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BACTERIOLOGICAL AND HISTOPATHOLOGICAL STUDIES ON *STREPTOCOCCOSIS* IN NILE TILAPIA, *OREOCHROMIS NILOTICUS*

(With 3 Table and 18 Figures)

By

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دراسات بكتيرية وباثولوجية عن مرض الميكروب السبحى المكور في أسماك البلطي النيلي

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الهدف من هذه الدراسة هو إجراء بحث لتسجيل الإصابة بمرض الميكروب السبحى المكور في أسماك البلطي النيلي في محافظة أسيوط. تم تجميع ١٠٠ سمكة بلطي نيلي وبالفحص تم عزل ٣١ ميكروب يتشابه في صفاته مع ميكروب *Streptococcus faecium* من عدد ٢٤ سمكة بلطي نيلي. تم التعرف علي الميكروب عن طريق سلوك النمو الخلوي علي أوساط التغذية الصناعية المختلفة والفحص المجهري والتفاعلات البيوكيميائية. تم عمل العدوي الصناعية بنجاح عن طريق الحقن بالتجويف البريتوني ، ومن العلامات المرضية التي ظهرت علي الأسماك المحقونة أنزفة نقطية علي منطقة الفم والزعانف وغطاء الخياشيم وكذلك لوحظ جحوظ في العين بالإضافة إلي نزيف داخل العين. ظهرت الصفة التشريحية في صورة أحتقان في الطحال والكلى والخياشيم. وظهر الكبد شاحب اللون. بينما كانت التغيرات النسجومرضية الموجودة على شكل تحلل وتنكرز لخلايا الكبد ونقص فى الخلايا الليمفاوية فى الطحال واحتقان الاوعية الدموية فى الخياشيم مع وجود استماتة بالخلايا الطلائية المبطنة للقنوات البولية بالكلى. وقد أظهرت الدراسة أن الأريثيروميسين هو من أفضل المضادات الحيوية تأثيراً علي البكتيريا معملياً.

SUMMARY

The aim of this study was to investigate streptococcosis in Nile tilapia, *Oreochromis niloticus*, in Assiut, Egypt. Thirty-one isolates of *Streptococcus faecium* could be recovered from 24 fish out of 100 Nile tilapia that were randomly collected from the River Nile and El-

Ibrahemia canal at Assiut governorate. Identification was based on colony morphology, culture behaviour in various media, microscopic examination, biochemical tests and carbohydrate fermentation. Experimental infection was successfully done through intraperitoneal injection. The pathognomic signs were petechial haemorrhages on the gill cover, mouth region and fins. Unilateral or bilateral exophthalmia were almost associated with eye haemorrhages. The most common post-mortem lesions were pale liver, congestion of spleen, kidneys and gills. The histopathological changes were hydropic degeneration and necrosis of the hepatocytes, depletion of the lymphocytes of the white bulb of the spleen, congestion of the blood vessels of the gills with degeneration of the lamellar epithelium and necrosis of the kidney tissue. Antibiogram sensitivity test for *Streptococcus faecium* proved that, erythromycin was the drug of choice.

Key words: *Streptococcosis, Nile tilapia, bacterial examination, histopathology.*

INTRODUCTION

Aquaculture is currently the largest source of fish supply in Egypt accounting for almost 65 percent of total fish production of the country, with over 99 percent produced from private farms. Total aquaculture production in 2009 reached 705.490 tonnes with a total marked value of 7,450,553 Egyptian pounds. Nile tilapia has become the most important aquaculture species with a total harvest of about 390.280 tonnes, more than 55 percent of the total aquaculture harvest in 2009 (GAFRD, 2010).

Bacterial pathogens are the most serious disease problems in tilapia production causing 80% of fish mortalities (Clark *et al.*, 2000; Shoemaker *et al.*, 2000). Streptococcosis was a bacterial infection among fresh and marine fish reared in aquaculture. Streptococcosis has recently created a major disease problem in cultured tilapia and considered of high importance in recent years due to increase reports of outbreaks and the high economic losses caused by gram-positive bacteria in both wild and culture fish (Domenech *et al.*, 1996). Outbreaks of streptococcosis have been reported in many parts of Egypt, Ismailia governorate (Badran, 1994), Kafr El-Sheikh governorate

(Khalil, 2000), El-Ibrahemia lake in Upper Egypt (Ebtesam, 2002), Fayoum governorate (Radwan, 2002), in monosex tilapia farm at Alexandria governorate (Safinaz, 2006), Suez Gulf and lake Qarun (Mostafa *et al.*, 2010).

