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Electron Microscopy (SEM-EDAX & TEM) Studies on Streptococcosis in Egyptian Nile Tilapia (*Oreochromis niloticus*)

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Abstract: A field study on Streptococcus infection as a major cause for epidemic infections in Nile Tilapia aquaculture in Kafr El-Shikh Governorate was conducted. This study was carried out to study the gross lesions and post mortem finding of Streptococcsis in Nile Tilapia. Streptococcus was isolated, then identified by Electron Microscopy (Scanning Electron Microscope (SEM), Energy Dispersive X-Ray Analysis (EDAX) and Transmission Electron Microscope (TEM)) to investigate the phenotypic characteristics, ultra-structure of the bacterial cells. Clinical signs of the infected fish showed presence of hemorrhagic patches on different external body regions, ascitis and exophthalmia. Post mortem examinations revealed presence of inflammation of the liver, spleen and kidney. Microbial identification by Gram stain showed Gram positive cocci in shape arranged in chains and the biochemical tests displayed negative catalase and oxidase tests. Electron Microscopy (SEM, EDAX & TEM) confirmed these results and showed the clear cocci shape of streptococcus species and the arrangement of the chains with a bacterial average length of 700 nm. The EDAX results revealed that the elemental structure of the bacterial sample were C,N,O with the concentration 46.93%, 16.61% and 36.46%, respectively.

Keywords: *Streptococcus species*, *Oreochromis niloticus*, SEM, TEM, EDAX

1. Introduction

Nile Tilapia (*Oreochromis niloticus)* is considered an important food source for human. In Egypt, Nile Tilapia is a valuable fish species and is cultured in both semi intensive and intensive culture systems. In 2007, Egypt produced 265,862 Ton from Tilapia culture. This amount was estimated to represent 41.8% of the total fish culture productions [1]. Tilapia is the most intensive cultivated freshwater fish in Egypt [2].

Streptococcosis in fish is a septicemic disease that has been reported around the world causing severe economic losses in fish production. Streptococcal infection can be introduced inside the farms through new imported fish, especially if the quarantine standards are missing, or by other animals, birds or insects, which cause spreading of the infection between different farms [3].

Streptococcosis is known as “Pop-eye disease”, due to the accumulation of mucopurulant exudates around the eye (exophthalmia). It induced erratic swimming as it affects the nervous system, lethargy, darkening of the skin, corneal opacity, hemorrhage inside or around eye, at gills, base of the fins, vent, or on the body, ascites, enlarged pale yellow liver (hepatomegally), congested inflamed kidney (nephritis) and spleen (spleenomegally) [4].

The usage of Electron Microscopy (EM) is a modern

method to study the microorganisms. Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) are useful equipments for the examination of external and internal structure of cells and studying the interaction between bacteria and host cells.

Therefore, the present study aimed to characterize Streptococcosis species in nile tilapia and identify the pathogen using SEM, EDAX & TEM.

2. Materials and Methods

2.1. Fish Samples

Fish samples, which showing streptococcal-expected clinical signs, were collected from different Nile Tilapia fish farms in Kafr El-Shikh Governorate. Ten fish samples were taken from 5 different farms with fish weight ranged between 100 and 120 g. Fish were kept on ice and transported to the laboratory.

Clinical examination:

The clinical examination of the fish was performed according to the method described by Conroy and Hermann [6].

2.2. Post Mortem Examination

The post mortem examination of the fish was performed according to the method described by Austin and Austin [7].

2.3. Isolation and Identification of the Causative Agent

Under a complete aseptic conditions, bacteriological samples were taken from different organs (gills, spleen, liver and kidney) with a sterile bacteriological loop; tryptic soy broth (TSB) was used for primary cultivation at 37°C for 48 hours. Then, inoculation was done from the broth on tryptic soy agar (TSA), brain heart infusion agar (BHI) and blood agar media (5% sheep blood) for isolation of streptococcus bacteria. The inoculated media were incubated at 22 - 37°C for 48 hours before examination of the isolated bacteria. Examination under light microscope was done using a pure colony from the incubated media and Gram stain test was done to identify the Streptococcus species. Oxidase and catalase test were also used for further identification of Streptococcus species.

