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Bacteriological and Molecular Studies on the Enterococcus Species Isolated From Diseased Fish and Its Effect on Fish Farm Profits

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Abstract

The study was *carried* out on *120 fish samples O. nitoticus collected from* Kafir El-Sheikh Governorate (60 diseased and 60 apparently healthy fish). The clinical picture of naturally infected *O. niloticus* showed haemorrhagic spots on *the operculum, base of fins and mouth edges, skin darkening, uni-or* bJJateraJ exophthalmia and skeletal deformity in some cases abdominal distension was observed. Postmortum lesions in *O. niloticus* revealed congested and enlarged liver or pale with grayish nodules in some cases. Spleen and kidneys were enlarged and congested and abdominal cavity contained serous fluid in some cases. Bacteriological examination revealed the isolation of (26) streptococcus isolates with an incidence of (43.3%) from diseased *O. niloticus,* and isolation of (17) isolates with an incidence of (28.3%) from the 60 apparently healthy . These isolates were biochemically tested. SDS-P AGE analysis of whole cell protein of selected serotyped strains revealed the presence of 7-13 protein bands and the most common characteristic bands were 36.67 KDa, 27.37 KDa and 44.0 KDa. Kb. DNA profile analysis of the 3 streptococcus species showed common band at 321 Serological examination of 37 selected isolates result in differentiation into 17 *Enterococcus faecalis,* 12 *Streptococcus iniae, 5 Streptococcus pneumoniae* and 3 untypeable strains. Experimental infection of 8 groups of *O. niloticus* (each of 10 fish) with bacterial suspension of 8 isolates (2 *Enterococcus faecalis, 5 Streptococcus iniae* and 1 Streptococcus *pneumoniae* result in mortality rate of 20%, 10% and 0%, respectively. While, inoculation of the bacterial filtrate of the same isolates result in mortality rate of 30%, 22% and 10%. Our results cleared that the enterococci causes a great economic losses to fish farm production and it differ according to the type of bacteria that infected the fish. In bacterial suspension infection the weight losses for each 100/fish were 450 gm, 262.5 gm and zero losses zero losses for S. fecalis, S. iniae and S. pnumoniae and the return losses reached to 4.5 LE, 2.62 LE and zero losses for S. fecalis, S. iniae and S. pnumoniae. While, in bacterial filtrate the weight losses for each 100/fish were 675 gm, 1237.5 and 112.5 gm for S. fecalis, S. iniae and S. pnumoniae and the return losses reached to 6.75 LE, 12.37 LE and 11.25 losses for S. fecalis, S. iniae and S. pnumoniae.

From these results we concluded that:

- Fish farms should avoid use of polluted water.
- Fish handlers with cut wounds should avoid fish handling without gloves as Streptococcus soft tissue causing sepsis, infection endocardities, urinary tract infections, labor pneumonia and meningitis.
- Human should keep water sources away from sewage pollution.

Keywords: 2 Enterococcus faecalis, 5 Streptococcus iniae , O. niloticus , Streptococcus pneumoniae , Aquaculture production

Contents

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1. Introduction

The intensive aquaculture production leads to the development of infectious disease outbreaks. Bacterial diseases are the most common diseases in intensive fish rearing raring facilities [1].

Streptococcus spp. is the most prevalent gram-positive fish bacterial pathogens. The disease has been well recognized with the intensification of aquaculture and has economic consequences on fisheries in many areas of the world. In Thailand, streptococcal infections are major threat to *Thai tilapia* industry and causes production lost in all regions of tilapia farming up to 75% in acute cases [2].

Streptoccosis caused by different species of streptococci including, *S. iniae* which has been reported as a significant contributor to the damage of aquacultured fish, *S. agalactiae [3], S. difficile* and *S. shiloi [4], S. faecium [5, 6], S. equi, S.equisimilis, S. pyogenes, S. zooepidemicus [7].*

Streptococcus is a gram-positive, facultative anaerobic, non-sporing cocci arranged singly, in pairs *(S. pneumoniae* arranged in pairs of lancet-shaped or flame-shaped cocci). [8] in chains (either short or long), variably haemolytic (α , ***β*** or non haemolytic) [3].

The main clinical signs detected in fish due to streptococcal infection were lethargy, loss of appetite, spine displacement, uni or bilateral exophthalmia (pop-eye), abdominal distension and haemorrhagic lesions on the skin [9, 10]. However, human disease is characterized by soft tissue infection following skin puncture [11], infective endocarditis, sepsis and urinary tract infections are serious infections caused by *S. fecalis [12]*. *S. pneumoniae* is an opportunistic gram-positive cocci in the upper respiratory tract in about 20% of healthy human, but may cause lobar pneumonia and meningitis [13]. The risk of the disease was increased in immunocompromised workers especially those suffer cuts or puncture wounds [14].