Streptococcus species are Gram-positive, non-acid fast, non-motile, oxidase, catalase and indole negative. Streptococcus cells are usually spherical or oval arranged in pair or in short chain (Austin and Austin, 2007). Several species of streptococcus spp. can be involved in fish infection including *St. iniae* (Shoemaker *et al.*, 2001). *St. faecium* (Minami, 1979), *St. agalactiae* (Kusuda and Komatsu, 1978), *St. fecalis*, unclassified streptococcus sp. (Kusuda and Salati, 1999) and *St. difficile* (Bunch and Bejerano, 1997). Streptococcosis characterized by erratic swimming, unilateral or bilateral exophthalmia, and general haemorrhages (Noga, 2010). Zoltkin *et al.* (2003) recorded that *Streptococcus iniae* was capable of causing disease in human who had recently handled infected fish.

The objective of this study was to isolate and identify the most common streptococcus species isolated from Nile tilapia in Assiut governorate. Pathogenicity of streptococcus species was studied and trial for the antibiogram of streptococcus sp. was also done.

MATERIALS and METHODS

1- Sampling and processing:

A total of one hundred Nile tilapia, *Oreochromis niloticus,* (100-350 g. of body weight and 18-26 cm in total length), were collected alive from the River Nile and El-Ibrahemia canal, Assiut city during the period from January, to May, 2011. Fish were transported alive to the Aquatic Animals Diagnostic Laboratory. Animal Medicine Dept, Faculty of Veterinary Medicine, Assiut University. Clinical and post-mortem examinations were carried out using the methods described by Buller (2004).

Bacterial examination:

Sterile swabs from liver, kidney, spleen and brain were streaked on brain heart infusion agar supplemented with 0.2 g /L sodium azide, blood agar supplemented with 5% sterile sheep's blood and tryptacose Soya agar. The inoculated plates were incubated at 37°C for 24-48

hours. Single colonies from plates with dense, virtually pure culture growth were re-streaked on the same media to obtain pure isolates (Kusuda *et al.*, 1991).

Identification of the isolates

Bacterial isolates were identified according to their cultural behaviour, colony morphology, and biochemical tests according to (Garrity, 2001; Austin and Austin, 2007).

Pathogenicity of *streptococcus faecium* to O. *niloticus*

A total number of 45 apparent healthy Nile tilapia, with an average body weight of 50±5 g. and total length 12-14 cm were obtained from fish farm in Assiut city. Apparent healthy Nile tilapia were acclimated to laboratory conditions for 15 days.

Bacterial strains:

Bacterial isolates from kidney of infected fish were identified as *Streptococcus faecium* was passed through Nile tilapia via intraperitoneal injection for three times and used for determination of pathogenicity. Streptococcus isolate was grown on BHI agar and suspended in sterile distilled water to be used for experimental infection.

Bacterial counts and dilutions:

A preliminary growth curve study was conducted to determine counts of colony forming units (CFU) of *Streptococcus faecium* in BHI broth at various growth phases using standard plate count method (ElKamel and Thune, 2003).

Experimental challenge:

Acclimated Nile tilapia were divided into three groups, 15 fish in each group, was placed in three aquaria. 0.33 ml of a bacterial suspension $0.3x10^8$ cfu/ml was I/P injected in all fish of one group. Second group remained un-injected as a control, while third group was I/P injected with 0.33 ml of distilled water. All experimental fish were distributed in glass aquaria supplied with sufficient chlorine free tap water. All inoculated fish were observed twice daily for any abnormal clinical signs and mortalities for 15 days. Specimens from liver, kidneys, spleen and gills were collected from injected fish fixed in 10% neutral buffered formalin for histopathological examination according to Roberts (2001). Re-isolation of the organism was carried out from liver, kidneys, spleen and brain of dead and sacrified fish (Perera *et al.*, 1997; Hussain, 2002).

Antibiotic sensitivity test:

The sensitivity of bacterial isolates to different antibiotics was carried out using the disc diffusion technique. The interpretations of zones were estimated according to the limits given by Carter and Cole, (1990).

RESULTS

Clinical examination:

The main clinical signs of naturally infected Nile tilapia with *Streptococcus faecium* were petechial pen-head haemorrhage on the mouth region, gill cover and fins. Some of fish showed unilateral or bilateral exophthalmia with eye haemorrhages (Fig. 1). Anus was inflamed as well as prolapsed were also observed (Fig. 2). Internal lesions included pale liver, marked congestion of kidney and spleen with splenomegaly. Gall bladder was enlarged and engorged with bile (Fig. 3).

