

Genotyping and pathogenicity of *Streptococcus iniae* strains recovered from cultured *Oreochromis niloticus* at Kafr El-Shiekh Governorate, Egypt.

Abdelgayed M. Younes¹, Alkhateib Y. Gaafar¹, Alaa El-Din Z. Abu-Bryka¹,
Laila A. Mohamed¹ and El-Sayed M. Bayoumy^{2&3}

1- Hydrobiology Department, National Research Centre, Dokki, Egypt.

2- Biology Department, College of Science, Imam Abdulrahman Bin Faisal University, Dammam City, Saudi Arabia.

3- Basic and Applied Scientific Research Center, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia

ARTICLE INFO

Article History:

Received: July 27, 2019

Accepted: Sept. 15, 2019

Online: Sept. 22, 2019

Keywords:

Streptococcus iniae
cultured fish
Oreochromis niloticus
rep-PCR
epizootiology

ABSTRACT

Streptococcus iniae cause high mortalities in aquaculture farms all over the world. This study aimed to isolate, identify pathogenicity and genetic relatedness of *S. iniae* obtained from cultured Nile tilapia (*O. niloticus*) at Kafr El-Shiekh Governorate. Fourteen isolates of pure *S. iniae* cultures were biochemically identified, and confirmed through amplification and sequencing of the 16S rRNA gene. Experimental challenge with six selected isolates resulted in marked clinical signs similar to those recorded in natural infections. The LD₅₀ values were ranged between 3.7×10^7 to 4.3×10^8 cfu/fish. All strains were sensitive to oxytetracycline and florfenicol. In repetitive sequence-based PCR (rep-PCR) analysis using the BoxA primer, isolates presented genetic heterogeneity and were divided into six genotypes banding patterns. Understanding the epizootiology and genetic variability between *S. iniae* isolates, could help for better control and vaccine development.

INTRODUCTION

Tilapia considers one of the most popular Freshwater fish worldwide. Nile tilapia (*Oreochromis niloticus*) is the most widely cultured Tilapia species because of its rapid growth and high adaptability to reproduce in different environments. However, intensive cultivation has led to bacterial diseases, which considered the main cause of high mortalities among fish (Younes *et al.*, 2015).

The first isolation of *Streptococcus iniae* was in 1976 from a subcutaneous lesion of a captive Amazon bottle nose dolphin (*Inia geoffrensis*) infected with acute 'golf ball disease' (Pier and Madin 1976). Since then, it has been reported in many cultured and wild fish species worldwide, especially warm water cultured fish. In recent years, these bacteria have become the most significant disease causing high mortality in tilapia industry. Fish can asymptotically carry *S. iniae*, which can also be associated with sporadic disease outbreaks. The infected fish show general signs of lethargy, ulcers, exophthalmia, and encephalitis (Agnew and Barnes 2007 and Zamri-Saad *et al.*, 2014). Moreover, the emerging zoonotic importance of *S. iniae* was recorded in humans who deal with affected fish (Baiano and Barnes 2009).

Typing of *S. iniae* bacteria into known groups is essential to understand the epizootiology and better control of these problematic organisms. Many molecular techniques have been used to study genotypic diversity of *S. iniae* (Dodson *et al.*, 1999 and Chou *et al.*, 2014). Therefore, this study describes isolation, pathogenicity and genotypic heterogeneity of *S. iniae* isolated from cultured tilapia (*O. niloticus*) at Kafr El-Sheikh Governorate.

MATERIALS AND METHODS

Sample collection and Bacterial isolation:

A total number of 100 of Nile tilapia (*O. niloticus*) showing diseases signs with a range of body weight 70-100 g were collected from private fish farms in Kafr El-Sheikh Governorate, Egypt. The fish specimens were collected during October 2018; the fish were transferred to the Hydrobiology Laboratory, National Research Centre, Giza for complete bacteriological and molecular workups. *S. iniae* isolates recovered from 25 diseased fish with signs of septicemia, corneal opacity, exophthalmia, eye hemorrhage, bleeding on the base of the fins.

