fish group - bilateral in 50%of the fish group) and 10% of the fish group with clear eye	dark discoloration in the form of strips	Tail fin and dorsal fin rot in the all fish in group	Distended abdomen (in 100% of the fish group)	Pale friable	Enlarged congested dark friable	Severely congested	Hemorrhagic ascitic fluid	Slightly congested







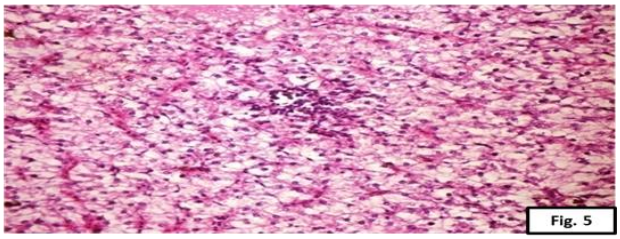
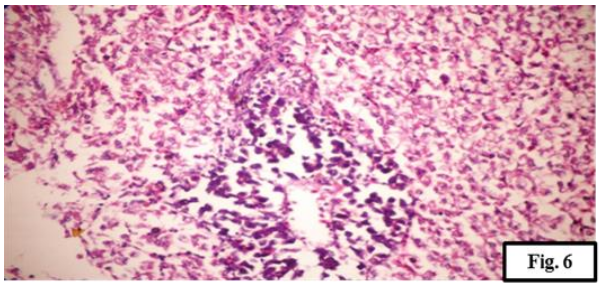


Figure 2: Liver of fish infected with *S. dysgalactiae* showing; thrombosis of the portal blood vessel.(H&E X400).
Figure 3: Liver of fish infected with *E. fecalis* presenting; massive necrosis of hepatocytes, most of them appeared without nuclei. (H&E X400)
Figure 4: liver of fish infected with *S. dysgalactiae* presenting marked fatty change. (H&EX400)
Figure 5: liver of *S. dysgalactiae* infected fish presenting focal area of inflammatory cells replacing necrotic hepatocytes. (H&E X400)



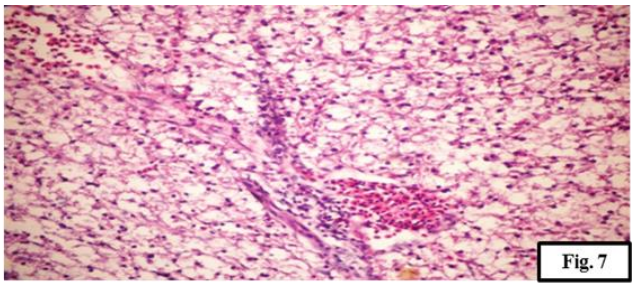


Figure 6: Hepatopancreas of fish infected with *L. garvieae* showing completely dissociated, necrotic and destructed acinar cells. (H&E X400)

Figure 7: liver of fish infected with *E. gallinarum* showing congestion of the portal triad vessels with moderat inflammatory cells infiltration (H&E X400)

Concerning renal alterations of all groups, it was characterized by congestion of the glomerular and intertubular blood vessels of variable severity together with inter tubular pockets of hemorrhage in some fish. Thrombosis of the interstitial blood vessel was an obvious finding in fish infected with *S. anginosus*. The renal tubular epithelial cells showed widespread degenerative changes in the form of granular and vacuolar degeneration (Figure 8). Marked fatty change was noticed in epithelial lining renal tubules of *S. anginosus* infected fish. While, hyaline droplets were found in *S. anginosus* and *E. fecalis* infected fish and complete hyalinization of some tubules was obvious in *S. dysgalactiae* infected fish (Figure 9). In addition, necrosis and desquamation of the epithelial lining some renal tubules leaving the basement membrane was also pronounced but was more severe in *S. anginosus* infected fish (Figure 10). Focal and diffuse interstitial inflammatory cells infiltration with congested interstitial blood vessels were observed in *S. anginosus*, and *L. garvieae* infected fish (Figures 11 and 12) as well as areas of liquefaction in the former fish, some of those inflammatory cells were replacing some necrotic renal tubules (*L. garvieae* infected fish). Wherever, the glomeruli showed variable changes, some glomeruli showed hypercellularity while others showed atrophied glomerular tuft (*L. garvieae* and *S. dysgalactiae* infected fish). Early fibroblastic interstitial proliferation was evident in *S. anginosus* infected fish and a basophilic bacterial colony (by Gram's stain) was observed in *S. anginosus* infected fish.

