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The effectiveness of protective measures against Streptococcosis and the immune responses triggered by the administration of live, live-attenuated, and killed vaccines were assessed in Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

The objective of this project was to develop live (LV), live attenuated (LAV), and autoclaved killed vaccines (AKV). The development of the vaccine involves utilizing two well-characterized strains of *Streptococcus iniae* (*S. iniae*), namely *S. iniae* 1 and *S. iniae* 2. *S. iniae* 2 was obtained from Department of Fish diseases, Faculty of Veterinary Medicine, Beni-Suef University, Egypt while *S. iniae* 1 strain was gifted from microbiology department, Egyptian Drug Authority, Dokki, Giza, Egypt. Pathogenicity test and lethal dose determination were performed. To conduct the experiment, apparently healthy Nile tilapia, *Oreochromis niloticus* (*O. niloticus*) of average weight 30±0.2g were divided into five experimental groups: T1 group, which served as a negative control and received saline; T2 group, which served as a positive control and received *S. iniae* 2; T3 group, which received an autoclaved killed vaccine for *S. iniae* 2; T4 group, which received a live attenuated vaccine for *S. iniae* 2; and T5 group, which received a live vaccine for *S. iniae* 1. At the end of the vaccination period, *S. iniae* 2 was introduced challenge to all groups. Serum samples were collected three weeks after vaccination to measure serum bactericidal activity, lysozyme activity, nitric oxide, alkaline phosphatase, and acid phosphatase. The findings demonstrated that the pathogenicity test reach 0 and 100% mortality rate for *S. iniae* 1 and *S. iniae* 2, respectively. Live attenuated vaccine had significantly higher protective rate than live vaccinations, while autoclaved vaccine had the best protective efficacy (88.2%). These results were confirmed through measurement some immune parameters as Serum bactericidal activity, lysozyme activity, nitric oxide, alkaline phosphatase and acid phosphatase.

Introduction

Aquaculture plays a crucial role in ensuring food security and reducing poverty by providing nutrition and livelihood opportunities to millions of individuals worldwide. In fact, approximately 50% of the world's seafood is now produced through aquaculture (FAO, 2020). Nile Tilapia, *Oreochromis niloticus* has been the most widely farmed species (Sharma *et al*., 2010). This species is also regarded as an appropriate fish for aquaculture development study due to its rapid generation period and highly developed immune system (Tan *et al*., 2017). However, inadequate health management practices and the absence of reliable diagnostic facilities pose challenges in the early detection of pathogens, leading to mass fish mortality (Aboyadak *et al*., 2016). Egypt has experienced outbreaks of mass fish mortalities caused by different pathogens affecting both cultured marine and freshwater fish. These mass mortalities are characterized by sudden and unexpected deaths within a short period among both wild and farmed fish populations (El-Mezayen *et al*., 2018). One of the main reasons for the mass mortalities of Nile tilapia and many other aquaculture species is streptococcosis. *Streptococcus* sp., is present all over the year in water column and mud and in all seasons with high occurrence in the warm seasons (Celik-Altunoglu *et al*, 2017). *Streptococcus iniae* and *Streptococcus agalactiae* are typically responsible for streptococcosis. Around the world, *Streptococcus iniae* is one of the major pathogen that infects *O. niloticus*. These bacteria are also zoonotic and handling and preparing contaminated food can lead to diseases in humans. Infections in humans were first noted in 1996, (Weinstein *et al*., 1997). The first discovery of *Streptococcus iniae* was by Pier and Madin (1976) that isolated from subcutaneous abscess in freshwater dolphin (I*nia geoffrensis*). The bacterium express β-hemolytic activity with blood agar containing 5% sheep blood; however it is difficult to be categorized using the Lancefield antigen method, which is generally used to classify *Streptococcus* species. The infected fish showed typical clinical signs of streptococcosis which include darkened skin, exophthalmia, corneal opacity, distention in abdominal cavity, and erratic swimming (Agnew and Barnes, 2007). Internally, affected fish may exhibit hemorrhages in all internal organs including brain and ascitic fluid in the abdominal cavity (Eldar *et al*., 1995). The virulence factors of *S. iniae* that contribute in pathogenesis are phosphoglucomutase enzyme, exopolysaccharide, M proteins, capsular polysaccharides, and cytolysin streptolysin S (Zhang, 2021). Streptococcal control in fish farms is achieved by combining management techniques with oral antibiotic administration (Abu-Elala *et al*., 2019). The development of an efficient, environmentally safe, and affordable vaccine against *Streptococcus* species is the key focus of disease control research at present. The reason why we used vaccine is emergence of antimicrobial resistance and jeopardizes public health (Zhang *et al*., 2020). To provide an extensive defense against *S. iniae*, it is necessary to develop a vaccine that efficiently manages outbreaks (El-daim *et al*., 2023a). Live-attenuated vaccines (LAV) may provide long-lasting protection by inducing strong cellular and humeral immunity (Taylor *et al*., 2022). In Egypt; there are no commercially available vaccinations for *S. iniae*, and the only inactivated whole-cell preparations with a minimal level of protection against heterogeneous strains. The main aim of this project was to develop live (LV), live attenuated (LAV), and autoclaved killed vaccines (AKV) against streptococcosis and measuring immune responses following the vaccinations in Nile tilapia, *Oreochromis niloticus*.