Samples for bacterial isolation were taken under aseptic condition from posterior kidney, hepatopancreas, and brain, then streaked on Brain Heart Infusion (BHI) agar (Oxoid, UK) supplemented with 5% horseblood. The inoculated plates were incubated at 28°C for up to 48 hr. The growing bacteria were subcultured to obtain pure isolates. The suspected colonies were identified by motility, oxidase and catalase tests. The isolates were then biochemically identified using commercial API20 STREP (BioMerieux, France) according to the manufacturer's instructions. Pure isolates of *S. iniae* were stocked in tryptic soya broth (TSB) containing 15% (vol/vol) glycerol at -80°C. **Genotypic Characterization:** 2×10^9 of overnight Gram positive cells culture were resuspend in 180 µl Digestion Buffer containing fresh Lysozyme (20 mg/ml). The extraction of genomic DNA was carried out using PureLink™ Genomic DNA Purification MiniKit (Invitrogen, USA), according to the manufacturer's protocol. Two sets of oligonucleotide primers sin1/sin2 were used for identification of specific *S. iniae* 16srRNA gene (Table 1). The PCR amplifications were performed in reaction mixtures containing 25 µL of DreamTaq Green PCR Master Mix (2X), 100 ng template DNA, and sterile distilled water for a total volume of 50 µL in a gradient thermal cycler (T100; Bio-Rad, USA). Additionally, all strains of *S. iniae* were investigated for the presence of capsular polysaccharide *cpsD* gene (Table 1) by PCR (Baums *et al.* 2013).

Table 1: Oligonucleotides used in this study

Gene	Primer name	Sequences (5'-3')	Annealing	Product size (bp)	Reference
16 S rRNA	Sin 1	CTAGAGTACACATGTAGCTAAG	50°C	300	(Zlotkin <i>et al.</i> , 1998)
	Sin 2	GGATTTTCCACTCCATTAC			
<i>cpsD</i> gene	<i>cpsD-F</i>	TGGTGAAGGAAAGTCAACCAC	58°C	534	(Baums <i>et al.</i> , 2013)
	<i>cpsD-R</i>	TCTCCGTAGGAACCGTAAGC			
rep-PCR	BoxA	ACGTGGTTTGAAGAGATTTTTTCG	40°C	-	(Malathum <i>et al.</i> , 1998)

The PCR products were electrophoresed on 1% ethidium bromide stained agarose gel in TAE buffer for 1 h at 100 V. The PCR products were purified using

GeneJET gel extraction kit (Thermo Scientific, UK) and sent for direct sequencing by using Sanger DNA sequencer (Applied Biosystems, USA). The nucleotides and amino acid sequences were blasted using NCBI nucleotide collection (nr/nt) databases <http://blast.ncbi.nlm.nih.gov/Blast.cgi>.

Antimicrobial Susceptibility:

The antimicrobial susceptibility of *S. iniae* isolates was conducted by the disc diffusion test on Mueller-Hinton agar as described by the Clinical and Laboratory Standards Institutes guidelines (CLSI, 2006). The antibiotics (Oxoid, UK) tested were: ampicillin (25 µg), amoxicillin (25 µg), oxytetracycline (30 µg), streptomycin (10 µg), erythromycin (15 µg), kanamycin (30 µg), novobiocin (30 µg), florfenicol (30 µg), nalidixic acid (30 µg) and cefotaxime (30 µg).

Experimental infection:

To evaluate the pathogenicity and to determine the (LD₅₀) of *S. iniae* isolates obtained from *O. niloticus*. Six selected isolates of *S. iniae* (Si1, Si2, Si3, Si4, Si5, and Si6) were used for the experimental challenge test. Experimental infection was conducted in apparently healthy *O. niloticus* (weight 50 ± 10 g) through intraperitoneal injection. Fish were divided into 19 groups in aerated 60-L aquaria (5 fish per aquarium), and acclimated at 26 ± 2°C for 10 days. The fish were challenged with 0.1 mL of serial diluted bacterial suspensions at a concentration of x10⁸ – x10⁶cfu/fish. The control group injected with sterile 0.8% NaCl solution. Fish mortality was recorded for 7 days. Re-isolation on tryptic soya agar plates from kidney of dead fish was performed to confirm *S. iniae*-caused mortality.

