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Investigation of Streptococcal micro-organism as a cause of mortality syndrome in cultured Nile tilapia

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Abstract
The objective of this study was the isolation and identification of Streptococcus spp. as one of the mortality syndrome causes in tilapia fish farms. One hundred apparently healthy and diseased cultured *Oreochromis niloticus* fish and 20 water samples (10 samples from the water inlet and 10 samples from farms water) were collected randomly from some private fish farms at Kafr El sheikh. Bacteriological examination revealed the isolation of Streptococcus spp. either from water or diseased fish then the isolates were identified microscopically, biochemically and finally confirmed by PCR. A total of 7 Streptococcus spp. isolates; 5 S. agalactiae isolates (3 from fish and 2 from water) and 2 isolates of *Streptococcus iniae* (1 from fish and 1 from water) were isolated from the examined samples. *S. agalactiae* isolates from fish were examined for the presence of virulence gene *Scp* B, results showed that all examined samples have this gene. Experimental infection was carried out using isolated Streptococcus spp. and treatment trial was done using Florfenicol according to the result of the sensitivity test.

Keywords: *Streptococcus* spp., *Oreochromus niloticus*, mortality

Introduction
Nile tilapia (*Oreochromis niloticus*) is widely cultured all over the world as a result of its characters as; fast-growing, can cultured with high stocking density, and is relatively can resistant to many stresses and several diseases [1]. In the last few years, fish farms suffered from severe mortality especially in tilapia fish farms in summer seasons which leads to high economic losses. The main cause of this syndrome was not clear but clinical signs on diseased fish matched with streptococcus infection. *Streptococcus* is a common disease affecting various species of fish worldwide. *Streptococcus* considered being the most devastating disease as it can cause massive kills of large size fish which leads to high economic losses [2, 3, 4]. Streptococcosis a complex disease caused by different genera [1]. *Streptococcus agalactiae* and *Streptococcus iniae* are important aquatic pathogens; they are Gram-positive, cocci-shaped bacteria that commonly occur in pairs or in long chains and beta-hemolytic. They can be grown in both aerobic and facultative anaerobic as described by [5]. *Streptococcus agalactiae* is the major cause of *Streptococcosis* in farmed tilapia. *S. iniae* also causes mortality but to a lesser extent [6]. *Streptococcus agalactiae* can causes severe damage to fish aquaculture worldwide. The mortality rate of 10-40% was observed. The infected fish exhibited a variety of symptoms typical of Streptococcosis, including lethargy, exophthalmia, corneal opacity, ascites, hemorrhage and erratic swimming [3]. The highest incidence for Streptococcus was in the summer followed by autumn, spring and winter. High water temperature over than 26ºC during summer season acts as a stress factor on fish, because it decreases the dissolved oxygen in water, increases the ammonia level, affects the immune system of fish and acts as a suitable media for growth and multiplication of bacteria [7, 8, 1]. Molecular diagnosis using the PCR technique is useful to identify streptococcus, which is faster and more reliable test than microbiologic culture [9]. Some important genetic virulence factors like C5a peptidase (SCP), serine protease (CSP), and many surface proteins have been found extensively in various streptococcus species [10, 11]. Many studies done in Egypt showed that PCR assay using scpB genes was more sensitive for detection of Streptococcus species group B than the culture method [12, 13, 14], as the sensitivities of SCPB PCR assays were 94.4% by [15]. The sensitivity can be higher by using selective and enriched broth media previous to performing the PCR [15].

The present study was carried out to investigate and determined the actual cause of severing mortalities of some cultured *O. niloticus* at Kafrelsheikh Governorate.

Materials and Methods

Fish: A total no. of 100 cultured *O. niloticus* fishes with average body weight (150±15g) showed apparent clinical signs of disease were collected randomly alive from10 private fish farms (10 fish per farm), from Kafrelsheikh Governorate and transported in battery aerated plastic pages to the wet lab. at Animal Health Research Institute, Kafr El sheikh lab, Egypt for examination.