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Materials and methods

Ethical approval

The Committee of Animals Welfare and Research Ethics of Benha University's Faculty of Veterinary Medicine, Egypt, number BUFVTM 09-06-23, provided recommendations for doing this research.

Bacterial strains

Well identified *Streptococcus iniae* 2 (*S. iniae* 2) strain was gifted from Department of fish Diseases, Faculty of Veterinary Medicine, Beni-suef University, Egypt. It was isolated from clinically diseased marine water fish (gilthead sea bream, *Sparus auratus*) that suffered from mass mortality in lower Egypt with an average weight 350±0.5g with accession number #:MT086601 while *Streptococcus iniae* 1 (*S. iniae* 1) strain was gifted from microbiology department, Egyptian Drug Authority, Dokki, Giza, Egypt.

Assessment of S. iniae isolates virulence

The preserved bacterial isolates at -80ºC were revived in Tryptic Soya Broth (TSB: Merck, Germany) at 30±1ºC for 24 h followed by streaking on TSA and incubated at 30±1ºC for 24 h. One colony from the isolates was aseptically picked, transferred to 10 ml of TSB separately, and incubated at 30±1°C for 24 h and then was suspended in PBS, and their count was adjusted to 1.5, 3, 6, 9×10^8 cells/ml, using Spectrophotometer with absorbance of 0.1, 0.257, 0.451 and 0.582 respectively, at wavelength 620 nm following Abu-Elala *et al*. (2019).

Experimental design

The intensity of *S. iniae* isolates virulence was evaluated as following; a total of 180 fish (30±0.2 g body weight) were divided into 9 groups (20 fish/ group). Each group from the first 8 groups were injected i.p. (0.2 ml) with different bacterial cell concentration (1.5, 3, 6, 9 $\times10^8$ cells/fish) for the two *S. iniae* isolates. Group No. 9 was kept as control (n=20 fish) which was inoculated with sterile saline. Following the challenge, food was provided twice a day for the duration of the trial at a rate of 4% of body weight. For seven days following the injection, daily records of the clinical signs and lethal rates among the infected and control groups were documented.

Histopathological examination

Fish groups injected with *S. iniae* 1 and 2 at a concentration of 9×10^8 cells/ml had autopsy samples obtained from their liver, spleen, gills, and muscles. These samples were then preserved in 10% neutral buffer formalin for 48 hours. After washing with tap water, the subjects were dehydrated using methyl, ethyl, and 100% ethyl alcohol dilutions in sequence. The specimens were cleaned in xylene and then immersed in paraffin for 24 hours at 56 degrees in a hot air oven. Using a sliding microtome, tissue blocks made of paraffin wax from bees were created for sectioning at a thickness of 4 microns. Following deparaffinization and staining with hematoxylin and eosin, the resulting tissue sections were placed on glass slides for routine examination using a light electric microscope (Banchroft *et al*., 1996).