Immunohistochemical localization of the bacteria in fish tissues:

Fish tissue specimens were daily obtained from different organs and were fixed in Davidson's fixative for 24 hours, then tissue specimens were dehydrated using ascending gradient of ethanol, cleared in chloroform and paraffin embedded, 4 µm sections were prepared using microtome and then mounted on positively charged glass slides. The slides were heated to 60°C for 1 hour to retrieve the bacterial antigen inside the tissue sections. After deparaffinization, immunohistochemical staining was performed according to Ramos-Vara (2005) using detection kits "Super Sensitive Polymer HRP QD400-60KE–BioGenex, USA) , with rabbit antiserum raised against *S. iniae* (1:100 dilution) as primary antibody. For negative controls, normal rabbit serum was used. The bound antibody was visualized using DAP as a chromogen, revealing brown precipitate as positive antigenic signal. Sections were counterstained with Gill's hematoxylin, and then blued with 0.1% ammonia water. The sections were visualized by a (CX41 Olympus) microscope provided with an (E-620 Olympus) camera.

Rep-PCR:

Primer BoxA 5'-ACGTGGTTTGAAGAGATTTTTTCG-3' (Table 1) was used for rep-PCR analysis. The rep-PCR conditions were 7 min at 95°C followed by 35 cycles of 30s at 95°C, 1 min at 40°C, 8 min at 72°C, and a final extension of 16 min at 72°C. Identical isolates showed identical PCR banding patterns. Isolates considered related if they different in one band. While, isolates considered in different clonal groups if they different in two or more bands (Malathum *et al.* 1998).

RESULTS

Bacterial identification

The results after incubation on (BHI) agar revealed that 14 bacterial *S. iniae* isolates were recovered from diseased *O. niloticus*. All colonies were white

transparent. The bacteria appeared non-motile, Gram-positive cocci arranged in pairs or chains; catalase and oxidase negative, and produced complete β -hemolysis on blood agar.

All isolates were confirmed by PCR- specific for 16S rRNA. The PCR produced a specific clear single *S. iniae* band of 300 bp (Fig. 1). The results were confirmed by the 16S rRNA gene sequences of three strains, which submitted to the GenBank database under the accession numbers MK757714, MK757715, and MK757716 respectively. The blast results of yielded sequences produced 100% homology with other *S. iniae* in the GenBank database.

Moreover, all the 14 *S. iniae* isolated were tested for the presence of capsular polysaccharide *cpsD* gene using PCR assay. PCR results showed amplification of 534 bp specific for *cpsD* genes in all isolates (Fig. 1).

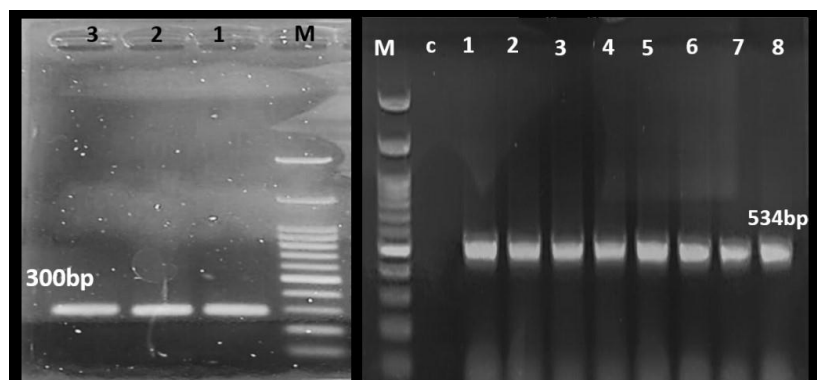


Fig. 1: Agarose gel electrophoresis showing amplification of 300bp for *S. iniae* 16S rRNA and 534bp for *cpsD* gene for capsular polysaccharide. M: 100 bp DNA molecular marker.