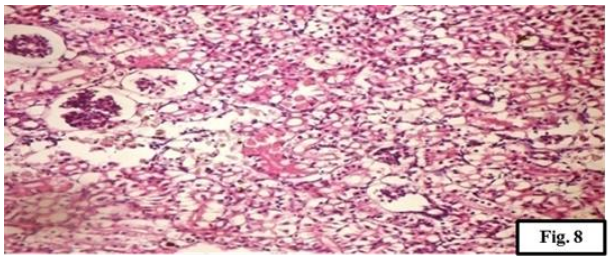
In regards to the histopathological changes that observed in the examined different sections of spleen of the different groups of fish, it revealed vascular congestion and variable degrees of lymphocytic depletion of the lymphoid follicles that was more severe in *S. dysgalactiae* and *L. garvieae* infected fish. On the other hand, thrombosis of the splenic blood vessels (Figure 13) was clearly seen in *S. anginosus*, and *L. garvieae* infected fish together with marked basophilic precipitate which proved to be bacterial colonies by Gram's stain (Figure 14). An increase in the melanomacrophage center was noticed in all groups but it was more conspicuous in *S. anginosus*, *L. garvieae*, and *S. dysgalactiae*, together with focal inflammatory cells infiltarates in the splenic parenchyma.

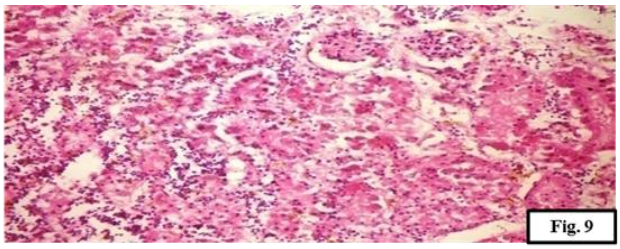
As regards to microscopical examination of brain specimens, the examined sections of different groups revealed congestion of cerebral and meningeal blood vessels with perivascular edema. Meningitis was an obvious finding especially of the meninges of the telecephalon that characterized by thickening with congested blood vessels and inflammatory cells infiltration. Lymphocytic infiltration was observed in the cerebral cortex of fish infected with *E. gallinarum*. Focal gliosis (Figure 15) as well as areas of liquifactive necrosis replaced by inflammatory cells (Figure 16) was observed in infected fish with *E. faecalis* and *L. garvieae*. Marked brain edema was observed in all groups mostly as perivascular and pericellular edema was noticed. Variable degrees of neuronal degeneration and necrosis were observed in all groups (Figure 17), the later appeared as ghost cells without nuclei accompanied with neuronophagia and satellatosis (Figure 18).

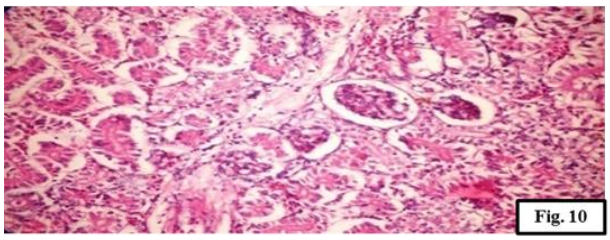
IV-Discussion

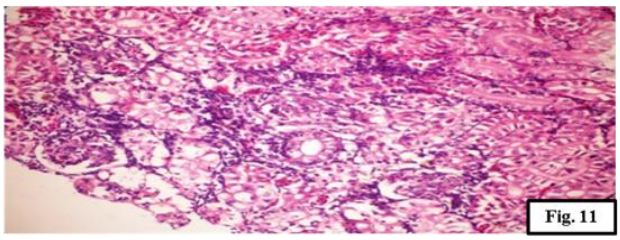
In the current study tilapia fish was challenged with different isolates of organisms causing streptococcosis in fish for studying their pathogenicity and detecting the clinicopathological findings. Six bacterial isolates were isolated from naturally infected tilapia fish from different lacations in Egypt including (*Streptococcus pluranimalium* (*S. pluranimalium*), *Streptococcus dysgalactiae* (*S. dysgalactiae*), *Enterococcus fecalis* (*E. fecalis*), *Enterococcus gallinarum* (*E. gallinarum*), *Lactococcus garvieae* (*L. garvieae*), *and Streptococcus anginosus* (*S. anginosus*)). Each isolate was experimentally inoculated in a separate group of tilapia fish. The isolates used in this study evoked natural disease and also were able to evoke an experimental disease supporting evidence of its pathogenisty. All infected

