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Virulence-associated genes profiling of *Streptococcus iniae* isolated from diseased Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

The present work was conducted to determine the occurrence of streptococcal infection in a disease outbreak (summer mortality syndrome) affected Nile tilapia farms located at Kafrelsheikh governorate, Northern Egypt. Affected farms suffered from increased mortalities with appearance of different signs indicating bacterial infection. One hundred and forty clinically diseased fish samples were collected from seven farms. Initial bacterial isolation on modified Edwards's medium indicated the presence of 30 *Streptococcus* isolates, half of them was further identified by polymerase chain reaction as *Streptococcus iniae* (*S. iniae*). Molecular study revealed the presence of six virulence-associated genes in the recovered *S. iniae* isolates. The recovered genes are *scpl*, *simA*, *pdi*, *SagA*, *pgmA* and *cpsD*, these genes were detected in 7, 7, 5, 4, 3 and 9 isolates representing 46.6, 46.6, 33.3, 26.6, 20.0, and 60.0%. Virulence-associated genes may have a potential role in disease severity and promoting the pathogenicity of bacterial infection.

1. INTRODUCTION

Fish is one of the most important protein sources, particularly in developing and high-population countries (Aboyadak et al., 2015; Ali et al., 2021a). Expansion in aquaculture is a must to meet the nutritional requirements of a rapidly growing human population. In the last two decades, finfish aquaculture has increased by more than four folds from 12.5 to 54.1 million tons (FAO, 2018).

Egypt is the leading African country in aquaculture, particularly in tilapia production. Egyptian tilapia production reached 1.22 million tons in 2019 (GAFRD, 2020), and about 88% of this figure comes from aquaculture.

Summer mortality syndrome represents a big challenge for expansion in Egyptian tilapia production, in the last few years, these frequent outbreaks resulted in severe economic losses estimated at about one billion Egyptian pounds. The diseased fish show the general signs of septicemia (Ali et al., 2018).

Ali et al. (2021b) reported that thermal stress is the main predisposing factor for the summer mortality syndrome associated with disease outbreaks affecting cultured fish, stressed fish became immune-compromised and highly susceptible to bacterial diseases.

Gram-positive cocci infection represents a big challenge for global Nile tilapia aquaculture contributing to a huge financial loss. *Staphylococcus aureus*, *Streptococcus inae* and *Streptococcus agalactia* were responsible for many disease outbreaks in cultured tilapia (Aboyadak et al., 2016a; Ali et al., 2019; Heckman et al., 2022).

Streptococcus iniae is one of the most important bacterial fish pathogens. It has been isolated from diseased cultured tilapia in Egypt (Aboyadak et al., 2016b; Saleh *et al*., 2019; Younes et al., 2019).

Studying bacterial virulence factors is the key to understand bacterial pathogenicity (El-Bahar et al., 2019). Pathogenic bacteria can induce disease in susceptible hosts through the expression of their virulence factors (Søborg et al., 2016). These factors act individually or in combination. Pathogenic bacteria produce many chemical substances, enzymes, or other factors which are toxic to host cells either directly or indirectly (Finlay and Falkow 1997; Wu et al., 2008).

Virulence genes including C5a peptidase (*scpl*), M proteins (*simA*), polysaccharide deacetylase (*pdi*), streptolysin S protein (*SagA*), Phosphoglucomutase (*pgmA*) and Capsule (*cpsD*) are important for *S. iniae* pathogenicity (Moustafa et al., 2021). *Scpl* is responsible for the production of certain peptidase which hydrolyze neutrophil chemoattractant complement factor and impairs host

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resistance (Baiano and Barnes, 2009). *simA* is a surface protein that protects against phagocytosis and facilitate adherence to fish epithelial cells (Aviles et al., 2013). *Pdi* gene contributes to bacterial resistance to lysozyme killing as it enhances the adherence and invasion of epithelial cells (Milani et al., 2010). Molloy et al. (2011) reported that Streptolysin S encoded the in *sagA* gene is a cytolytic toxin-induced β-hemolysis. Buchanan et al. (2005) found that *pgmA* gene play a role in resistance of *S. iniae* to innate immune response. This gene is also responsible for normal bacterial cell wall morphology and production of surface capsule, so this gene has a potential role in its virulence. Moreover authors reported that *cpsD* gene encodes for certain facilitates binding to host tissues, such as epithelial cells.