Bacteriological examination:

The bacteriological examination of 100 Nile tilapia, *O. niloticus*, revealed 31 isolates. These isolates were mainly recovered from kidneys, liver, spleen and brain. The percentage of infection among examined fish was 24%. These isolates were gram-positive cocci in pair or short chain, non motile, oxidase and catalase negative. Colonies grew on brain heart infusion agar + sodium azide with pen head, dull creamy, circular, translucent, slightly convex colonies. On blood agar colonies were pale grey rounded colonies with zone of α-haemolysis (greenish coloration). It grew well on trypticase soya agar and gave pen head colonies, white, creamy, circular, raised and glistening colonies. The biochemical characteristics of isolated bacteria were summarized in Table (1).The obtained isolates were displayed the identical morphology, culture and biochemical characters of *Streptococcus faecium*.

Streptococcus faecium was isolated from liver, spleen, kidneys and brain of Nile tilapia with percentage of 19.35, 16.13, 38.71, and 25.81% respectively. These results are shown in Table (2). The percentage of isolates at different body weights revealed that the percentage of infection on fish with body weight (50-150), (155-250), (255-350) gram were 23.81, 21.88, 26.9% respectively. These results are demonstrated in

Table (3). It is notable that 7 isolates out of 12 isolated from kidneys were recovered from larger body weight fish.

Experimental challenge:

No bacterial pathogens were isolated from any of the fish screened prior to the experimental fish. The experimental infection was successfully induced by intrapretoneal infection with *Streptococcus faecium*, with the same clinical signs and post mortem changes similar to that of naturally infected fish (Fig. 5, 6), in addition to abnormal swimming pattern, darkness of the skin (Fig. 4). Fish took vertical position. In some cases, necrosis appears in liver and spleen (Fig. 7). Hyperemia was observed on the ovary (Fig. 8). Fibrinous peritonitis can be observed in the peritoneal cavity (Fig. 9). Mortality rate was 20%. *Streptococcus faecium* was re-isolated in pure form from dead and sacrified fish. No mortality occurred in any of the control fish and the bacterium was not isolated from any organ.

Histopathological alteration

Severe hydropic degeneration of hepatocytes as well as, deposition of hemosidrin pigment were observed Fig. (10). These degenerative changes progressed in some cases to focal coagulative necrosis of the hepatic cells with acidophilic cytoplasm and pyknotic and karyolytic nucleus Fig. (11). The gills showed fusion of the epithelial lining of the secondary lamellae and congestion of the lamellar blood vessels Fig. (12). Also vacuolar degeneration and necrobiotic changes in the epithelial cells and pillar cells were seen Fig. (13, 14). The branchial blood vessels in the gill arch were congested Fig. (15). The spleen of infected fish showed granulomas of varying size Fig. (16). Depletion of the lymphocytes in the white bulb of spleen and increase of the melanomacrophages were observed Fig. (17). The kidneys showed periglomerular oedema with mild infiltration of mononuclear inflammatory cells Fig. (18).

Antibiogram sensitivity:

Regarding to antibiotic sensitivity of the *Streptococcus faecium*, it was found that, the organism was highly sensitive to erythromycin (E_{15}), while it was resistant to Tobramycin (ToB_{10}), Neomycin (N_{30}) and sulfamethazole (SMZ_{100}).

Table 1: Cultural and biochemical characters of the isolated bacteria:

Biochemical test	Response
Gram-stain	G+ve Cocci in pair or short chain
Motility	-
Oxidase	-
Catalase	-
Growth on MacConkey agar	-
10°C	+
37°C	+
45°C	+
6.5% NaCl	+
Vogus proskauer	+
Indole	-
Citrate utilization	-
TST	-
Urease	-
Orgnine decarboxylase	+
Ornithine decarboxylase	+
Acid produce from:	
Glucose	+
Raffinose	+
Sucrose	+
Lactose	+
Arabinose	+
Mannitol	+
Sorbitol	-

Table 2: Organ susceptibility of *O. niloticus* to *streptococcus faecium* infection.

Organ	Isolated strains = 31	
	No.	%
Liver	6	19.35
Kidney	12	38.71
Spleen	5	16.13
Brain	8	25.81

Table 3: Body weight susceptibility of Nile tilapia to *streptococcus faecium* infection.