Lethal dose determination

O. niloticus (n=180) that appeared healthy and weighed an average of 30±0.2 g were acclimated for two weeks with a temperature of 25±1°C. Three groups of 20 fish each were randomly assigned to one of the three distributions. *S. iniae* isolates that had been inoculated were grown overnight on BHI medium at 30°C. Bacterial cells were obtained by centrifugation in phosphate buffer saline (PBS) (pH 7.4) at 4°C at 4000×g for 15 min and then calibrated with a spectrophotometer at an OD600 of 0.3± 0.005. Under these conditions, there were roughly 5×10^8 CFU/ml of bacteria present. In order to assess the infectivity of the isolated bacterial strain, fish were inoculated with 0.1 ml of bacterial suspension from 5×10^4 to 5×10^7 CFU/fish. Fivefold serial dilution steps were used to prepare these bacterial solutions. With the exception of the control group, which received sterile saline injections (60 fish), two groups totaling 120 fish were injected with each *S. iniae* isolate. The dead fish were documented every 12 hours during the seven-day observation period for all groups. Following, Reed and Muench (1938), the LD_{50} dosage was calculated. To confirm the pathogenicity of the bacterial isolates, a challenge test was conducted at a concentration of 1×10^7 CFU/fish. Over the course of seven days, *O. niloticus* were observed every 12 hours, and the mortality rate (MR) was computed.

Vaccine preparation

As stated by Li *et al*. (2011), killed, live attenuated, and live vaccines from *S. iniae* were developed. To put it briefly, the *S. iniae* 1 and *S. iniae* 2 strains were grown until an OD600 of 0.3 was reached, at which point the bacteria were collected to develop various kinds of vaccines. By autoclaving the bacterial culture in the BHI broth at 121°C for 15 minutes, the autoclaved killed vaccination (AKV) from the *S. iniae* 2 strains was produced. After treatment, the culture was centrifuged for 30 minutes at 7000 ×g. PBS was used to wash the cell pellets twice before they were resuspended in it following (Bactol *et al*., 2018). The *S. iniae* 2 strain live attenuated vaccine (LAV) was made by repeatedly freezing and thawing the bacterial culture in the BHI broth at a temperature of -20°C. After treatment, the culture was centrifuged for 30 minutes at 7000 ×g. PBS was used to wash the cell pellets twice before they were resuspended in it following (Bactol *et al*., 2018). The strain *S. iniae* 1 that was selected to make the live vaccination was diluted using sterile saline solution until the bacterial concentration was adjusted to 1×10^8 CFU/ml, with an OD600 of 0.3. Sterile conditions were maintained throughout the whole procedure. Until they were used, vaccines were stored at 4°C. Safety and sterility studies were performed on these vaccinations. For three weeks, fish were fed at a rate of 4% body weight twice a day, at 9:00 and 16:00. To account for the increased fish biomass, biweekly feed modifications were implemented. Treated with well-aerated, dechlorinated water, around half of the original water was changed three times a week. Groups T3, T4, and T5 each got 0.1 ml of the previously stated produced vaccinations. In contrast to the T1 control group, which received sterile saline as a negative control, the T2 control group received 0.1 ml of *S. iniae* 2 as a control positive. Three additional weeks were added to the vaccine schedule. At 8:00 and 15:00 h, the water temperature and dissolved oxygen (DO) levels were measured twice daily. Fish were kept in water that was kept at a constant temperature of (26±2°C), pH of (7.3±0.24), and dissolved oxygen level of (5.1±0.2mg/L) throughout the experiment following (Aly, 1981; Anderson *et al*., 1970).

Experimental setup

T1 group: Control sterile saline, control negative
T2 group: Control *S. iniae* 2 control positive
T3 group: Autoclave killed vaccine *S. iniae* 2
T4 group: Live attenuated vaccine *S. iniae* 2
T5 group: Live vaccine *S. iniae* 1

Serum collection

The blood was drawn from each group of fish (3 fish/replicate) 21 days post vaccination. Fish was anesthetized using MS222 at a dose of 30 mg/l then three samples from each group of fish were obtained from

caudal blood vessels using a 1mL syringe with a 25 G needle. Serum samples were collected, placed in Eppendorf tubes, and allowed to clot overnight. Centrifuging and clot bordering were completed. The obtained serum was stored until use at -20°C.