2.4. Identification of Streptococcus Species Using Scanning Electron Microscope (SEM)

A pure culture of Streptococcus bacteria on blood agar was prepared. Then, a cube of agar approximately 0.3 to 0.5 cm contains colonies were excised from the plates by a sterile scalpel and fixed on the aluminum stub of the SEM. The sample was processed for electron microscopy following the method of J. Goldberg. *et al*. [8].

2.5. Analysis of Streptococcus Species Using Energy Dispersive Analysis X-Ray (EDAX)

Energy Dispersive Analysis X-Ray (EDAX) is an analytical technique used to specify the elemental analysis or chemical characterization of a sample. This technique is associated with the SEM test and confirms the nature of the samples. The bacteria were examined by EDAX according to the method described previously by J. Goldberg. *et al*. [8].

2.6. Identification of Streptococcus Species Using Transmission Electron Microscope (TEM)

Bacteria were prepared for TEM as previously described by J. Goldberg. *et al*. [8]. Briefly, a pure culture of streptococcus bacteria on blood agar was prepared. By using a sterilized bacteriological loop, a pure colony was taken and emulsified in a tube contains saline solution and shacked well. With a special forceps, a special copper grid was picked up and immersed in the tube which has the saline with the bacterial cells then shacked well and pulled out of the tube. Copper grid then lifted to dry for 2 minutes before inserted inside the TEM.

Scanning of the sample was done with high tension (100000 KV). Magnification powers were (9300 X and 11000 X).

3. Results and Discussion

Clinical examination of diseased fish collected from the fish farms showed hemorrhages on the fins, gills (Figure 1) and corneal opacity (Figure 2), protrusion of vent and ascites. These results are matched with the result obtained by Nguyen *et al.* [9] who examined *S. iniae* infection in Japanese flounder (*Paralichthys olivaceus*) and reported that the gross lesions in the dead fish were small hemorrhagic lesions on the dorsal and pectoral fins, clouding of the cornea, exophthalmia, hemorrhagic gills, hemorrhage in abdominal wall and ascites. Also, Karsidani *et al*. [10] studied Streptococcosis in farmed rainbow trout (*Oncorhynchus mykiss*) and reported that the clinical signs were bilateral exophthalmia which may be associated with cataract, hemorrhage and ascites. Vent was prolapsed with hyperemia/hemorrhage in most affected fish.

Post mortem lesions of examined fish showed enlargement of the spleen (spleenomegally), pale enlarged liver (hepatomegaly) (Figure 3) and hemorrhage with inflammation in the kidney. These results were agreed with the observations recorded by Nguyen *et al.* [9], Salvador *et al.* [11] and Abuseliana *et al.* [12].

Microbiological tests were positive for streptococcus, culturing on BHI media revealed the presence of small non pigmented colonies (1 mm), culturing on TSA media showed translucent to slightly opaque, round, convex and whitish colonies (1.50±0.25 mm in diameter). Culturing on blood agar showed small whitish colonies that were surrounded by beta heamolysis. Catalase and oxidase test were negative which confirmed the presence of streptococcus bacteria. Gram stain test showed the presence of Gram

SEM results revealed that the Streptococcus colonies have variations in arrangement and length of the organisms in different areas of the same colony. Organisms in the central depressed area of the colony were arranged in chains grouped

together to form communicated chains which stick together to form a homogenous surface of the same shape and size with average length 500 nm (Figure 4). In the periphery of the colony, the organisms appeared as slight spherical shape. The cells attached to each other forming short chains which had distances in between and were not adjacent to each others. Chains were not grouped together as in the central area of the colony (Figure 5, Figure 6). The length of Streptococcus bacteria ranged between 500 nm and 900 nm. These results matched with the results of Whittaker *et al.* [13] who examined streptococcus bacteria under SEM, the results revealed that organisms in the central depressed area of the colonies were arranged in chains grouped together to form broad intercommunicating buttresses with spaces between producing a sponge like effect. In the raised periphery of these colonies, the organisms lengths ranged between 1.4 um and 0.8 um.