Body Weight (g)	Number of examined fish	No. of infected fish	%
50-150	42	10	23.81
155-250	32	7	21.88
255-350	26	7	26.9

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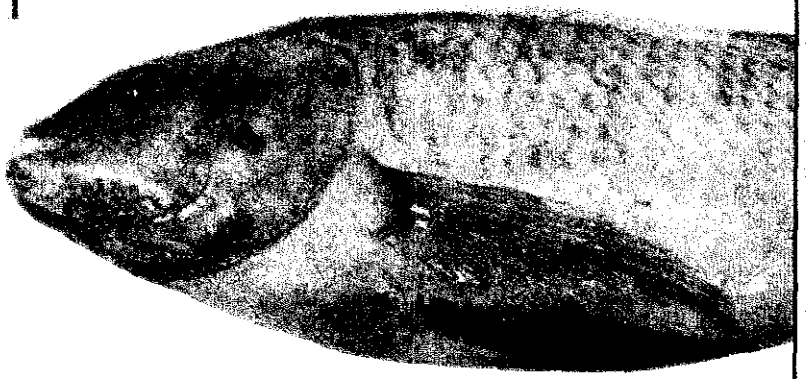

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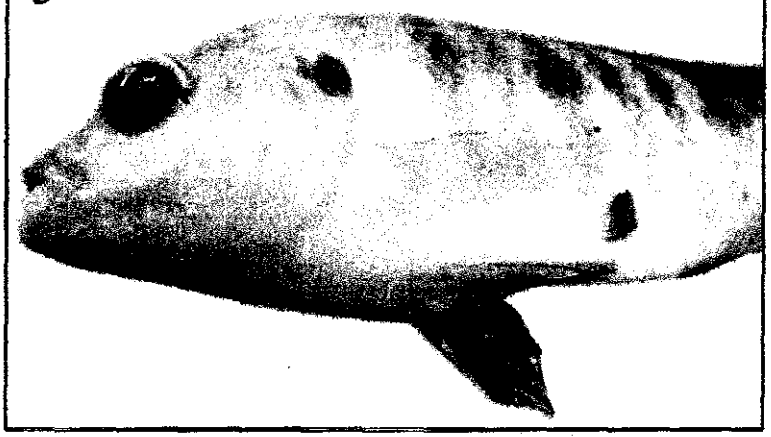

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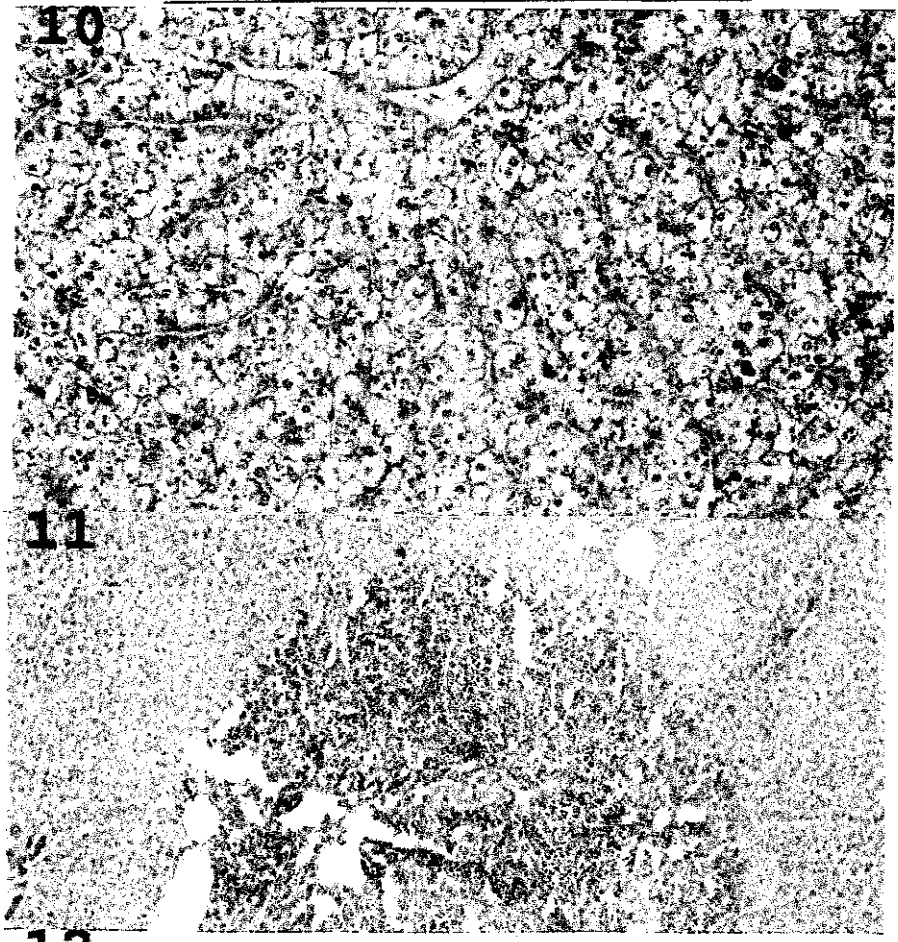