Anti-microbial Susceptibility

The results of *in-vitro* antibiotic sensitivity test on *S. iniae* isolates showed that all isolates tested were susceptible to oxytetracycline, novobiocin, florfenicol, nalidixic acid and resistant to ampicillin, amoxicillin, and cefotaxime. All isolates but three-showed susceptibility to erythromycin and Kanamycin.

Experimental infection

In the challenge experiments, LD₅₀ values of six selected isolates of *S. iniae* were 1.4×10^8 , 2.3×10^8 , 3.7×10^7 , 4.3×10^8 , 3.2×10^8 , 5.1×10^7 cfu/fish respectively. No mortality was observed in the control group. The challenged fish exhibited clinical signs of listless, lethargy, sluggish movement near the surface, skin depigmentation, uni-or bilateral exophthalmia, haemorrhages on eye and body surface, abnormal curvature of the dorsal spines. Necropsy revealed ascitis, pale liver, splenomegaly, dark and enlarged kidney. Results of re-isolated bacteria from kidney and spleen revealed the same injected *S. iniae* strains by PCR.

Immunohistochemical localization of the bacteria in fish tissues

The immunohistochemical investigations revealed the appearance of *S. iniae* antigenic signals in various fish organs such as hepatopancreas, spleen, posterior kidney, as soon as 24 hours post infection, while brain tissue revealed antigenic staining from the 4th day post infection (Fig.2).

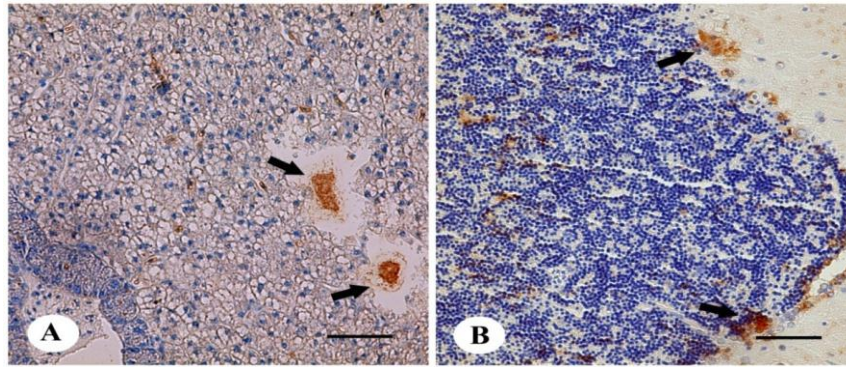


Fig. 2: Immunohistochemical picture of *O. niloticus* experimentally infected with *S. iniae*, 6 days post infection (A) hepatopancreas showing clusters of bacterial antigens (arrows) present inside necrotic tissue spaces (B) Brain showing presence of bacterial antigenic staining (arrows) within both grey and white matter, IHC, HRP/DAP chromagen, Hematoxylin counter stain, bar = 50 μ m.

Rep-PCR

The results of rep-PCR analysis on agarose gels revealed presence of six banding patterns (Fig. 3). PCR products generated from 4 to 8 bands. The generated PCR bands were mainly in the range from 0.5 kb to 3.0 kb. All genotypes but genotype 2 produced an intensive band of about 1,400 bp. Two clones, genotype 1 and 3, were identified among the 10 isolates. Four isolates of *S. iniae* in the clone 1 had the same DNA banding pattern, while clone 3 contained six *S. iniae* isolates with the same DNA banding pattern. Other four isolates were identified as distinct four clonal groups.