Challenge test

Three weeks post vaccination, all groups of fish (10 fish/duplicates) were challenged with a 0.1-ml intra-peritoneal injection (I/P) of strain *S. iniae* 2, which contained 1.0×10^8 CFU/ml, (Zhang *et al*., 2019; El-daim *et al*., 2023b). Seven days after the challenge, necropsy findings, cumulative mortality, and relative percentage survival (RPS) were recorded. RPS was determined using the formula mentioned by Amend (1981):

RPS=[1−(mortality in vaccinated group/mortality in control group)]×100%.

Bacterial re-isolation from kidneys of the moribund challenged fish was carried out to achieve Koch postulate.

Determination of immune parameters

Serum bactericidal activity

The *S. iniae* strain (2.7 x 10^6 CFU), which was used for the serum bactericidal activity investigation, was graciously donated by the Central Laboratory for Aquaculture Research of the Department of Fish Health and Management. In order to evaluate the bactericidal activity, then Tris buffer with pH 7.5 was used to diluted serum samples three, four, and five times according to Iida *et al*. (1989). The dilutions were mixed with a bacterial suspension (0.001 g ml^{-1}, *S. iniae*) and then incubated on TSA for 24 hours at 25°C in a volume of 50 microliters. The plate counting method was used to tally colony forming units (CFU). Survival index (SI) was used to represent the findings (Ward law and Unless, 1978). Determined by the Process SI= x (CFU at start / CFU at end). Value, calculated as follows: SI= (CFU at end / CFU at start) x 100.

The serum lysozyme activity was assayed by ELISA micro-well technique using ELISA kit (Sunlong Biotech co. China.) at wavelength of 450 nm following the manufacturer's instructions.

Acid phosphatase (Biodiagnostic co. Egypt.) was determined according to Kind and King, (1954) at wavelength 510 nm. Alkaline phosphatase, (Biodiagnostic co. Egypt.) was determined in accordance with Belfield and Goldberg (1971) at the wavelength 510 nm. Nitric oxide assay, (Biodiagnostic co. Egypt.) was determined in accordance with Montgomery and Dymock (1961) at wavelength 540 nm.

Statistical analysis

The statistical significance between the control and vaccine groups was investigated using Tukey's tests and one-way analysis of variance (ANOVA), both of which were performed using the statistical analysis program SPSS 26.0. The mean ± SEM was used to present the data. A p-value less than 0.05 were deemed noteworthy.

Results

Virulence of S. iniae isolates

Starting from the 1st day until the 3rd day, the mortality rates in infected fish showed high rate then decreased gradually or stopped till the 7th day post injection with *S. iniae* 2 especially at high dose. In some groups, fish died without any obvious signs, but most fish were presented at bottom in the glass aquaria, eye turbidity and bilateral exophalmia, redness in skin especially at base of fins, and detached scales, with abdominal distention and darkness in skin (Fig. 1A, B). Postmortem examinations revealed sever congestion in all internal organs, musculature and gills, with presence of ascetic fluid in abdomen (Fig. 1C). As presented in (Table 1), the highest percentage morality was observed in *S. iniae* 2 strain (100%) with dose 9×10^8 cells/ml. No mortalities were recorded in *S. iniae* 1. Kidney streaking from all dead fish gave rise to the growth of *S. iniae* isolates. Briefly, out of the 2 tested *S. iniae* isolates; *S. iniae* 2 was found to be virulent strain while, *S. iniae* 1 recorded no mortalities (a virulent strains).

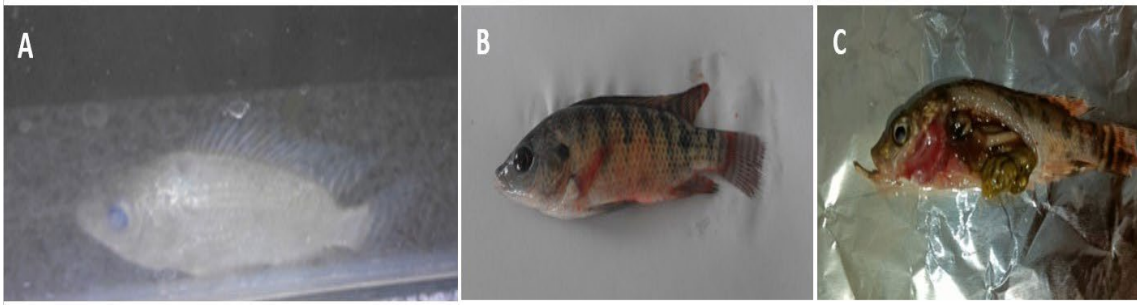

Fig. 1. Experimentally infected *O. niloticus* with *S. iniae* 2; presented at bottom in the glass aquaria, eye turbidity (A) and bilateral exophthalmia, redness in skin especially at base of fins (B). Postmortem examinations revealed sever congestion of all internal organs, musculature and gills and presence of ascetic fluid in abdomen (C).