Streptococcosis was recorded in Egypt among tilapia [15-20].

Streptococci cause a significant economic losses to the world aquaculture industry [21]. These have been estimated to exceed U.S $ 150 million annually, the economic losses attributed to increasing mortlity of the fish, increasing weight sale losses of the fish and the economic returns [14].

The object of this work is:

1. Isolation *of Streptococcus* spp. from tilapia fish.
2. Identification of the isolated *Streptococci* biochemically, serologically.
3. Studying the pathogenicity of isolated *Streptococcus* spp. through experimental infection.
4. Studying the molecular characters of different Streptococcal spp. isolated.
5. Evaluation the economic losses resulted from the infection of the fish with enterococcus bacteria.

2. Material and Methods

2.1. Materials

2.1.1. Fishes

2.1.1.1. Naturally Examined Fishes

A total number of 120 fish (60 clinically diseased and 60 apparently healthy Nile tilapia fish), *Oreochromis niloticus (O. niloticus)* were collected from different localities in Kafr El-Sheikh Governorate for bacterial examination. The alive fishes were transported with sufficient amount of water and oxygen (from the pond which the fish were harvested). The fishes were kept in well prepared aquaria till the time of examination. While, the freshly dead fishes were labeled and transferred in ice boxes and immediately subjected to bacteriological and post mortem examination [22].

2.1.1.2. Experimental Fish

One hundred and seventy apparently healthy *O. niloticus* weighted 60 ± 5g. The fish were transported in polyethylene bag containing 30% of its volume fish and water and the remaining volume of the bag were pumped with oxygen [22].

Five fish were randomly selected and subjected for bacteriological examination to ensure the avoidance from any pathogenic bacteria, the remaining fish were subdivided into 17 groups each of 10 fish (8 groups injected I/M with the bacterial suspension 0.1 ml/fish approximately contained 1.5 x 10^8 cfu., 8 groups injected with 0.1 ml/fish bacterial filtrate and the control group injected with 0.1 ml/fish sterile saline).

All experimental fish were kept in well prepared aquaria and left 7 days for acclimation to the laboratory conditions and fed with pelted food in a rate of 3% of their body weight. Excreta and uneaten food were siphoned daily. The temperature was adjusted at 25 ± 1°C along the experimental period [23].

2.1.2. Aquaria Used

Clean glass aquaria with dimensions of 100 x 30 x 40 cm were used. All aquaria were supplied with sufficient amount of dechlorinated tap water, as described by Innes [24] with continuous aeration by electric air pump (Renna, Italy). The water temperature were adjusted to 25 ± 1°C.

2.1.3. Media Used

2.1.3.1. General Media Used for Isolation: [25].

1. MacConkey agar (Biolife).
2. Trypticase soya agar (Becton Dickinson company). BBL™

3. Brain heart infusion agar (Biolife).
4. Brain heart infusion broth (Biolife).
5. Sheep blood agar 5%: Used for isolation and determination of the haemolytic activity of the isolated organisms [26].

2.1.3.2. Specific Medium Used for Isolation of Streptococci

1. Streptococcal Selective Agar (Biolife)

2.1.3.3. Media Used for Biochemical Identification

1. Simmon's citrate agar (BBL): Used for detection of the ability of recovered isolates for citrate utilization [26].
2. Triple sugar iron agar (Lab. 53): Used for the detection of hydrogen sulphide production and sugar fermentation by the obtained isolates [25].
3. Semi-solid agar medium (0.5%): Used for detection the motility of the examined isolates [26].
4. Starch agar: It was used to determine the ability of isolated microorganisms to hydrolyze starch [27]
5. Gelatin agar medium: It was used to determine the ability of the isolated bacteria to liquefy gelatin [27].
6. Indole test medium: It was used for detection the ability of isolated bacteria for indole production [26].
7. Glucose phosphate broth: Used in methyl red and voges proskauer test [26].
8. NaCl media 6.5%: Used to show the sensitivity of bacteria to 6.5% NaCl [26].
9. Peptone water: Used in sugar fermentation test [27].
10. Sugar media (Peptone water with 0.01% phenol red as indicator and 1% sugar (arabinose, lactose, maltose, mannitol, fructose, glucose and sucrose). Also, the tubes contained inverted Durham's tube, it was used for detection the ability of microorganisms to produce acid and gases [26].