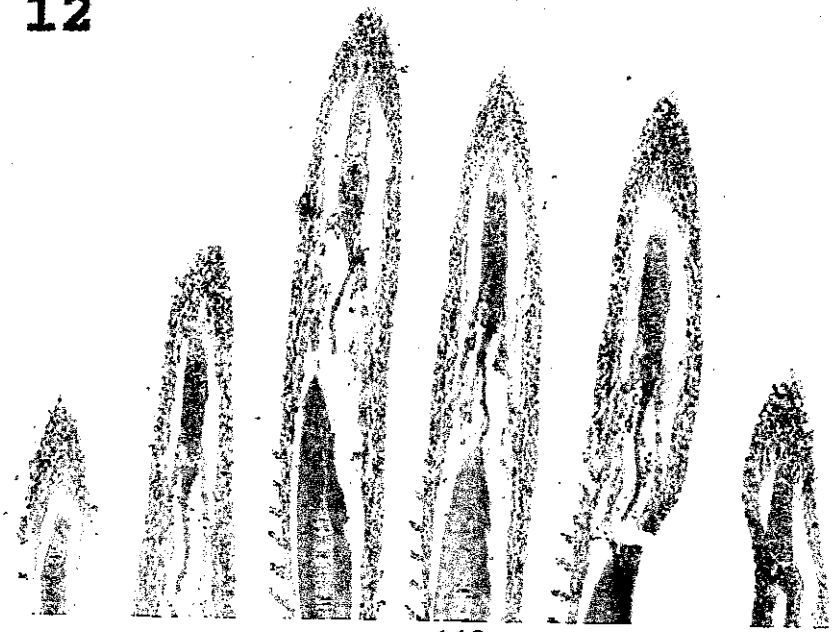

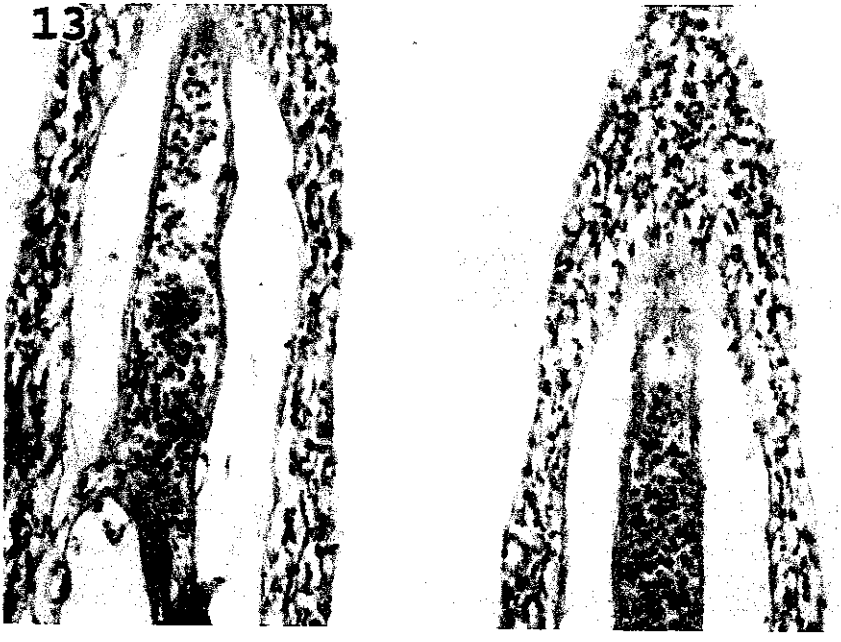

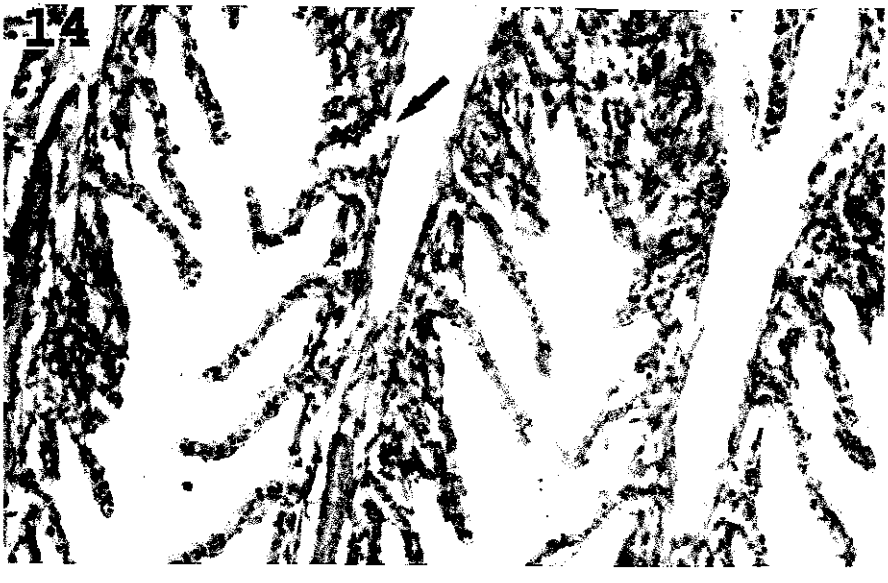

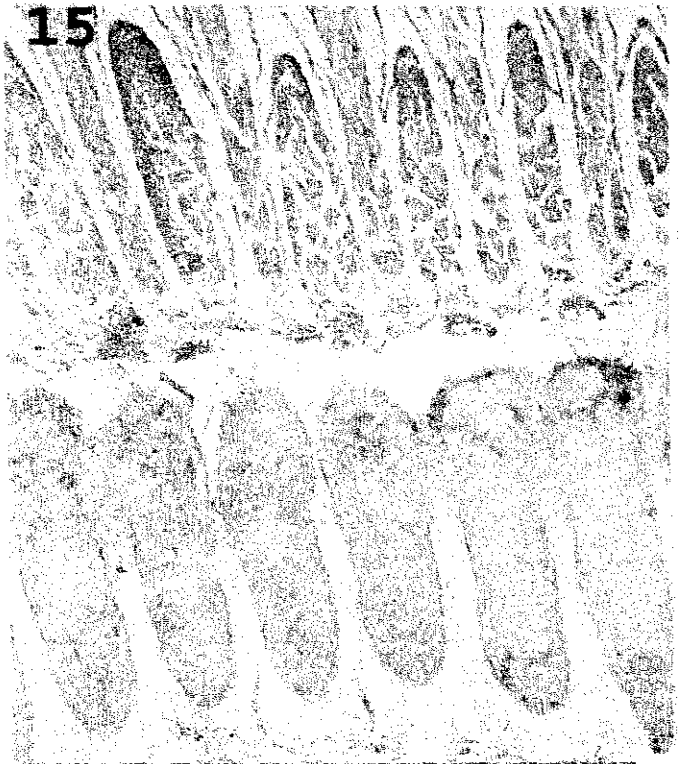
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