fish showed clinical signs similar to the signs described in other natural and experimental streptococcal infections (Ingilis *et al.*,1993, Kitao, 1993, Ferguson *et al.*,1994 and Zeid, 2004).The most prominent clinical signs, and postmortem findings of the experimentally infected tilapia fish in the current study were; lethargy, loss of appetite, hemorrhage at the base of the dorsal and pectoral fins, congested and protruded vent(was rare), darkening of the skin, ophthalmic lesions as unilateral or bilateral opacity, and eye hemorrhage. In addition, enlargement and congestion of liver (sometimes it was pale), kidney and spleen, few showed congested brain, and clear or hemorrhagic ascites. The previous observations were more or less similar to those observed by Abuseliana *et al.*(2011), Yanong and Francis-Floyd (2013), and Abdullah *et al.*(2013)who demonstrated the pathogenicity of *S. agalactiae* isolated from a fish farm in Selangor on red Tilapia. Our results of streptococcal infection of cultured tilapia is systemic infection in several internal organs(Eldar *et al.*,1996). In addition, our current results were in agreement with many recently published reports which have shown that naturally infected fish with streptococcal disease showed a variety of pathological conditions, including congestion of the internal organs particularly in the liver, spleen, kidney, and brain (Austin and Austin, 2007, Haghighi, 2010 and Moustafa *et al.*,2010).









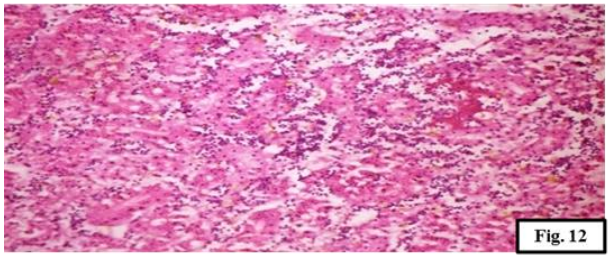
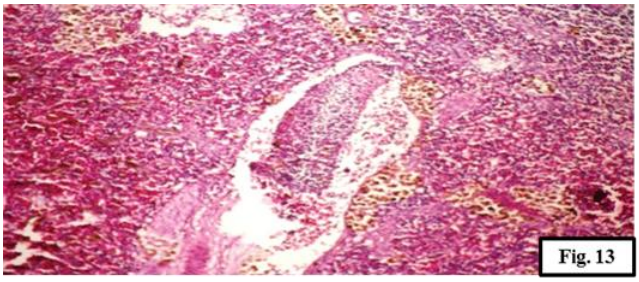


Figure 8: kideny of fish infected with marked granular and vacuolar degeneration as well as necrosis of the renal tubular epithelial lining, some of which appeared completely hyalinized and atrophied glomerular tufts. (H&E X400)
Figure 9: Kideny of *S. dygalactiae* infected fish showing completely necrosed and hyalized renal tubules some of which replaced by inflammatory cells. (H&E X400)
Figure 10: Kideny of *E. gallinarum* infected fish showing; marked desquamation of the epithelal lining leaving the basement membrane, notice the congestion, atophy and hypercellularity of the glomerular tufts. (H&E X400)
Figure 11: kideny of fish infected with *E. gallinarum* showing marked hypercellularity of the glomerular tufts and focal interstitial mononuclear inflammatory cells with few esinophilic cells infiltration in the interstitial tissue. (H&E X400)
Figure 12: kideny of fish infected with *L. garvieae* presenting; diffuse interstitial inflammatory cells infitation among the necrosed tubules. (H&E X200)



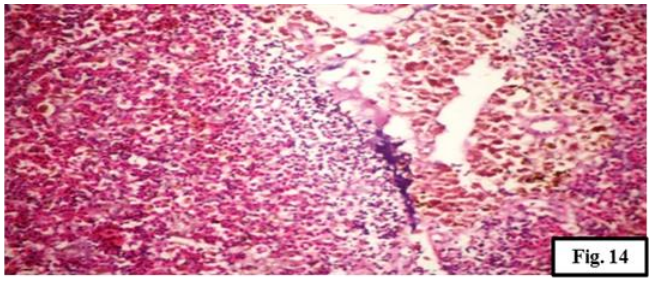
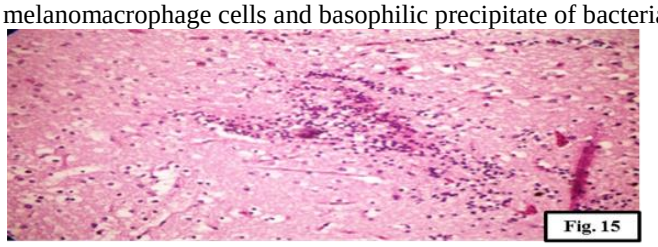
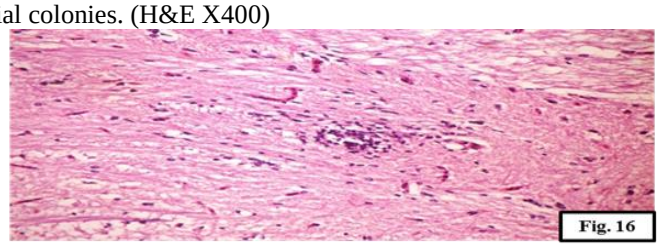
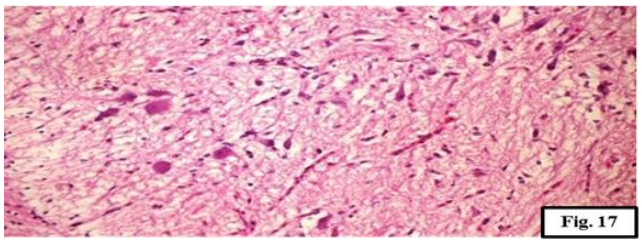