Molecular methods such as PCR is not only enabling rapid and accurate identification of bacterial pathogens but also it is considered a valuable tool for detection of virulence genes which cannot be detected by any other conventional microbiological method (Kingombe et al. 2010; Søborg et al., 2013 & 2014; Aboyadak et al. 2015).

The aim of the present study was isolation and identification of *Streptococcus iniae* from cultured diseased *O. niloticus* and determining the prevalence of virulence genes in the recovered isolates for understanding the clinical picture of the disease.

2. MATERIAL AND METHODS

2.1. Study area and fish sampling:

Seven tilapia farms located at Trompat seven, Alriad district, Kafrelsheikh province were studied for determining the cause of increased mortality during the summer season of 2019. One-hundred and forty diseased Nile tilapia ranged between 100 – 250 g were collected (20 fish/farm). Each fish sample was preserved in a sterile bag in an ice box and immediately transported to laboratory for further analysis as described by Aboyadak et al., (2017).

2.2. Clinical signs and postmortem examination:

Diseased fish were inspected in the affected farms before sample collection to determine any external and behavior abnormalities as well as gross internal lesions then report for each fish was carried out during the microbiological examination as described by Ali et al. (2019).

2.3. Bacterial isolation and identification:

The initial bacterial isolation was performed as described by Aboyadak *et al*. (2016a) on brain heart infusion agar in which isolates were incubated at 35 °C for 24 h. The recovered isolates were subjected to gram staining, and Gram-positive cocci isolates were cultured on modified Edwards Medium enriched with 5% bovine blood and colistin sulphate 5 mg/L for selective isolation of streptococci. Grown colonies were preserved at -86 °C in glycerol for further molecular identification.

2.4. Molecular identification of the recovered isolates and virulence genes:

The molecular study was conducted for identification of *S. iniae* isolates and determining certain virulence genes by PCR. Genomic DNA was extracted using G-spin™ total DNA extraction kit, Intron, Korea. All the PCR reactions were performed in 25 µl reaction volume consisting of 12.5 µl of 2xMaster Mix (Intron, Korea), 3 µl of DNA extract, 1.25 µl of each forward and reverse primer after that 7 µl of nuclease-free water was added.

All the streptococcus isolates recovered from modified Edwards Medium were subjected to PCR amplification of 16S rRNA gene to determine *S. iniae* isolates as described by Zlotkin *et al.* (1998).

PCR identified *S. iniae* isolates were screened for the presence of *scpl*, *simA*, *pdi*, *SagA*, *pgmA* and *cpsD* virulence genes. Primers used in the molecular study are represented in table (1). PCR reactions were performed in Peltier Thermal Cycler model MG 960T using programs showed in table (2).

PCR reaction products were electrophoresed on 1% molecular grade agarose gel supplemented with 0.5 µg/ml of ethidium bromide, 5 µl of DNA marker was used for determining bands size. Electrophoresis was performed at 80 V for 1 h in tris EDTA buffer, after that DNA bands were visualized by gel documentation system.

Table 1 Primers used in the present study.

Target		Oligonucleotide Sequence (5' - 3')	Size (bp)	Reference
S. iniae 16S rRNA	F	CTAGAGTACACATGTACTAAG	300	Zlotkin *et al.,* (1998)
	R	GGATTTTCCACTCCCATTAC		
scpl	F	GCAACGGGTTGT CAAAAATC	822	Baums *et al.,* (2013)
	R	GAGCAAAAGGAGTTGCTTGG		
simA	F	AATTCGCTCAGCAGGTCTTG	٩٩٤	Aviles et al., 2013
	R	AACCATAACCGCGATAGCAC		
Pdi	F	TTTCGACGACAGCATGATTG	381	Baums *et al.,* (2013)
	R	GCTAGCAAGGCCTTCATTTG		
SagA	F	AGGAGGTAAGCGTTATGTTAC	190	Baums *et al.,* (2013)
	R	AAGAAGTGAATTACTTTGG		
pgmA	F	TATTAGCTGCTCACGGCATC	713	Buchanan et al., (2005)
	R	TTAGGGTCTGCTTTGGCTTG		
cpsD	F	TGGTGAAGGAAAGTCAACCAC	534	Baums *et al.,* (2013)
	R	TCTCCGTAGGAACCGTAAGC		

Table 2 PCR reaction conditions used in the molecular study.