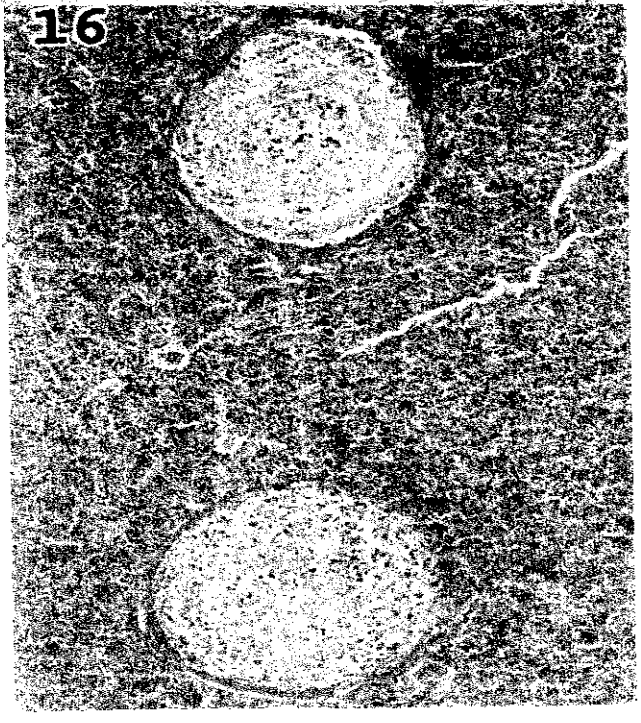
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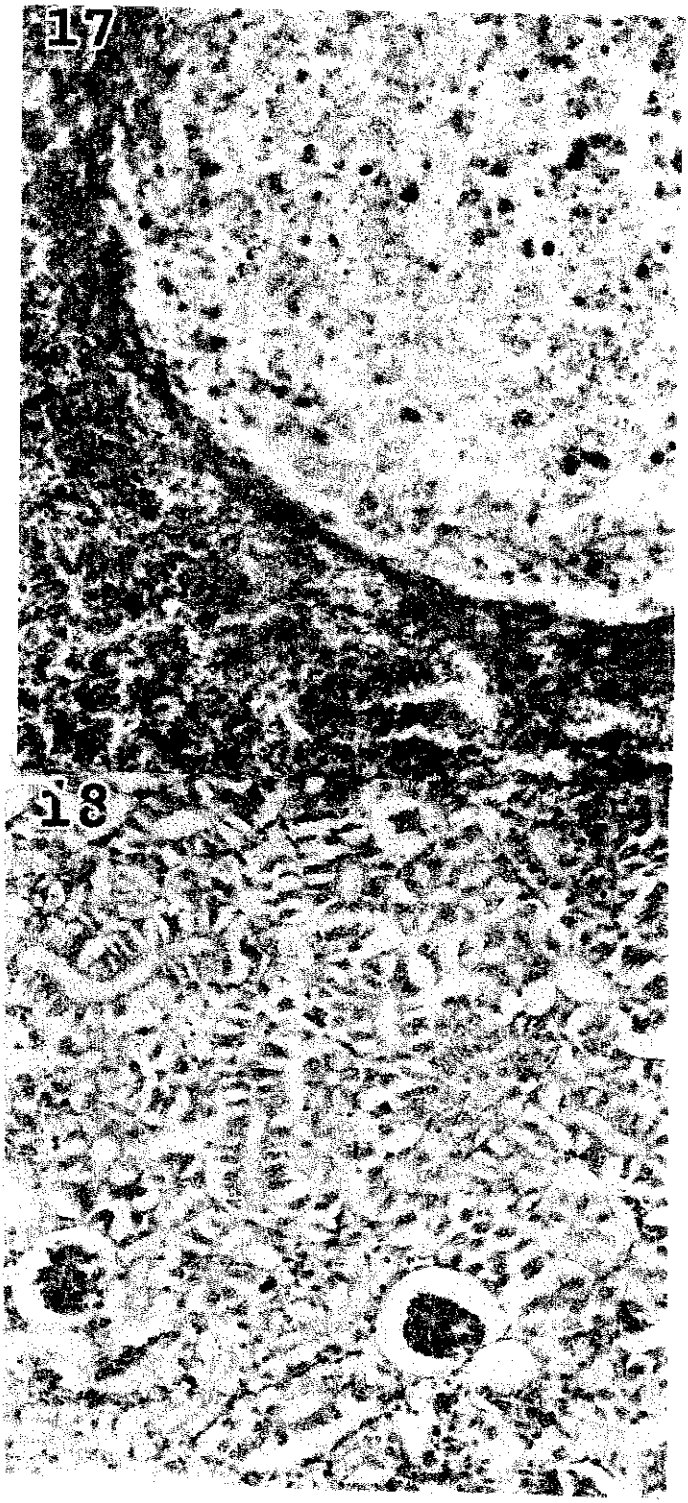
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Fig. 17: Spleen of experimentally infected fish showing activation of the melnomacrophages H&E x400.

Fig. 18: Kidney of experimentally infected fish showing degeneration in the renal Tubules and congestion in the glomerular capillaries H&E x400.