Bacteria was tested by using the EDAX and the results showed that the elemental structure of it was composed of carbon (C), nitrogen (N) and oxygen (O) with relative concentration of 46.9%, 16.6% and 36.4%, respectively. This result confirmed the presence of sample of an organic nature as bacteria (Figure 7). Hydrogen is an essential element of the organic materials. In the present study hydrogen did not appear in the analysis because it is the lightest element in the periodic table and has the lightest energy among the all elements which requires special detectors to analyze its light energy.

Regarding the TEM results for the tested bacteria (magnification powers were 11000 X and 9300 X and high tension was 100000 KV), the bacterial cells were cocci in shape and appeared as chains connected to each others. The outer polysaccharide capsule and some internal structures of cells appeared as the beam penetrates the bacterial cells. Diameter of the cells ranged between 600 nm and 900 nm (Figures 8, Figure 9, Figure 10). Barnes *et al.*, Buchanan *et al.* and Locke *et al.* [14-16] examined bacteria strains by using the TEM with high tension 80 KV and magnification power of 25000 X, 16000 X and 15500 X, the TEM analysis represented the characteristic cocci shape of streptococcus and the presence of the capsule surrounding the bacterial cells.

Figure 1. *Naturally infected Nile Tilapia (O. niloticus) with hemorrhages on the gills and fins.*

Figure 2. *Naturally infected Nile Tilapia (O. niloticus) showing a corneal opacity.*

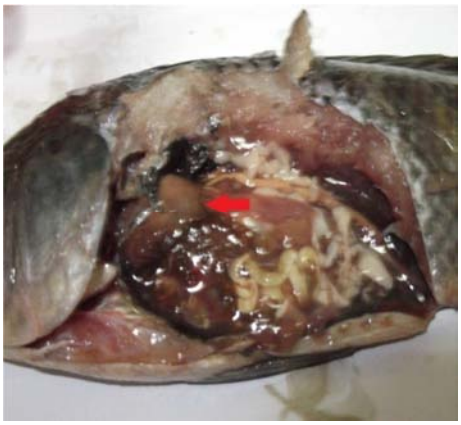


Figure 3. *Naturally infected Nile Tilapia (O. niloticus) havinh enlarged enlarged pale liver (hepatomegally) (arrow).*

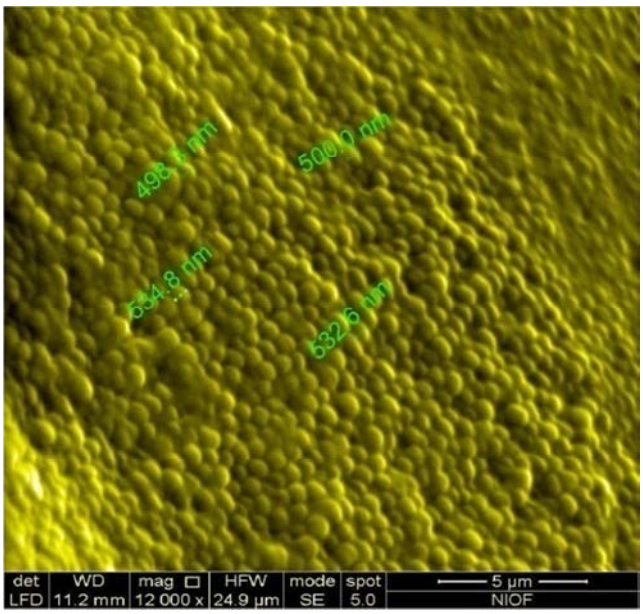


Figure 4. *Streptococcus under the SEM in the centre of the colony (magnification power 12000 X and High tension 20000 KV). Bacterial cells were cocci in shape, arranged in long chains grouped together forming connected chains. The average length of bacteria is 500 nm.*