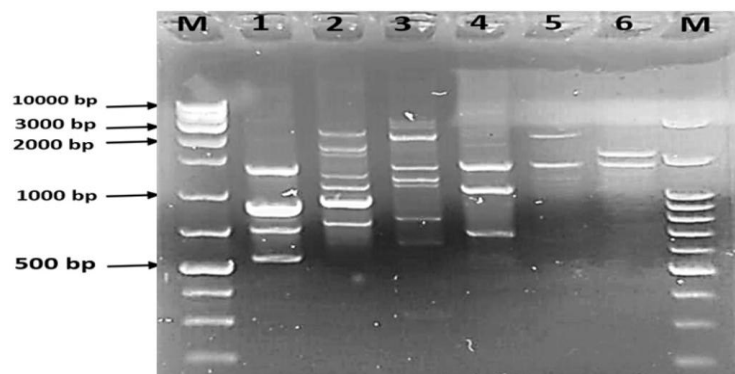


Fig. 3: Agarose gel showing six rep-PCR patterns of *S. iniae* isolates generated with the BoxA primer. M: 1kb and 100 bp molecular marker. Numbers to the right show the molecular size marker in bp.

DISCUSSION

Outbreaks of *S. iniae* recently considered as one of the most important bacterial infections causing significant economic impacts in cultured and wild populations of tilapia and catfish aquaculture farms. Acute infection of Streptococcosis can cause more than 50% mortality rates (Zamri-Saad *et al.*, 2010 and Saleh *et al.*, 2019). Streptococcus spp. are widely distributed in aquatic environments (Evans *et al.*, 2006). Like other opportunistic bacteria, the bacterial pathogenicity is associated with the sharp increase of physicochemical parameters of water (ammonia, salinity, temperature), low dissolved oxygen and stress factors (Younes *et al.*, 2016).

In this study, 14 strains were identified as *S. iniae*. Strains further confirmed by molecular analysis and sequencing of 16S rRNA gene. The results indicated that all of the tested isolates were *S. iniae* by their characteristic band at 300 bp and

confirmed by sequencing. These findings were in accordance with other reports with higher or lower percentage recorded in Egypt due to difference in the sampling time and the studied area. Consequently, this pathogen has become of great concern in Egypt due to the direct economic impacts on the local fish culture industry in several geographical areas, notably in *Nile tilapia* cultured in brackish water in Fayoum Governorate (Radwan 2002), and from cultured *Nile tilapia* in Kafr El-Sheikh Governorate (Saleh *et al.* 2019). Moreover, streptococcosis infections were reported in lake El-Ibrahimia in Upper Egypt (Ebtsam 2002).

Some virulence factors have been identified by *S. iniae* that contribute to the virulence such as phosphoglucosyltransferase enzyme, exopolysaccharide, M proteins, capsular polysaccharides, and cytolysin streptolysin S (Baiano and Barnes 2009). The PCR confirmed the presence of *cpsD* gene in all isolates tested in this study. The *cpsD* gene plays a crucial role in the formation of streptococcal species capsule that determine virulence and leads to fish kills (Locke *et al.*, 2007 and Eyngor *et al.*, 2010).

Effective antibiotic treatments are essential for controlling the bacterial infection. The results obtained from susceptibility assays showed that *S. iniae* isolates can be mainly controlled by antibiotics especially oxytetracycline and florfenicol. Oxytetracycline and florfenicol were recommended to the fisheries in USA, resulting in the powerful control of the bacterial fish outbreaks (Chou *et al.* 2014).

The pathogenicity test results and the calculated LD₅₀ of the recovered *S. iniae* isolates were ranged between 3.7×10^7 to 4.3×10^8 cfu/fish for isolates respectively. Many authors recorded different values of LD₅₀ for *S. iniae* isolates; this can be explained by the difference in virulence of isolates (Saleh *et al.* 2019). The clinical signs noticed in this study are consistent with septicaemic signs caused for *S. iniae* infection in tilapia, results in meningitis, exophthalmia, eyes turbidity, petechial haemorrhages and in some cases curvature of the spinal cord (Agnew and Barnes 2007, Zamri-Saad *et al.* 2010 and Baums *et al.* 2013).

Typing processes are important to understand bacterial relationship, pathogen outbreaks, routes of disease transmission, virulence, and vaccine development (Olive and Bean 1999). The BOX repetitive element is highly conserved repeated DNA element identified in the chromosome of *Streptococcus pneumoniae*. They dispersed in noncoding regions throughout the entire genome and involved in bacterial competence, virulence, colony opacity and genetic transformation (Van Belkum *et al.*, 1996).