Table 1. Mortality rates and Relative percent survival (RPS) estimations from intra-peritoneally injected *O. niloticus* with 0.2 ml *S. iniae* at 1.5, 3, 6, 9×10^8 cells/ml.

Group no.	No. of fish/ group	*S. iniae* strain	Doses Cells/ml	Number of dead fish							Total mortality	Mortality %	RPS
				1st day	2nd day	3rd day	4th day	5th day	6th day	7th day			
1	20	*S. iniae* 1	1.5×10^8	0	0	0	0	0	0	0	0	0	100
2	20	*S. iniae* 1	3.0×10^8	0	0	0	0	0	0	0	0	0	100
3	20	*S. iniae* 1	6.0×10^8	0	0	0	0	0	0	0	0	0	100
4	20	*S. iniae* 1	9.0×10^8	0	0	0	0	0	0	0	0	0	100
5	20	*S. iniae* 2	1.5×10^8	5	1	1	0	0	0	0	7	35	65
6	20	*S. iniae* 2	3.0×10^8	7	2	2	0	0	0	0	11	55	45
7	20	*S. iniae* 2	6.0×10^8	7	3	4	1	0	0	0	15	75	25
8	20	*S. iniae* 2	9.0×10^8	9	5	4	1	1	0	0	20	100	0
9	20	Sterile saline	-	-	-	-	-	-	-	-	-	0	100

Histopathological findings for experimentally infected O. niloticus

As presented in Fig. 2; highly pathogenic strain (*S. iniae* 2) exhibited pathological lesions in gills as entire necrosis of secondary lamellae (arrow), interstitial hemorrhages were observed (asterisk) (a). Branchitis associated with leukocytic cellular infiltrations (b). Liver showed marked congestion of hepatic blood sinusoids (c), diffuse hemorrhage in hepatic parenchyma (d), clear vacuolation of the cytoplasm of hepatocytes (e), multiple necrotic nodules in the hepatic parenchyma (f), and disappearance of hepatocytes and replaced by eosinophilic substances (g), extensive degeneration in the lymphocytic population with lymphoid depletion with accumulation of hemosiderin (h). Spleen showed extensive hemorrhage in the splenic tissue (i), destruction of splenic tissues, extensive degeneration in the lymphocytic population with lymphoid depletion and accumulation of hemosiderin (j, k). Musculature showed sever intermuscular hemorrhage with sever degeneration in musculature (l). While a virulant strain (*S. iniae* 1) as presented in Fig. 3; exhibited mild pathological lesions in liver as hemolysis (Hs), destruction in pancreatic structure (D) and congestion in blood sinusoids (a). Gills showed some hemorrhage and degeneration in primary lamella, hemorrhage and hyperplasia in secondary lamellae and separation in epithelium of secondary lamella (b). Musculature showed mild degeneration in musculature and edema in between muscle fibrils (c). Spleen showed activation in the red pulp (R), activation in white pulp (W) (d), mild degeneration in splenic tissue, hemorrhage in blood vessels (Hr) and hemosidrinosis (Hn) (e).

Challenge trial

Clinical signs of streptococcal infection were exhibited in all challenged groups with less pronounced in the vaccinated groups. These include corneal opacity, darkness of skin, lethargy in swimming, exophthalmia, loss of appetite. Moreover, re-isolation of bacteria from kidneys of experimentally infected fish revealed Gram positive streptococci. Necropsy finding of challenged Nile tilapia revealed congested spleen, liver and kidney with presence of abdominal fluids in some fish. The RPS% of AKV group (*S. iniae*2) was significantly higher (88.2 %) than the RPS% of the LAV group (*S. iniae*2) (82.4%) and higher than the RPS% of LV group (*S. iniae*1, 76.5%). The RPS% was 100% in the non-vaccinated control group (Table 2).