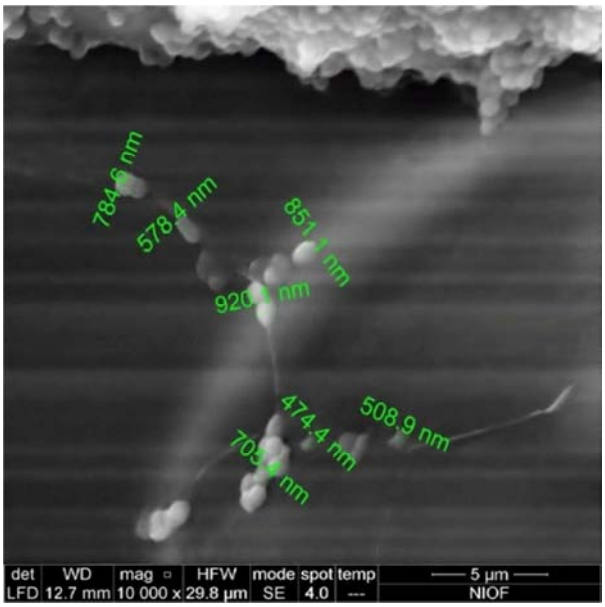


Figure 5. *Streptococcus under the SEM at the periphery of the colony (magnification power 10000 X and High tension 15000 KV). Bacterial cells appeared cocci in shape, attached to each other forming short chains which had distances in between and were not adjacent to each others. The length of bacteria ranged between 500 nm and 900 nm.*

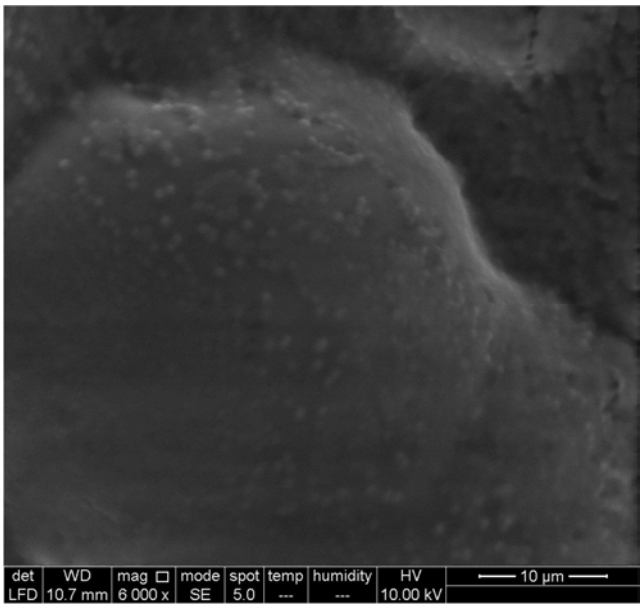
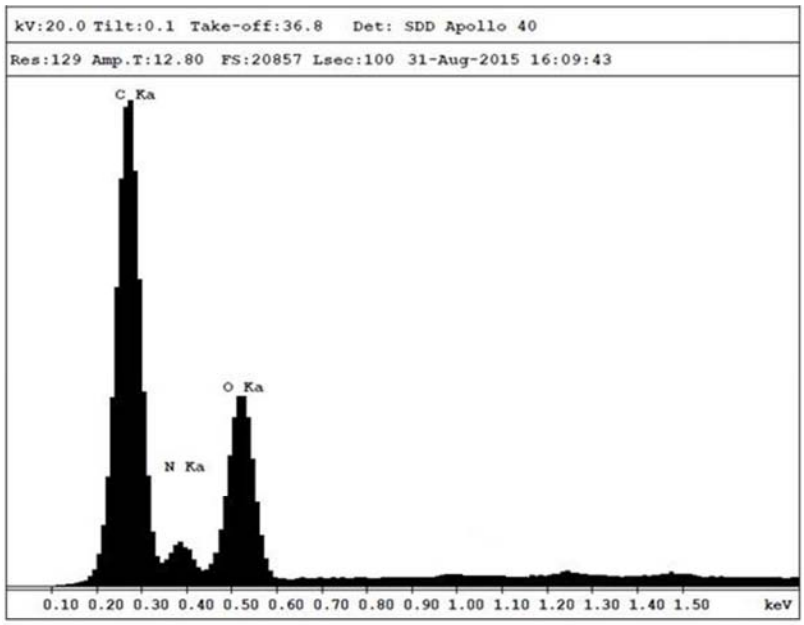


Figure 6. *Streptococcus under the SEM at the periphery of the colony. Bacterial cells appeared cocci in shape, rounded and attached in short chains. (magnification power 6000 X and High tension 1500 KV).*



EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : Default

Element	Wt %	At %	K-Ratio	Z	A	F
C K	46.93	53.00	0.2865	1.0075	0.6056	1.0005
N K	16.61	16.09	0.0251	0.9987	0.1514	1.0007
O K	36.46	30.91	0.0664	0.9908	0.1839	1.0000
Total	100.00	100.00				

Element	Inte. Ratio	Bkgd Inte.	Inte. Error	P/B
C K	1.000	6.24	0.32	0.16
N K	0.083	8.67	1.21	0.01
O K	0.399	11.20	0.52	0.04

Figure 7. *EDAX analysis results of Streptococcus species showed that the main elements of the tested Streptococcus colonies were C, N and O. The concentrations of the elements in the colonies were 46.93%, 16.61% and 36.46%, respectively (High tension was 20 KV and the Count per Second (CPS) was 100 seconds.*

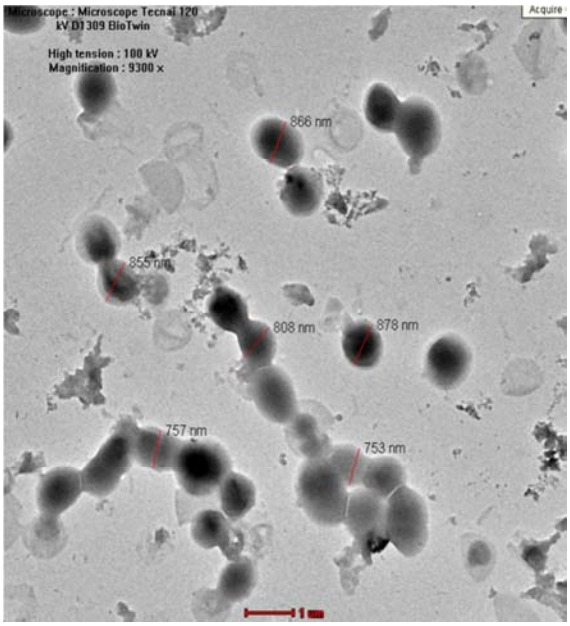


Figure 8. *Streptococcus species under the TEM (magnification power 9300 X and High tension 100 KV). The bacterial cells were cocci to elongated cells and appeared as chains connected to each others.*

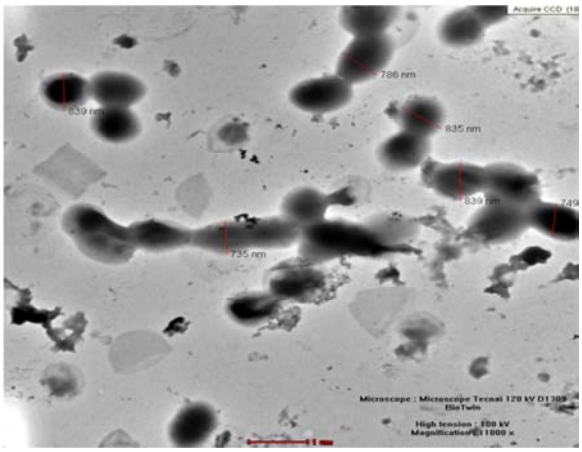


Figure 9. *Streptococcus under the TEM (magnification power 11000 X and High tension 100 KV). The cells were cocci to elongated and appeared as chains connected to each others.*

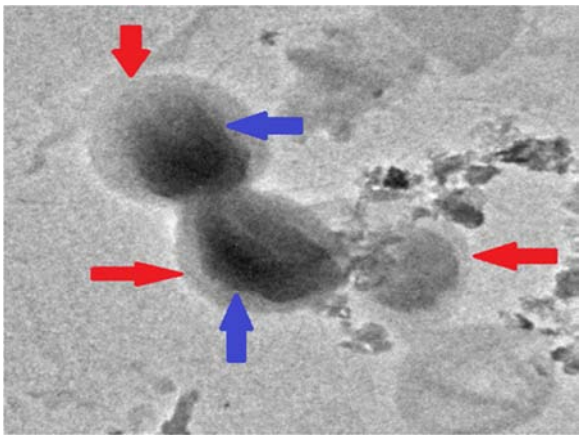


Figure 10. *Streptococcus cells showed the outer polysaccharide capsule (red arrows) and the internal. structures of the cells (blue arrows).*

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