Target gene	Initial Denaturation	Cycles	Denaturation	Annealing	Extension	Final Extension
16S rRNA	94 ◦C/4 m	30	94 °C/1 m	58 ◦C/1 m	72 °C/1 m	72 °C/10 m
scpl	94 °C/2 m	30	94 °C/1 m	58 ◦C/1 m	72 °C/2 m	72 °C/10 m
simA	95 °C/10 m	45	95 °C/15 s	57 °C/1 m	60 °C/15 s	72 °C/10 m
Pdi	94 °C/2 m	30	94 °C/1 m	58 ◦C/1 m	72 °C/2 m	72 °C/10 m
SagA	94 °C/2 m	30	94 °C/1 m	58 ◦C/1 m	72 °C/2 m	72 °C/10 m
pgmA	94 °C/2 m	30	94 °C/30 s	55 ◦C/30 s	72 °C/1.5m	72 °C/10 m
cpsD	94 °C/2 m	30	94 °C/1 m	58 ◦C/1 m	72 °C/2 m	72 °C/10 m

3. RESULTS

3.1. Clinical and postmortem examination:

Naturally infected fish showed hemorrhagic skin ulcers, scales desquamation, fin, and tail erosions. Internally liver, spleen, and posterior kidney were congested, enlarged with presence of hemorrhagic spots in severely affected fish. Elementary tract was also congested and partially empty as represented in figure (1).

3.2. Initial bacterial isolation:

Thirty Streptococcus isolates were recovered during the initial bacterial isolation on Edward's media, Streptococcus isolates grown as small grayish rounded colonies as showed in figure (2).

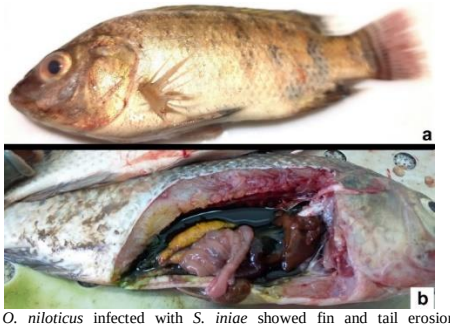

Figure 1 *O. niloticus* infected with *S. iniae* showed fin and tail erosions, scale desquamation (a), congested enlarged liver and empty intestine (b).

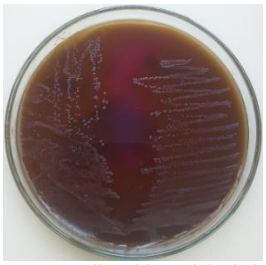

Figure 2 Streptococcus grown as small grayish rounded colonies on modified Edward's Medium enriched with 5% bovine blood supplemented with 5 mg/L colistin sulphate.

3.3. Molecular study:

Out of the recovered *Streptococcus* isolates, 15 *S. iniae* were identified through the presence of the characteristic bands at 300 bp based on amplification of 16SrRNA gene. PCR screening of six virulence genes among *S. iniae* isolates indicated the presence of *scpl*, *simA*, *pdi*, *SagA*, *pgmA* and *cpsD*, genes in 7, 7, 5, 4, 3 and 9 isolates representing 46.6, 46.6, 33.3, 26.6, 20 and 60% respectively as represented in table (3) and figures (3&4).

Table 3 Prevalence of virulence genes in the recovered *S. iniae* isolates

Gene	No.	%
16S rRNA	15	50
scpl	7	46.6
simA	7	46.6
Pdi	5	33.3
SagA	4	26.6
pgmA	3	20
cpsD	9	60

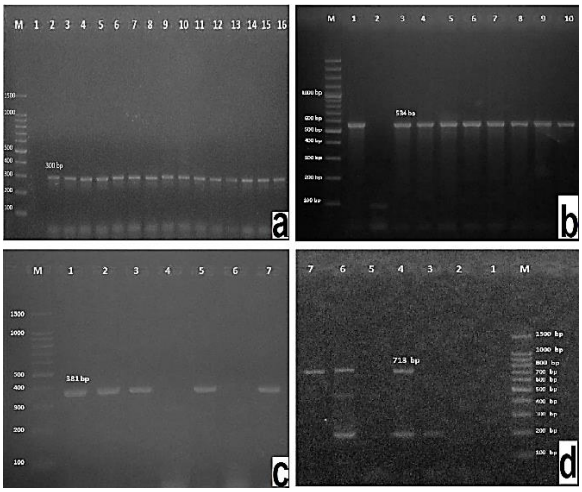

Figure 3 Gel electrophoresis of PCR amplicon Lane M: 100 bp DNA ladder. a)15 *S. iniae* isolates at 300 bp. b) 9 isolates positive for *cpsD* gene at 534 bp. c) 5 isolates positive for *Pdi* gene at 381 bp. d) 3 isolates positive for *pgmA* gene at 713 bp.