2.1.4. Reagents and Stains

1. Hydrogen peroxide 3% (Piochem).
2. Kovac's reagent (Biolife). 1% soln. of tetramethyl-P-phenylene diamine dihydrochloride.
3. Gram's iodine solution 1% (Egyptian Diagnostic Media, EDM) used for starch hydrolysis test.
4. Methyl red reagent [26].
5. Phenol red.
6. Kovac's reagent for indole test.

2.1.5. Kits for Serological Identification

Streptex kits (Welcome Diagnostic. A Divsion, Dartford, England) for serological grouping *of Streptococcus* sp. including group A, B, C, D, E and N) [28].

2.1.6. Buffers and Reagents for Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

The whole cell protein extracts of streptococcus strains were subjected to discontinuous SDS-PAGE according to the methods of Laemmli [29] *and Bollag, et al. [30]*.

2.2. Methods

2.2.1. Clinical and Post Mortem Examination

The naturally infected fishes were subjected to clinical and post mortem examination according to the methods described by Stoskopf [31] and Noga [32].

2.2.2. Bacteriological Examination: Bacteriological Samples

The fishes were cleaned and swabbed with a piece of cotton moistened with ethyl alcohol 70%, then the surface was scorched with flame. The fish was opened aseptically as described by Stoskopf [31]. Samples for bacteriological examination were taken from skin and underlying musculature, liver, spleen, kidneys, heart, and brain.

Isolation and Purification of Bacteria:

Tissue specimens inoculated into Brain heart infusion broth and incubated at 30°C for 24 h. A loopful from 24 hr inoculated broth was streaked on MacConkey agar, Brain Heart Infusion Agar (BHIA), Trypticase Soya Agar (TSA) and Streptococcus selective agar. The plates were incubated at 30°C for 24-48 h. Suspected colonies were picked up and re-streaked on a new plates of Streptococcus Selective Agar medium and re-incubated at the same temperature for 24 hrs. Pure isolates were inoculated into a nutrient agar slant as stock culture for further identification into semisolid agar for detection of motility and preservation.

Haemolysis on Sheep Blood Agar 5%:

The bacteria were streaked on 5% sheep blood agar and incubated at 30°C for 24 hr. The plates were examined for the presence of bacterial colony growth and the zone of haemolysis around them [33].

Biochemical Characterization:

The suspected isolates of streptococci were subjected to schemes of biochemical reactions characterization (cytochrome oxidase reaction, catalase reaction, gelatin liquefaction, starch hydrolysis, esculin hydrolysis, indole production, methyl red and voges-proskauer reaction, citrate utilization, hydrogen sulphide production, sugar fermentation activities and ability to grow in 6.5% NaCl as described by Cowan and Steel [34]; Cruickshank, et al. [25]; Koneman, et al. [35]; Bergey, et al. [36] and Elmer, et al. [37].

2.2.3. Serotyping of the Isolated Streptococci

A total of 37 recovered isolates were selected and subjected to serological identification by slide agglutination using Streptex kits [28].

2.2.4. Molecular Characterization

Bacterial Isolates:

A total of 8 biochemically and serologically identified streptococcus isolates including (2 *Enterococcus faecalis*, *5 Streptococcus* iniae and 1 *Streptococcus pneumoniae)* were selected randomly for molecular characterization for the similarity.

Extraction of Whole Cell Protein:

The whole cell protein of the selected streptococcus isolates were separated using the method described by Elliot, et al. [38] as follow:

2.2.4.1. Electrophoresis of Whole Cell Protein Antigens

Carried out according to [39].

2.2.4.2. Isolation of DNA from the Isolated Streptococci

Eight different serotypes of streptococcus (5 *Streptococcus iniae, 2 Streptococcus faecalis* and 1 *Streptococcus pneumoniae)* isolates were tested using the technique of [40].

2.2.5. Experimental Infection

Preparation of Inocula:

Twenty four hours of pure culture of chosen isolates of streptococcus (2 *Enterococcus faecalis, 5 Streptococcus iniae* and 1 *Streptococcus pnemoniae)* as they were the most common isolated species were suspended in sterile saline. The preintended bacterial suspensions concentration of 1.5 x 10^8 cfu was estimated using McFarland's 0.5 standard tube.

Each fish were challenged with their corresponding bacterial isolates as described by Rasheed and Plumb [41].

Preparation of Culture Extract According to Hamilton [42]:

Experimemal Design:

Each 0.1 ml of bacterial suspension contain approximately 1.5 x 10^8 cell/ml. The other 8 groups were injected I/M with 0.1 ml of the bacterial filtrate of the same previously tabled isolates.

Both infected and control groups were kept under observation for 30 days. The morbidity, clinical signs, P.M. changes and mortality rates were recorded daily in both experimentally infected and control groups. Reisolation of the inoculated bacterial strain was done and the isolated bacteria were reidentified as previously described.