Immunological parameters

After three weeks post-vaccination, Serum bactericidal activity against the pathogenic *S. iniae* 2 strain was the highest for AKV (2.11±0.075) followed by LAV and LV; 1.5±0.0176 and 0.99±0.079 ; respectively they were still rather higher than the control group (Table 3). Also, lysozyme activity level was high for AKV (3.48±0.17) followed by LAV and LV, 2.104±0.04 and 1.58±0.032; respectively. Alkaline phosphatase recorded significant increase in AKV group (5.99±0.089) compared to LAV and LV, 3.98±0.17 and 2.94±0.11; respectively. Acid phosphatase concentration was also significantly increased in AKV group (0.459±0.012). A similar trend was also recorded for Nitric oxide concentration in AKV group in comparison with LAV and LV groups (Table 3).

Table 2. Relative Percentage of Survival (RPS) of *O. niloticus* challenged by *Streptococcus iniae* 2 after 7th days post vaccination.

Item	Control −ve	Control +ve	AKV	LAV	LV
Fish No.	20	20	20	20	20
1st day	0	8	0	1	1
2nd day	0	4	0	1	0
3rd day	0	5	1	0	1
4th day	0	0	0	0	0
5th day	0	2	0	0	1
6th day	0	1	1	1	2
7th day	0	0	0	0	0
Mortality %	0	100	10	15	20
RPS	100	0	88.2	82.4	76.5

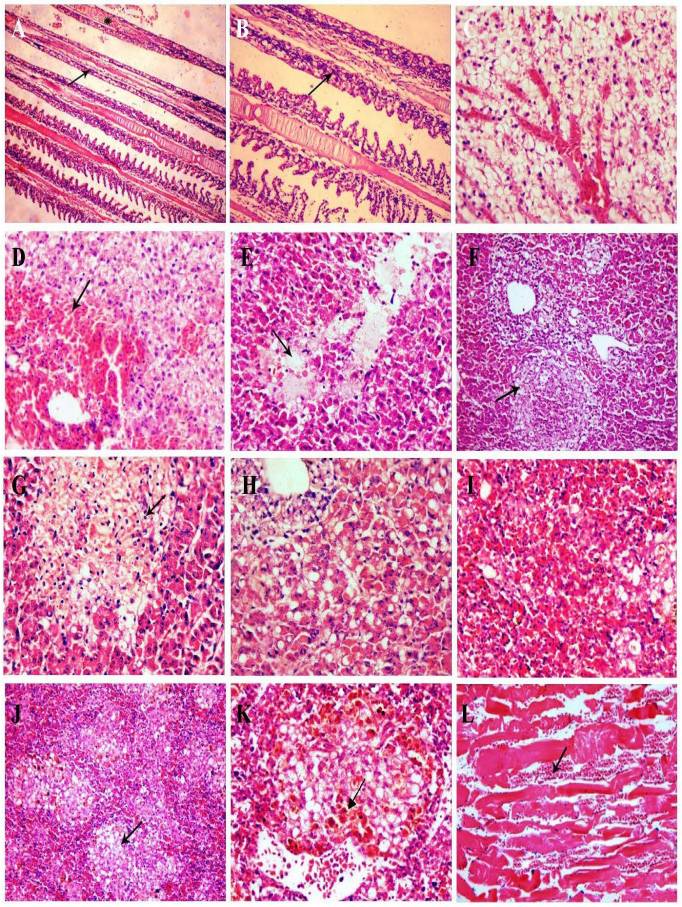

Fig. 2; highly pathogenic strain (*S. iniae* 2) exhibited pathological lesions in gills as entire necrosis of secondary lamellae (arrow), interstitial hemorrhages were observed (asterisk) (a). Branchitis associated with leukocytic cellular infiltrations (b). Liver showed marked congestion of hepatic blood sinusoids (c), diffuse hemorrhage in hepatic parenchyma (d), clear vacuolation of the cytoplasm of hepatocytes (e), multiple necrotic nodules in the hepatic parenchyma (f), and disappearance of hepatocytes and replaced by eosinophilic substances (g), extensive degeneration in the lymphocytic population with lymphoid depletion with accumulation of hemosiderin (h). Spleen showed extensive hemorrhage in the splenic tissue (i), destruction of splenic tissues, extensive degeneration in the lymphocytic population with lymphoid depletion and accumulation of hemosiderin (j, k). Musculature showed sever intermuscular hemorrhage with sever degeneration in musculature (l).