Water samples: Twenty water samples (10 samples from the water inlet and 10 water samples from farm water) collected by inverting a 500ml sterilized glass bottle, 30cm under the pond water surface. 30ml of water samples were centrifuged at 5000 rounds per minute for 5 minutes, 1 ml of sediment was incubated into the test tube containing 9 ml of trypticase soy broth at 37^{O}C for 24 hrs and completed for bacteriological examination [16].

Fish examination: Naturally infected *O. niloticus* was carefully examined in ponds of the fish farms for swimming, feeding, and any abnormal clinical signs on the body. Also, any post mortem lesions were examined and recorded according to [17].

Bacteriological Examination of *Streptococcus* spp.:

A-Isolation: Under complete aseptic conditions, bacteriological isolation was carried out from 100 apparently healthy and diseased fish. Tissue samples (eyes, brain, kidney, liver, spleen, and skin) of fish and prepared water samples were inoculated into trypticase soy broth at 37 ^{O}C for 24 hrs. The prepared samples were streaked on the surface of Trypticase Soy agar supplemented with 3% NaCl and on Blood agar. The inoculated plates were incubated at 37 °C for 24-48 hours and examined for bacterial growth. Suspected streptococcal colonies sub cultured purified and preserved in semisolid agar tubes for further identification [18].

B- Identification [17].

I-Bio-chemical testes: The isolates were initially identified by characteristic morphology and catalase-negative before being subjected for further identification by using the following tests: hemolysis of 7% sheep blood agar, smears of suspected bacterial colonies of cultured samples were prepared, stained with gram stain and examined microscopically finally API test for the suspected purified isolates were carried.

II-Polymerase chain reaction (PCR)

DNA extraction: DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{0}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligo-nucleotide Primer: Primers used were supplied from Metabion (Germany) are listed in Table (1).

Table 1: Primers sequences, target genes, applicant sizes and cycling conditions

Microbial agent	Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final Extension	Reference
					Secondary denaturation	Annealing	Extension		
Streptococcus *agalactiae*	*ScpB*	ACAACGGAAGGCGCTACTGTTC ACCTGGTGTTTGACCTGAACTA	255	94 ˚C 5 min.	94 ˚C 30 sec.	47 ˚C 30 sec.	72 ˚C 30 sec.	72 ˚C 7 min.	[19]

PCR amplification: primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied bio-system 2720 thermal cycler.

Analysis of the PCR Products: The PCR products were separated by using electrophoresis (1% agarose gel; Applichem, GmbH, Germany) in 1x TBE buffer using gradients of 5V/cm at room temperature. For gel analysis, 40 µl of the products were loaded in each gel slot. Gelpilot100 bp (Qiagen, Germany, GmbH) and general 100 bp ladder (Fermentas, Thermo, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Antibiotic Sensitivity testing: It was applied according to guidelines stipulated by the international recommendations given by the National Committee for Clinical Laboratory Standards [20], using Muller Hinton agar. Bacterial isolates were tested for their susceptibility to 11 different antimicrobial discs (Disc diffusion method) included T: Oxytetracycline (30µg), Flo: Florfenicol (30µg), CT: Celestine (10 µg), CIP: Ciprofloxacin (5µg), CTX: Cefotaxime (30µg), EX: Enrofloxacin(5µg), Af: Flummox (10µg), E: Erythromycin (15µg), CN: Gentamycin (10µg),Ax: Amoxicillin (15µg), and Sp:Spiramycin (100µg).