Economic losses:-

The economic losses of the fish due to exposure to enterococci were determined from dead fish, weight of dead fish and the losses in return due to dead fish according to the following equations [43]:

a-Weight of dead fish = Number of dead fish X Average weight of the fish (gm).

b-Losses in returns (L.E) = Weight of the fish (Kg) X Price of Kg fish (L.E).

Statistical analysis. The incidences of enterococci among fish, incidence of dead fish was statistically analysed using Chi^2-test according to [44].

Results: 1.Clinical signs and PM. lesions of naturally infected fishes:

Naturally infected *O. niloticus* revealed signs including haemorrhages on the body surface especially on the operculum, base of the fins and mouth edges. Some fishes showed exophthalmia (unilateral or bilateral), skin darkening and skeletal deformity, while, post mortem examination revealed pale liver with distended gall bladder, congested kidney and spleen.

The abdominal cavity contained serous or serosinguinous fluid. Meanwhile, apparently health examined fish didn't have any signs or post mortem lesions in the internal organs (Fig. 1 and 2)

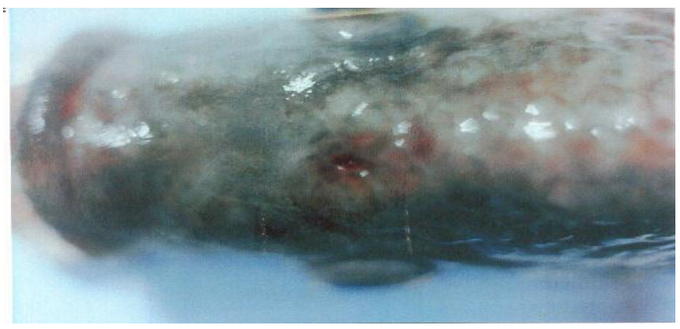

Fig-1. Fish showing bilateral exophthalmia and haemorrhage on head.

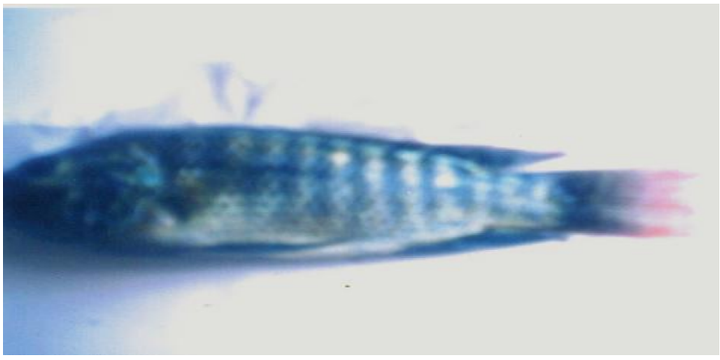

Fig-2. Fish showing skin darkening.

3. Results of the Bacteriological Examination

3.1. Diseased Fish

Table (1) indicated the result of the bacteriological examination of the naturally infected fish for the streptococcal isolation. Suspectec streptococci were isolated in pure culture from 26 samples out of 60 examined samples with an incidence of (43.3%). On the other hand, the other 34 samples didn't gave suspected streptococcal colonies even mixed with other bacterial growth with a percentage of 56.7%.

Table-1. Results of the bacteriological examination of diseased fish samples. % according to the No. of examined samples.

No. of examined fish	No. of positive samples		No. of negative samples	
	No.	%	No.	%
60	26	43.3%	34	56.7%

3.2. Apparently Healthy Fish

From Table (2), out of 60 examined apparently healthy fish (17) isolates were recovered from samples with an incidence of (28.3%). While, the other (43) samples gave negative suspected streptococcus isolation results.

Table-2. Results of the bacteriological examination of apparently healthy fish samples % according to the No. of examined samples.

No. of examined fish	No. of positive samples		No. of negative samples	
	No.	%	No.	%
60	17	28.3%	43	71.7%

3.3. Identification of the Isolated Streptococcus Species

3.3.1. Colonial Characteristics and Gram's Staining

The bacterial isolates of suspected Streptococci grew on TSA, BHIA appeared as small, pin head, creamy white, circular and glistening. On streptococcus selective agar appeared as very small (dew drop like colonies), grayish white or large colonies (2-3 m), creamy in colour and circular. Meanwhile, the results of dry/heat Gram stained smears revealed gram positive cocci in pairs or chains short or long. *Streptococcus pneumoniae* appeared as lanced shape or double flame cells.

3.4. Motility

Stabbing of all recovered isolates into semisolid nutrient agar medium revealed that all isolates were non-motile.