In this study, we classified 14 *S. iniae* strains from diseased *O. niloticus* by genetic characteristics rep-PCR into six clonal patterns. Previous work has demonstrated that rep-PCR is fast, simple, less costly and frequently used for the genomic fingerprinting of Gram-positive pathogens (Malathum *et al.* 1998 and Romalde 2006). In a Korean study, *S. iniae* strains had been clustered into two genotypes in the flounder using BoxA primer and virulence (Kim *et al.*, 2014). While, other works demonstrated the limited utility of using the Box primer to discriminate between *S. iniae* isolates recovered from different host's species from either fish or mammals in North America (Dodson *et al.* 1999) or recovered from different geographic locations in the Americas and Caribbean islands (Chou *et al.* 2014) which generated one homogenous group using the Box primers. By contrast, *S. iniae* isolates formed three distinct clusters using the GTG5 primer (Chou *et al.* 2014).

Two clones; clone 3 (3.7×10^7 cfu/ml) and clone 6 (5.1×10^7 cfu/ml) have one fold lower in pathogenicity challenge than other. Therefore, to initiate the *S. iniae*

outbreak it needs high bacterial load in the farm water and sediment or fish subject for stress factors. Moreover, further studies with more isolates collected from other sites of country should be conducted to study the genotypic relationship and pathogenicity.

In conclusion, *S. iniae* pathogen has become of great concern in Egypt due to direct impacts and high mortalities among cultured *O. niloticus* in several geographical areas. Rep-PCR analysis of *S. iniae* strains recovered in this study gave genetic heterogeneity and classified into six different clusters. Therefore, *S. iniae* outbreaks caused with more than one genotypically distinct strain. Development of an effective vaccine for reducing the devastating outbreaks should confer broad protection against genetic heterogeneity of strains. This approach requires understanding the epizootiology and genetic variability of *S. iniae* in Egypt.

ACKNOWLEDGMENT

This work was supported by the fund of National Research Centre in Egypt.

REFERENCES

- Agnew, W. and Barnes, A. (2007). *Streptococcus iniae*: an aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination. *Vet Microbiol*, 122: 1-15.
- Baiano, J. and Barnes, A. (2009). Towards control of *Streptococcus iniae*. *Emerg infect diseases*, 15: 1891.
- Baums, C.; Hermeyer, K.; Leimbach, S.; Adamek, M.; Czerny, C.P.; Hörstgen-Schwark, G.; Valentin-Weigand, P.; Baumgärtner, W. and Steinhagen, D. (2013). Establishment of a model of *Streptococcus iniae* meningoencephalitis in Nile tilapia (*Oreochromis niloticus*). *J. compar pathol*, 149: 94-102.
- Chou, L.; Griffin, M.J.; Fraites, T.; Ware, C.; Ferguson, H.; Keirstead, N.; Brake, J.; Wiles, J.; Hawke, J.P.; Kearney, M.T.; Getchel, R.G.; Gaunt, P. and Soto, E. (2014). Phenotypic and genotypic heterogeneity among *Streptococcus iniae* isolates recovered from cultured and wild fish in North America, Central America and the Caribbean islands. *J Aquat Anim Health*, 26: 263-271.
- Dodson, S.; Maurer, J. and Shotts, E. (1999). Biochemical and molecular typing of *Streptococcus iniae* isolated from fish and human cases. *J. Fish. Dis.*, 22: 331-336.
- Ebtsam, S.H. (2002). Studies on streptococcosis in Nile tilapia, *Oreochromis niloticus*, in Assuit (Upper Egypt). Faculty of Veterinary Medicine, Assuit University, M. V. Sc. Thesis.
- Evans, J.; Klesius, P. and Shoemaker, C. (2006). An overview of *Streptococcus* in warmwater fish. *Aqua Health Internat*, 7: 10-14.
- Eyngor, M.; Lublin, A.; Shapira, R.; Hurvitz, A.; Zlotkin, A.; Tekoah, Y. and Eldar, A. (2010). A pivotal role for the *Streptococcus iniae* extracellular polysaccharide in triggering proinflammatory cytokines transcription and inducing death in rainbow trout. *FEMS Microbiol Lett*, 305: 109-120.
- Kim, M.S.; Jin, J. W.; Han, H.J.; Choi, H.S.; Hong, S. and Cho, J.Y. (2014). Genotype and virulence of *Streptococcus iniae* isolated from diseased olive flounder *Paralichthys olivaceus* in Korea. *Fish. Sci.*, 80: 1277-1284.
- Locke, J.B.; Colvin, K.M.; Datta, A.K.; Patel, S.K.; Naidu, N.N.; Neely, M.N.; Nizet, V. and Buchanan, J. T. (2007). *Streptococcus iniae* capsule impairs phagocytic clearance and contributes to virulence in fish. *J. Bacteriol*, 189: 1279-1287.
- Malathum, K.; Singh, K.V.; Weinstock, G.M. and Murray, B.E. (1998). Repetitive sequence-based PCR versus pulsed-field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. *J Clin Microbiol*, 36: 211-215.