Experimental infection: 60 apparently healthy *O. niloticus* Fish 80 -100 gm body weight was divided into 4 groups, 15 fish in each Group in glass aquarium supplied with de-chlorinated water and source of aeration. Injection of each fish Intraperitoneal with 0.3 ×10^{8}CFU/ml from *S. agalactiae* suspension [21]. Recording for clinical signs and mortality rate on injected fish [22]. Re-isolation of *S. agalactiae* from these experimentally infected fish was carried

Treatment trial: Usage of the highly sensitive antibiotics Oxytetracycline, Florfenicol, and Colistine according to result of the antibiogram for treatment of experimentally infected fish for 5 days each antibiotic was mixed well to fish ration in concentration of 3gm antibiotic/kg ration and feeding was 3% from fish weight in divided amount per day [8].

Results

The clinical picture of the collected fish exhibited loss of escape reflex; skin darkness; bilateral exophthalmia with corneal opacity and ulcers varied in its degrees (Figure 1).

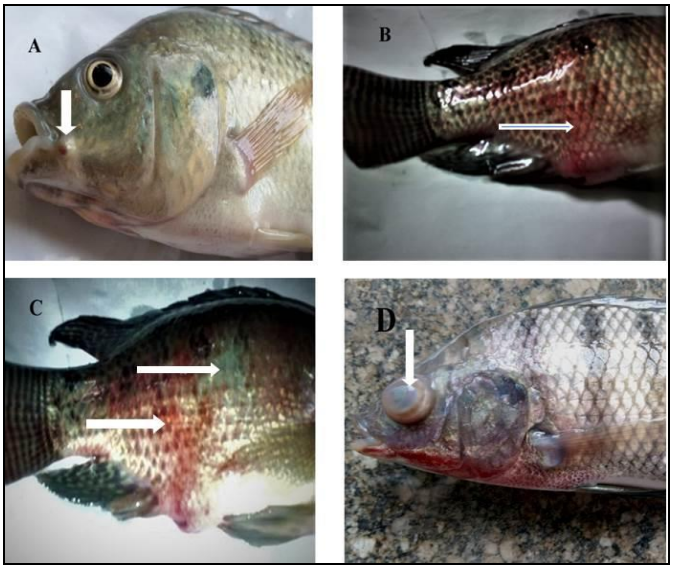

Fig 1: A: Naturally infected tilapia fish with streptococcus spp. showing abscess around head. B & C: Naturally infected tilapia fish with streptococcus spp. showing hemorrhagic patches progressed to be ulcerative areas. D: Naturally infected tilapia fish with streptococcus spp. showing exophthalmia and corneal opacity.

The post mortem examination of naturally infected *O. niloticus* revealed pale or congested liver with distended gall bladder, abdomen filled with bloody fluid, and congestion of the internal organs with congested kidney (Figure 2).

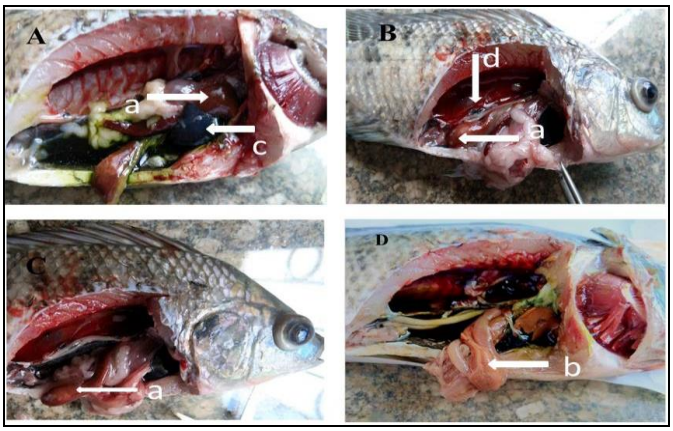

Fig 2: P.M lesions of naturally infected fish A, B ,C and D:Naturally infected tilapia fish with streptococcus spp. showing hepato-splenomegaly with congested (arrow a) or pale liver (arrow b) with distended gall bladder(arrow c), abdominal cavity filled with bloody fluid(arrow d)and congested, enlarged kidney.