The biochemical characterization of the isolated *Streptococcus* spp.:

In Table (3) the biochemical tests showed variable results and were not definitive or can differentiate it in general. But, some tests were indicative for enterococci *(Enterococcus faecalis)* as growth on MacConkey agar, 6.5% NaCl broth bile esculin medium hydrolysis and growth at 45°C.

On 5% sheep blood agar there were α or β haemolytic or non haemolytic colonies.

Table-3. Biochemical characteristics of the suspected *Streptococcus* isolates

Test	Enterococcus faecalis	Streptococcus iniae	Streptococcus pneumoniae
• Catalase	-	-	-
• Oxidase	+	-	+
• Growth on blood agar	α / β	β or non haemolytie	α
• Growth on TSA, BHI	+	+	-
• Growth on MacConkey	+	-	-
• Growth at 45°C	+	-	-
• Growth on 6.5% NaCl	+	-	-
• Starch hydrolysis	+	+	-
• Gelatin liquefaction	±	-	-
• Esculin hydrolysis	-	+	-
• Indole test	+	±	-
• Citrate utilization	-	-	±
• Methyl red	±	-	-
• Sugar fermentation			

			Continue
- Mannitol	+	-	-
- Glucose	±	±	+
- Sucrose	±	±	-
- Lactose	±	-	-
- Maltose	±	±	+
• Lipase activity	-	-	-

3.5. Serological Identification

The thirty seven *Streptococcus* isolates and were differentiated into 17 *Enterococcus faecalis*, 12 *Streptococcus iniae*, 5 *Streptococcus pneumoniae* and 3 unidentified strains (isolates).

3.6. Molecular Characterization

8 isolates were selected from the 3 species. (5 isolates *Streptococcus iniae, 2* isolates *Enterococcus faecalis* and 1 isolate *Streptococcus pneumoniae.*

3.6.1. Molecular Characterization of Whole Cell Protein of 8 Streptococcus Isolates

8 Streptococcus isolates (2 *Enterococcus faecalis, 5 Streptococcus iniae* and 1 *Streptococcus pneumoniae)* were characterized using SDS-PAGE method. SDS-PAGE revealed several protein bands of different molecular weights ranging from 18-138.35-KDa. The more common recovered bands among the tested strains were 25 KDa, 31.67 KDa, 36.67 KDa and 52.36 KDa. While, the minor bands were 18, 29.02, 48.51, 62.72, 93.63, 102.78, 105.92 and 138.35 KDa. The results showed that SDS-PAGE analysis of whole cell protein of Streptococcus spp. Had 713 bands. The present study revealed difference in minor bands among the tested strains of the same *Streptococcus* spp. (Fig. 2) and Table (4).

3.6.2. Molecular Characterization of DNA

The DNA profile examination for 5 *Streptococcus iniae*, 2 *St. fecalis* and 1 *Streptococcus pneumoniae* revealed common band at 321 Kb. (Fig. 4).

Table-4. Protcin analysis of streptococcus species

Band	Marker		Pneumoniac		E. faecalis								S iniae					
			Lane 1		Lane 2		Lane 3		Lane 4		Lane 5		Lane 6		Lane 7		Lane 8	
	MW	RF	MW	RF	MW	RF	MW	RF	MW	RF	MW	RF	MW	RF	MW	RF	MW	RF
1	17.00	0.875	19.04	0.585	18.66	0.865	18.56	0.866	18.37	870	18.46	0.868	18.46	0.868	18.95	859	19.24	854
2	26.00	0.802	29.02	0.715	21.10	0.823	25.13	0.767	25.79	755	25.52	0.759	25.79	0.755	25.65	0.757	28.58	0.720
3	34.00	0.688	32.33	0.679	23.39	0.788	27.28	0.736	27.85	729	28.28	0.724	27.85	0.729	28.93	0.722	31.83	0.684
4	43.00	0.566	34.29	0.653	27.43	0.734	30.24	0.701	30.55	698	31.67	0.686	31.67	0.686	31.67	0.686	35.70	0.651
5	55.00	0.444	37.52	0.628	31.34	0.689	33.68	0.665	35.64	846	36.01	0.642	336.01	0.642	35.82	0.644	39.30	0.613
6	72.00	0.372	42.88	0.583	34.56	0.656	37.91	0.625	40.32	604	40.53	0.602	40.32	0.664	40.32	0.604	49.01	0.538
7	95.00	0.299	47.44	0.547	37.91	0.625	42.66	0.585	45.37	564	46.08	0.559	45.37	0.564	45.61	0.562	59.27	0.474
8	130.00	0.299			43.32	0.586	47.52	0.549	50.54	528	51.33	0.523	51.07	0.524	50.80	0.526	69.86	0.418
9	170.00	0.167			48.51	0.542	58.06	0.787	62.72	455	63.04	0.453	63.69	0.450	63.04	0.453		
					53.48	0.509			75.07	394	75.07	0.394			72.42	0.406		
					61.13	0.484			105.92	278	102.76	0.288			99.58	.0299		
					68.79	0.424												
					138.35	0.188												