- Olive, D.M. and Bean, P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol*, 37: 1661-1669.
- Pier, G.B. and Madin, S.H. (1976). *Streptococcus iniae* sp. nov., a beta-hemolytic streptococcus isolated from an Amazon freshwater dolphin, *Inia geoffrensis*. *Int J Syst Evol Microbiol* 26: 545-553.
- Radwan, I. A. (2002). Microbiological studies on characteristics of *Streptococcus iniae* isolated from diseased *Tilapia nilotica* and aquatic environments. *Vet Med J Giza*, 50: 273-279.
- Ramos-Vara, J. (2005). Technical aspects of immunohistochemistry. *Vet Pathol*, 42: 405-426.
- Romalde, J. L. (2006). Application of DNA fingerprinting techniques to the study of fish and shellfish pathogens (pp. 99– 127). In M. M. Read (Ed.), *Focus on DNA Fingerprinting Research*. New York, NY: Nova Science Publishers Inc.
- Saleh, H.; Ali, N.; Aboyadak, I. and Saber, N. (2019). Subcellular degenerative changes in hepatopancreas and posterior kidney of *Streptococcus iniae* infected Nile tilapia using Transmission Electron Microscope. *Egy J. Aquat. Biol. Fish.*, 23: 305-316.
- Van Belkum, A.; Sluijter, M.; De Groot, R.; Verbrugh, H. and Hermans, P. (1996). Novel BOX repeat PCR assay for high-resolution typing of *Streptococcus pneumoniae* strains. *J Clin Microbiol*, 34: 1176-1179.
- Younes, A.; Eldin, A.; and Ellatif, A. (2016). A contribution of crustacean isopoda, bacterial infections and physicochemical parameters in mass mortalities among fishes in Lake Qarun. *Res J Pharma Biol Chem Sci.*, 7: 1906-1911.
- Younes, A.; Mohamed, L.A.; Eida, M. and Gaafar, A. (2015). Characterization and pathogen challenge of *Pseudomonas* species from *Oreochromis niloticus* in Egypt. *Res J Pharma Biol Chem Sci.*, 6: 312-317.
- Zamri-Saad, M.; Amal, M. and Siti-Zahrah, A. (2010). Pathological changes in red tilapias (*Oreochromis* spp.) naturally infected by *Streptococcus agalactiae*. *J compar pathol*, 143: 227-229.
- Zamri-Saad, M.; Amal, M.; Siti-Zahrah, A. and Zulkafli, A. (2014). Control and Prevention of Streptococcosis in Cultured Tilapia in Malaysia: A Review. *Pert J Trop Agri Sci* 37.
- Zlotkin, A.; Hershko, H. and Eldar, A. (1998). Possible transmission of *Streptococcus iniae* from wild fish to cultured marine fish. *Appl Environ Microbiol*, 64: 4065-4067.