A total of 7 *Streptococcus* spp. isolates were recorded; 5 *S. agalactiae* isolates (3 from examined fish and 2 from examined farm water) and 2 isolates of *Streptococcus iniae (*1 from examined fish and 1 from examined farm water). S. *agalactiae* isolates found in 30% of examined fish samples and 20% of examined water farms and not detected in examined water farm inlets, while *S. iniae* isolates found in 10% of examined fish samples and 10% of examined water farms and not detected in examined water farm inlets (Table 2).

Table 2: number and percentage of isolation of *S. agalactiae* and *S. iniae* from fish and water samples.

Streptococcus spp.	**Fish samples**			**Water samples**					
				inlet			**Farm water**		
	+ve	No	%	+ve	No	%	+ve	No	%
S. agalactiae	3	100	3	0	10	0	2	10	20
S. iniae	1		1	0		0	1		10
Total	4		4	0		0	3		30

% calculated according to the No. of examined samples

The three examined *Streptococcus agalactiae* isolated from fish samples had the Streptococcal C5a peptidase-like gene; SCP B (Fig 3).

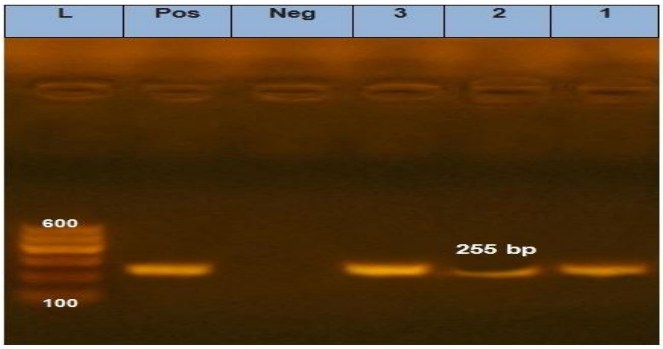

Fig 3: Showing Agarose gel electrophoresis of PCR amplification of (255 bp) for characterization of virulence gene (scpP) S. *agalactiae*.

Lane M: 100 bp ladder as molecular size DNA marker.
Pos: control positive for *S. agalactia*
Neg: control negative for *S. agalactiae*
Lane 1-3: positive virulence gene (scp P) for *S. agalactiae*.

Table 3. show that *Streptococcus agalactiae* exhibited resistance to enrofloxacin and erythromycin while, *Streptococcus iniae* resistance to Flummox, erythromycin and Spiramycin.

Table 3: Antibiogram of *S. agalactiae* and *Streptococcus iniae*.

Antibiotic	***Streptococcus agalactiae***	***Streptococcus iniae***
Oxytetracycline(Ox_{30})	S	S
Florfenicol(f_{30})	S	S
Colistine (Ct_{10})	S	S
Ciprofloxacin (Cip_5)	R	I
Cephotaxime (Ctx_{30})	I	S
Enrofloxacin (Ex_5)	R	I
Flummox (Af_{10})	I	R
Erythromycin (E_{15})	R	R
Gentamycin (cn_{10})	I	I
Amoxicillin (Ax_{25})	S	I
Spiramycin (Sp_{100})	S	R

S: Sensitive R: Resistance I: Intermediate

Treatment trial of experimentally infected tilapia with *Streptococcus agalactiae* shows that (as shown in Table 4), the group treated with Florfenicol had the lowest mortality rate and higher Survival rat flowed by Oxytetracycline group then Colistine group

Table 4: Mortality rate and Survival rate of experimentally infected tilapia with *Streptococcus agalactiae* (15 fish of each group).

group	Oxytetracycline group		Florfenicol group		Colistine group	
	+ve No.	%	+ve No.	%	+ve No.	%
mortality	5	33.3	3	20	8	53
Survival	10	66.6	12	80	7	47

Discussion

Bacterial diseases can affect cultured fish causing severe economic losses that resulted from fish mortality that can be as high as 80% [23]. Streptococcal infections in fish are septicemic diseases that have been reported worldwide causing severe economic losses in fish production [24, 25]. This disease is now recognized as a highly important infectious disease in several aquatic animal species associated with significant morbidity and mortality in farmed fish [26].