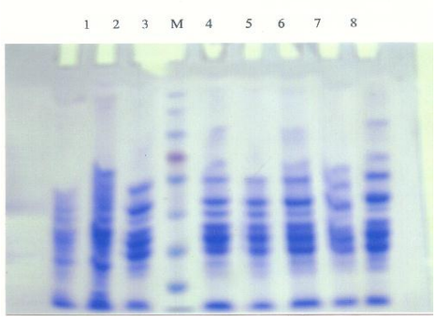

Fig-3. Electrophoretic protein patterns of 8 Streptococcus species from 1-Streptococcus pneumoniae, from 2-3 Enterococcus faecalis and from 4-8 Streptococcus iniae .

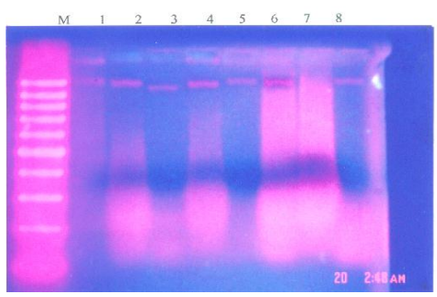

Fig-4. Electrophoresis of Streptococcus DNA profile

4. Results of Pathogenicity

4.1. Results of Inoculation of Bacterial Cells Suspension

The inoculation of bacterial suspension of 8 isolates from different species in 8 groups each of 10 fishes result in clinical signs as skin darkening, red eye with or without mild exophthalmia, ulcers on the side of body (pale ulcer), haemorrhages on body surface, operculum and around base of fins and distended abdomen. The P.M. lesion showed serous fluid in abdomen, pale liver, congested spleen and kidney. The morbidity and the mortality were indicated in Table (5). The inoculated microorganism was reisolated from the internal organs of the inoculated fish. (Fig. 5)

Table-5. Mortality rate of fish inoculated with bacterial suspension.

Bacterial suspension	Group No.	No. of fish/group	Morality rate/week				Total No.	Total %
			1st w	2nd w	3rd w	4th		
S. faecalis	1	10	2	0	0	1	3	30
	2	10	0	0	1	0	1	10
S.iniae	3	10	0	0	0	1	1	10
	4	10	0	1	0	0	1	10
	5	10	0	0	0	0	0	0
	6	10	0	0	1	0	1	10
	7	10	0	1	0	1	0	20
S. pneumoniae	8	10	0	0	0	0	0	0

4.2. Results of Inoculation of Bacterial Filtrate

Inoculation of the filtrate in 8 groups each of 10 fishes revealed as abovementioned signs but more severe. For example exophthalmia (unilateral or bilateral) were more prominent, ulcers were deeper and may reach to bone, fins eroded and haemorrhages were more severe. There were no Streptococci recovered from the internal organs (Table 6).

The control group showed no signs (normal). The Streptococci were reisolated from the experimentally infected fish and retested morphologically, biochemically and compared with the inoculated strains were identical. (Fig. 6)

Table-6. Mortality rate offish inoculated with bacterial filtrate.

Bacterial filtrate	Group No.	No. of fish/group	Morality rate/week				Total No.	Total %
			1st w	2nd w	3rd w	4th w		
S. faecalis	1	10	1	1	0	1	3	30
	2	10	0	1	2	0	3	30
S. iniae	3	10	0	1	1	0	2	20
	4	10	1	0	2	0	3	30
	5	10	0	0	1	1	2	20
	6	10	0	1	2	0	3	30
	7	10	0	0	1	0	1	10
S. pneumoniae	8	10	0	0	0	1	1	10

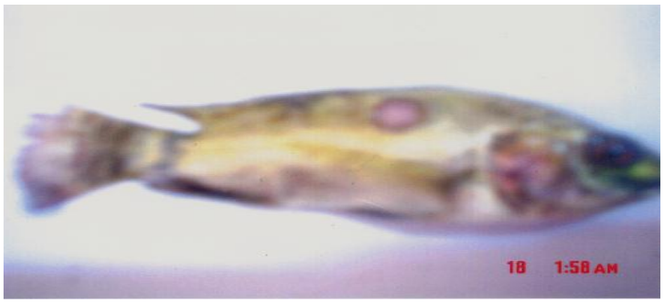

Fig-5. Fish showing skin darkening, small body ulcers with haemorrhage .