DISCUSSION

Streptococcus species is gram-positive cocci that cause streptococcal infection in fish and cause significant economic losses in fish farm industry (Baeck *et al.*, 2006). The bacteria recovered from the internal organs and brain of Nile tilapia showed pen head, dull creamy, circular, translucent, slightly convex colonies on brain heart infusion agar supplement with 0.2 g/L sodium azide. These results agree with Edwards (1932) who published a report on the isolation of mastitis streptococci from mixed cultured with sodium azide as a selective media. *Streptococcus faecium* grew well on blood agar and gave pale grey colonies with greenish zone (α-hemolysis). These results agree with Badran (1994); Zeid (2004). The isolated bacteria from Nile tilapia were gram-positive cocci in pair or short chain, non-motile, catalase and oxidase negative, Indole urease, TSI and citrate utilization negative,in addition to the results of carbohydrate fermentation. From the results, the isolated bacteria could be identified as *Streptococcus faecium* as guided by Garrity (2001) and Austin and Austin (2007). Also,the results coincide with the findings of Baya *et al.* (1990); El-Bouhy (2002). *Streptococcus faecium* did not grow on MacConkey agar. These results similar to the report of Torkey *et al.* (2006) and disagree with Bragg and Broere (1986); El-Bouhy (2002) who reported that *Streptococcus faecium* had variable growth on MacConkey agar. These differences may be due to strain difference. The isolates grew well at 6.5% NaCl, these results agree with Garrity (2001); Torkey *et al.* (2006); disagree with Ebtsam (2002).

The percentage of infection among the examined fish (100 fish) revealed that 24 fish (24%) were naturally infected with *Streptococcus faecium*. These results were higher than those reported by Badran and Eissa (1991) (1.7% in Tilapia), Ebtsam (2002) (10% in Nile tilapia) and Torkey *et al.* (2006) (22.33% among cultured freshwater fishes). These differences may be related to the small tributaries of E-Ibrahemia canal in which fish live where there are subjected to sewage and waste water which is considered the source of infection, in addition to the difference

in the level of pollution in different sites of the River Nile, as well as the increase of contamination of the River Nile and El-Ibrahemia canal by sewage waste and bad habits of human.

Out of the 96 biological materials from the 24 infected fish, the numbers of isolates of *Streptococcus faecium* were 31 (8 samples of brain, 6 of liver, 12 of kidney and 5 of spleen). The results may be due to septicaemia and pathogenicity of the isolating *St. faecium* (Kimura and Kusuda, 1982) and those observed by El-Bouhy (2002); Zeid (2004). This pathogenicity may attribute high capacity for tissue invasion and toxic products.

The present study revealed that, *Streptococcus faecium* recovered from large size fish (255-350 gram) were more than medium and smaller size fish. Noga (2010) recorded that streptococcosis can theoretically affect all fish sizes. However, bigger fish are usually more susceptible to the disease.

Fish infected with *Streptococcus faecium* exhibited some abnormal behaviour as abnormal movement pattern, lethargy. These signs may be due to the tropism of the bacteria for the central nervous system (Noga, 2010). Clinical signs of experimentally infected fish were similar to those described by other authors (Robert, 2001; Radwan, 2002; Safinaz, 2006), including pen head, haemorrhage on the mouth region, gill cover and fins. There were unilateral or bilateral exophthalmia and haemorrhages either in one or both eyes and this may be attributed to the haemolytic effect of bacterial toxins (Kusuda and Salati, 1999). Postmortem lesions observed in the experimentally infected fish were congested kidney. Spleen was darker than normal and lager in size. These results are related to the period of acute infections, bacteria rapidly reach the blood system and disseminated to all internal organs. Major clinical signs associated with this septicemic condition are haemorrhages and inflammation in kidneys, spleen and eye. Gall bladder was enlarged and distended with bile, as a result of minimizing the food intake (Plumb, 1999). Postmortem lesions of experimentally infected fish were similar to those described by Badran (1994) and Moustafa *et al.* (2010).

The histopathological changes were Hepatocyte vacuolization and necrosis and splenic congestion. These findings agreed to that recorded by Evans *et al.* (2002); Safinaz (2006); Ali *et al.* (2011). The gross pathological changes seen in this study were typical of a septicemic infection and the observed lesions and clinical abnormalities corresponded to histopathological findings. The increased in

Melanomacrophages Centers (MMC) in the liver and spleen in the infected tilapia was possibly related with the cellular effective immune response of fish. Filho *et al.* (2009) described the relevant role of MMC and *S. agalactiae* infection in Nile tilapia and suggested that the macrophages were associated with lymphocytes for antigen trapping. The other lesions found in the gills and kidney could be attributed to the strong action of alfa haemolysis of Steptococcus (Zeid 2004; Safinaz 2006; Main *et al.* 2009)

Results of antibiogarm sensitivity of *Streptococcus faecium* isolates to different antibiotic discs similar to the results of Domenech *et al.* (1996); Franks *et al.* (1998); Khalil (2000); Torkey *et al.* (2006).

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