The present study was carried out to investigate the actual cause of sever mortalities of cultured *O. niloticus* fish in some fish farms at Kafr El Sheikh Governorate. The clinical picture of the collected fish exhibited loss of escape reflex; skin darkness; bilateral exophthalmia with corneal opacity and ulcers varied in its degrees (Figure 1) matched with previously observed by [27, 28, 29, 30]. The post mortem examination of naturally infected *O. niloticus* revealed pale or congested liver with distended gall bladder, abdomen filled with bloody fluid, and congestion of the internal organs with congested kidney (Figure 2) previously observed by [31, 32, 33]. Streptococcosis isolates Colonies on Trypticase Soy agar supplemented with 3% Na CL were small pinpoint whitish round and slightly raised and on Blood agar with β hemolysis, Gram-positive cocci arranged in short chains, catalase and oxidase were negative, these results were confirmed with API test. As showed in Table 2, A total of 7 Streptococcus spp. isolates were recorded; 5 *S. agalactiae* isolates (3 from examined fish and 2 from examined farm water) and 2 isolates of *Streptococcus iniae (*1 from examined fish and 1 from examined farm water). S. *agalactiae* isolates found in 30% of examined fish samples and 20% of examined water farms and not detected in examined water farm inlets, while *S. iniae* isolates found in 10% of examined fish samples and 10% of examined water farms and not detected in examined water farm inlets, these show that the incidence of *S. agalactiae* is greater than the incidence of *S. iniae* , this result is due to *S. agalactiae* is the major cause of Streptococcosis in farmed tilapia than other Streptococcosis spp. [6], we can notice that farm water samples were more contaminated with Streptococcosis spp. than the examined water farm inlets, which may be due to that the main source of Streptococcosis spp. were the infected fecal matter used as fertilizer in farm pounds.

As shown in Fig 3, the three examined *Streptococcus agalactiae* isolated from fish samples had the Streptococcal C5a peptidase-like gene (SCP B). *Streptococcus agalactiae* is a significant aquaculture pathogen, though its surface-anchored streptococcal virulence factors as Streptococcal C5a peptidase-like gene (SCP), which is located elsewhere on the chromosome (Locke *et al*. 2008). SCP gene may be detected in other streptococcal species [34, 35, 3]. As a result of Several limitations of diagnosis of streptococcal, which make to control and prevention of this disease is difficult [4], *In vitro* antibiotic sensitivity pattern of Streptococcus spp. isolates was conducted by disc diffusion method according to [23], for 11 antibiotic discs where *S. agalactiae* isolate was found to be sensitive to Oxytetracycline, Florfenicol, Colistine, Amoxicillin, and Spiramycin and resistant to Ciprofloxacin, Enrofloxacin, and Erythromycin, *Streptococcus iniae* isolates were found to be sensitive to Oxytetracycline, Florfenicol, Colistine, and Cefotaxime and resistant to Flummox, Erythromycin and Spiramycin, these results are matching with that recorded with [36, 37, 38, 8, 3], in other hand it differs than that recorded by [39]. Treatment trial of experimentally infected tilapia with *Streptococcus agalactiae* shows that (as shown in Table 4), the group treated with Florfenicol had the lowest mortality rate and higher Survival rat flowed by Oxytetracycline group then Colistine group.

Conclusions

Streptococcus spp. infection considered as one of the main causes of mortality syndrome in tilapia fish farms, leading to high economic losses and the source of infection with *Streptococcus* spp. in fish farms may be due to using of sewage polluted water in fish farms also using of animal fecal matter as fish pond fertilizer.

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