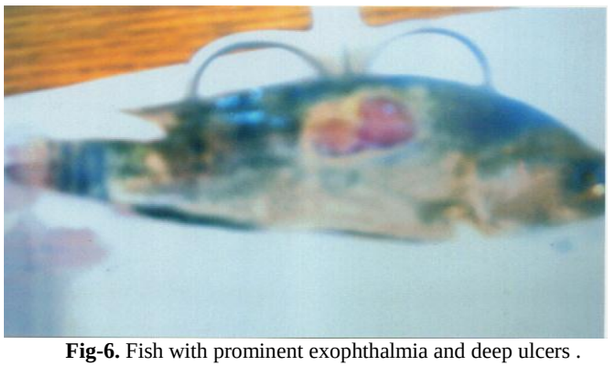

Fig-6. Fish with prominent exophthalmia and deep ulcers .

Table (7): cleared that, the enterococci causes a great economic losses to fish farm production and it differ according to the type of bacteria that infected the fish.

In bacterial suspension infection the weight losses for each 100/fish were 450 gm, 262.5 gm and zero losses zero losses for S. fecalis, S. iniae and S. pnumoniae and the return losses reached to 4.5 LE, 2.62 LE and zero losses for S. fecalis, S. iniae and S. pnumoniae.

While, in bacterial filtrate the weight losses for each 100/fish were 675 gm, 1237.5 and 112.5 gm for S. fecalis, S. iniae and S. pnumoniae and the return losses reached to 6.75 LE, 12.37 LE and 11.25 losses for S. fecalis, S. iniae and S. pnumoniae.

Table-7. Economic losses resulted from Streprococci.

Bacterial isolates	Bacterial suspension				Bacterial filtrate			
	No. of dead fish	Weight losses/gm/ 80fih	Weight losses/gm / 100fih	Return losses/ LE/100 fish	No. of dead fish	Weight losses/gm /80fih	Weight losses/gm / 100fih	Return* losses/ LE/100 fish
S. fecalis	4	360	450	4.50	6	540	675	6.75
S. iniae	5	450	262.5	2.62	11	990	1237.5	12.37
S. Pnumoniae	0	0	0	0	1	90	112.5	11.25

* Calculated depend on the price of Kg fish = 10 LE

5. Discussion

Streptococcosis is considered one of the most economically important bacterial infections in warm water aquaculture. The problem is world wide [45] and has occurred both sporadically and epizootically in fresh and salt water stocks of both farmed and wild fish [46]. The disease has increased with intensification of cultured fish [47] and considered the largest problem in intensive tilapia rearing systems throughout the world [48].

Clinical examination of naturally infected *O. niloticus* showed haemorrhagic spots on the external body surface particularly on the operculum, base of the fins and mouth edges, skin darkening, uni-or bilateral exophthalmia, cornsal turbidity, skeletal deformity and abdominal distension. The internal lesions were pale or congested liver with distended gall bladder, congested and enlarged kidney and spleen, hi some acute cases the abdominal cavity was filled with ascetic fluid.

These findings agreed with those mentioned by Kitao [49]; Eldar, et al. [4]; Ebtsam [17], Dena [18]; Salvador, et al. [50], Safinaz [51]) and Eman [20]. Moreover, nearly similar results were observed in tilapia species suffered from Streptococcosis [46, 52-54].

Exophthalmia and haemorrhages on the external body surface were the common detected sings in *O. niloticus.* Exophthalmia may be due to retrobulbar congestion and odema. While, haemorrahges on the external body surface may be attributed to the haemolytic effect of the exotoxins produced by a-or p-haemolytic *Streptococcus* species. These results were also described by Rasheed and Plumb [41],and Kitao [49].

Data from this work revealed that *Streptococcus* spp. were isolated from 60 diseased *O. niloticus* fish as (26) samples with an incidence of (43.3%). While, the other 60 apparently healthy *O. niloticus* samples show (17) positive streptococcus isolation with an incidence of (28.3%). These results are higher than those mentioned below

In biochemical characterization it was noticed that all isolates were catalase, oxidase indole, gelatin liquefaction are negative and with variable haemolyic pattern *(a* or P). These observations agreed with those recorded by Boomker, et al. [55]*; Bergey, et al. [36]*, *Ebtsam [17]*, El-Bouhy [56], Dena [18] and Eman [20] and partially agreed with Baya, et al. [57] who isolated non-haemolytic group B *Streptococcus* spp. which were catalase positive and El-Bouhy [56] who found that *Enterococcus faecalis* was positive gelatin liquefaction.

The isolated Enterococcus faecalis showed ability to grow in the presence of 6.5% NaCl, on MacConkey agar, at 45°C and to exhibit black zone round colonies on bile esculine agar. These findings were recorded by Foo, et al. [58];Carson, et al. [59]; El-Bouhy [56]; Dena [18]; Safinaz [51] and Eman [20].