Fig. 13: Spleen of fish infected with *E. gallinarum* presenting; large thrombus in the splenic blood vessel and increased melanomacrophage center. (H&E X200)

Fig. 14: Spleen of *L. garvieae* infected fish showing; focal inflammatory cells infiltration, inceared melanomacrophage cells and basophilic precipitate of bacterial colonies. (H&E X400)







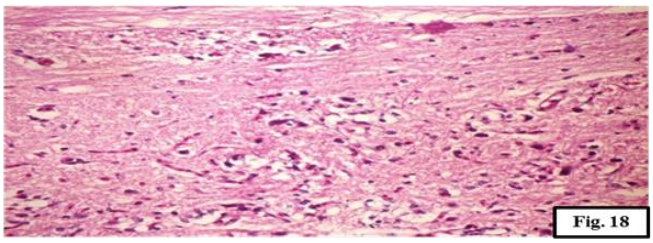


Figure 15: Brain of fish infected with *E. fecalis* showing; focal gliosis in the cerebral cortex, notice; the perivascular and pericellular edema. (H&E X400).

Figure 16: Brain of fish infected with *E. fecalis* presenting; area of liquifactive necrosis replaced by inflammatory cells.(H&E X400)

Figure 17: Brain of fish infected with dys presenting; neuronal degeneration and necrosis, notice the necrotic cells appeared ghost like without nuclei.(H&E X400)

Figure 18: Brain of fish infected with *L. garvieae* showing marked satellosis and neuronophagia of the necrotic neurons. (H&E X400)

These macroscopic findings were also matching with the histopathological examination of liver, kidney, spleen, and liver; in which the congested organs examined showed congested congested central veins, hepatic sinusoids, and hepatic blood vessels, focal areas of haemorrhage in liver, congested glomerular and intertubular blood vessels, intertubular pockets of hemorrhage in kidney specimens, and cerebral and meningeal blood vessels congestion with perivascular edema, also the inflammatory cells infiltration; all these findings were evidence for septicemia occurrence resulted from streptococcosis which is observed in natural and experimental infection (Romalde and Toranzo, 1999, Moustafa *et al.*,2010, Abuseliana, 2011, Azami, 2011 and Abdulla *et al.*,2013) he thrombi observed in liver portal vien, splenic blood vessels, and renal interstitial blood vessels, resulted in edema and degenerative and necrotic changes in these organs. The former lesion could be attributed to the disseminated intravascular coagulation that resulted from streptococcal infection. This explaination is agreed with that of Ferguson *et al.*,1994 who attributed the cause of death in the streptococcal infection to the disseminated intravascular coagulation. In addition, Ferguson *et al.*,1994 also estimated that spleen and kidney are the target organs of the pathogens causing streptococcosis for the severe histopathological changes occurred in them, while Syuhaidah Abdullah*et al.*(2013) concluded that the brain is the primary target organ in case of streptococcosis.

Regarding histopathological examination of the tissue sections taken from liver, kidney, spleen, and brain revealed marked histological alterations in the examined organs. The most prominent lesions were; congested hepatic blood vessels with focal areas of

heamorrhage, congested glumerular and intertubular blood vessels as well as intertubular pocket of haemorrhage, wide spread degenerative changes in hepatic and renal parenchyma that progressed to necrotic changes, and inflammatory cells infiltration. Variable degrees of neuronal degeneration, gliosis, areas of liquefaction replaced by inflammatory cells, lymphatic depletion of the splenic lymphoid follicles as well as thrombosis of the splenic blood vessels. In addition to marked increase in the melanomacrophage center in liver, spleen of the all infected fish groups was a conspicuious lesion. The observed histopathological alterations are more or less similar to those observed by many authors such as: Ferguson *et al.*,1994, Abuseliana *et al.*(2011), and Abdullah*et al.*(2013).

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