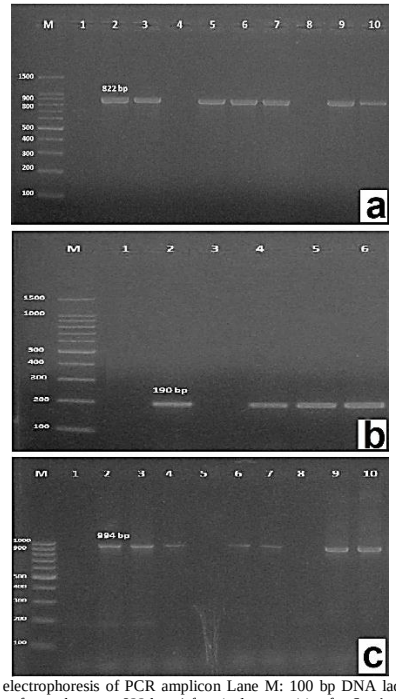

Figure 4 Gel electrophoresis of PCR amplicon Lane M: 100 bp DNA ladder. a) seven isolates positive for *scpl* gene at 822 bp. c) four isolates positive for *SagA* gene at 190 bp. c) seven isolates positive for *simA* gene at 994 bp.

4. DISCUSSION

In the present study, the most frequent clinical signs appeared on infected fish were hemorrhages at fin bases, inflammation, and redness of the abdominal wall and around the anal opening, fin and tail erosions. Few fishes showed corneal opacity and ascites. The behavioral abnormalities include swimming near water surface in a circular movement pattern. Our findings were in harmony with that reported formerly (Suanyuk et al., 2008; Figueiredo et al., 2012; Baums et al., 2013; Hossain et al., 2014; Saleh et al., 2019; Heckman et al., 2022). They recorded high mortality, exophthalmia, lethargy and diffused hemorrhagic spots on the external body surface of *Streptococcus iniae* infected fish.

Gross internal examination of diseased fish revealed the presence of enlarged congestion internal organs (hepatopancreas, posterior kidney, spleen, and intestine). These findings were similar to that shown previously (Abuseliana et al., 2011; Saleh et al., 2019; Moustafa et al., 2021; Heckman et al., 2022).

Streptococcosis is the most frequent Gram-positive infection affects cultured fishes. 15 *S. iniae* isolate were identified by amplification of 16S rRNA gene producing the characteristic bands at 300 bp. This result matched to the results described earlier (Zlotkin *et al.,* 1998; Dangwetngam *et al.*, 2016; Saleh *et al.*, 2019; Karen *et al.*, 2021).

The ability of pathogenic microorganisms to induce any disease condition in the susceptible host is associated mainly with the presence of virulence factors (Wu et al., 2008) and so, identification of virulence associated genes can give a good explanation about the potential pathogenicity of *S. iniae*. In the present study six different virulence genes have been identified from the recovered *S. iniae* isolates are *scpl*, *simA*, *pdi*, *SagA*, *pgmA* and *cpsD*. The forementioned genes were responsible for the pathogenicity of *S. iniae* through impairment of fish immune response, avoid phagocytosis and resisting the lysozyme killing mechanism. On the other hand, these

virulence factors help *S. iniae* to adhere and invade host cells and also to hemolyze RBCs and phagocytes (Buchanan et al., 2005; Baiano and Barnes, 2009; Milani et al., 2010; Molloy et al., 2011; Aviles et al., 2013).

Diversity of the recovered virulence genes in the present research illustrated the observed clinical findings and postmortem lesions, which are mostly induced by both invading bacteria and their circulated toxins during septicemia, with expression of virulence factors. During septicemia, presence of pathogenic bacteria and their extracellular toxic products are a potent inflammatory inducer giving rise to the clinical and necropsy findings (Saleh et al., 2019; Younes et al., 2019).

5. CONCLUSION

In this study fifteen *S. iniae* isolates were identified using 16SrRNA specific primer, *scpl*, *simA*, *pdi*, *SagA*, *pgmA* and *cpsD*, virulence genes were identified in 7, 7, 5, 4, 3 and 9 isolates representing 46.6, 46.6, 33.3, 26.6, 20 and 60% from the fifteen *S. iniae* isolates. Our findings indicated that the presence of virulence genes is contributing to bacterial pathogenicity.

CONFLICT OF INTEREST:

The authors declare that they have no conflicts of interest for current data.

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