Results of serological identification of 37 isolates revealed that there are 17 *Enterococcus faecalis,* 12 *Streptococcus iniae, 5 Streptococcus pneumoniae* and 3 unidentified isolates. These results were in agreement with those reported by Kitao [60]; Koneman, et al. [35]; Volk and Wheeler [61] and Baya, et al. [57]. The high isolation percentage of *E. faecalis* than *S. iniae* and *S. pneumonia* may be due to enter pollution with animal and/or human excreta.

Molecular characterization of isolated *Streptococcus iniae, Enterococcus faecalis* and *Streptococcus pneumoniae* revealed that the whole cell protein profile of those *Streptococcus spp.* Possessed 7-13 protein bands of molecular weights ranged from 18.37-138.35 KDa. The most characteristic bands were 36.67 (5 isolates), 27.37 (2 isolates) and 44 KDa (1 isolate). These results agreed with that recorded by Sacilik, et al. [62] and EI-Refaee [19]. They found that the most common characteristic bands of isolated *Streptococcus spp.* were 18 and 35 KDa. The results revealed that each *Streptococcus spp.* had characteritsitcs profiles of protein. These results go hand with hand with the results stated by Ulrich, et al. [63] who studied *St. pneumoniae* whole cell protein and found that the major band was at 35 KDa. The molecular characterization of DNA for the previously mentioned *Streptococcus spp.* revealed the presence of common band at 321 Kb. These results were nearly similar to that of Camargo, et al. [64] who found common bands at 339.5 Kb. The results of molecular characterization (whole cell protein and DNA profile) can be used in diagnosis and confirmation of isolated streptococcal infection in fish.

Experimental infection of *O. niloticus* with bacterial cell suspension 2 *Enterococcus faecalis,* 5 *Streptococcus iniae* and 1 Streptococcus pneumoniae) via I/M injection result in signs of lethargy, loss of appetite, emaciation, comeal turbidity, fins rot, uni-or bilateral exophthalmia with skin darkening and ulceration in some cases which may reach to bone. Post mortem examination of fish revealed enlarged, pale liver with distended gall bladder, enlarged and congested kidney and spleen, congested gills and ascites in abdominal cavity. While, the signs and post-mortem findings of fish groups injected with bacterial liquid culture filtrate were more sever than those of bacterial cell

suspension inoculation. These results were similar to those recorded by Tung, et al. [65];*Khalil [16]; El-Bouhy [56]; Ebtsam [17]; Dena [18]; EI-Refaee [19] and Eman [20]*. These results may be due to the presence of exotoxins produced in culture media during growth of the microorganism. .

The mortality rate of *O. niloticus* inoculated intramuscularly with bacterial suspension of *Enterococcus faecalis*, *Streptococcus iniae* and *Streptococcus pneumoniae* were 20%, 10% and 0%, respectively. These results were lower than those recorded by Dena [18]*; Safinaz [51] and Eman [20]* which were 100%, 100%, 30% and 70%, respectively. While, the mortality rate *of O. niloticus* inoculated with bacterial filtrate of *Enterococcus faecalis*, *Streptococcus iniae* and *Streptococcus pneumoniae* were 30%, 22% and 10%, respectively.

Further studies should applied on the toxic substances present in the streptococcal liquid cultures filtrates to know the different kinds of these exotoxins, also presence or absence of invasive enzymes. However, studies on the application of PCR technique must be tried for the detection of streptococcus species DNA in tissues of infected fish as a rapid and specific diagnostic technique for streptococcal infection in fish.

Our results in economic losses cleared that, the streptococci causes a great economic losses to the fish farms the higher economic losses observed with bacterial filtrate infection than the bacterial suspension and the higher economic losses observed with infection with S. fecalis, S. iniae in bacterial suspension while, in bacterial filtrate the higher economic losses observed in S. iniae, S. pnumoniae and S. faecalis. The economic losses of streptococci attributed to the higher mortality rate of the fish that causing increasing losses in body weight sale and decreasing of the economic returns.

Our results agreed with those of Klesius, et al. [21] and Locke, et al. [66]**,** where they reported that, the streptococci causes a high mortality to the fish farms with increasing to the body weight losses and increasing the return losses.

This results concluded that, the external symptoms, P.M examinations, laboratory examinations that includes bacterial isolation and biochemical characterization, serological examination and molecular characterization are the main methods for detection of streptococci in fish farms. Also, the results cleared that, the streptococci causes a great economic losses to fish production farms through increasing mortality, increasing the weight losses and return losses.

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