Table 3. Assesement of some immunological parameters for the tested groups after three weeks post vaccination.

Groups	Lysozyme activity (ng/mL)	Serum bactericidal activity (ng/mL)	Acid phosphatase (mU/ml)	Alkaline phosphatase (mU/ml)	NO (mM/ml)
C -	0.9^{d}±0.154	0.57^{d}±0.063	0.124^{e}±0.011	2.069^{d}±0.12	0.58^{d}±0.09
C +	0.9^{d}±0.032	0.55^{d}±0.107	0.184^{d}±0.017	2.19^{d}±0.026	0.71^{d}±0.004
Autoclaved killed vaccine (AKV)	3.48^{a}±0.17	2.11^{a}±0.075	0.459^{a}±0.012	5.99^{a}±0.089	2.45^{a}±0.155
Live attenuated vaccine (LAV)	2.104^{b}±0.04	1.55^{b}±0.0176	0.371^{b}±0.026	3.98^{b}±0.17	1.59^{b}±0.088
Live vaccine (LV)	1.58^{c}±0.032	0.99^{c}±0.079	0.3^{c}±0.089	2.94^{c}±0.11	1.28^{c}±0.024

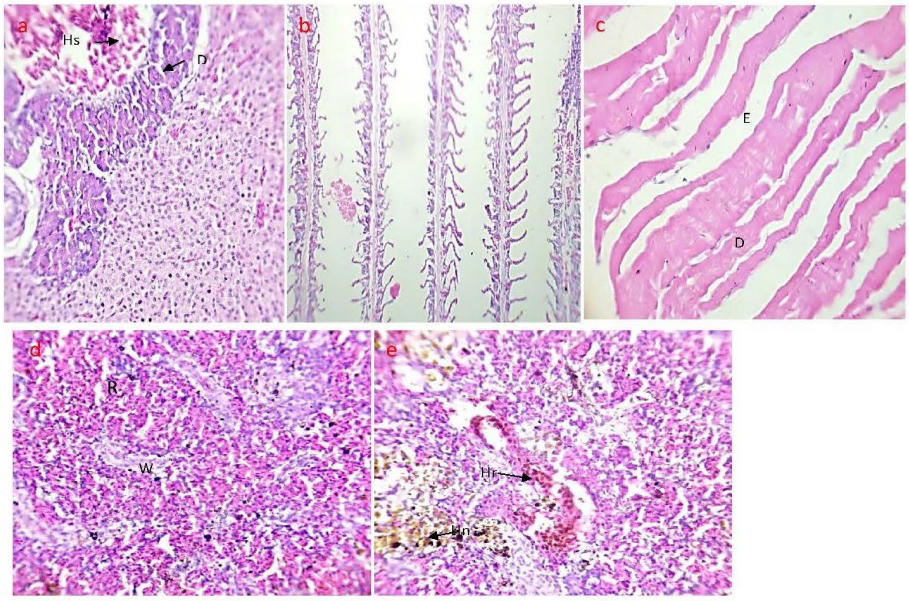

Fig. 3. Exhibited mild pathological lesions in liver as hemolysis (Hs), destruction in pancreatic structure (D) and congestion in blood sinusoids (a). Gills showed some hemorrhage and degeneration in primary lamella, hemorrhage and hyperplasia in secondary lamellae and separation in epithelium of secondary lamella (b). Musculature showed mild degeneration in musculature (D) and edema in between muscle fibrils (E) (c).Spleen showed activation in the red pulp (R), activation in white pulp (W) (d), mild degeneration in splenic tissue, hemorrhage in blood vessels (Hr) and hemosidrinosis (Hn) (e).

Discussion

One of the most significant bacterial fish diseases is *Streptococcus iniae*. It was isolated in Egypt from diseased cultured tilapia (Aboyadak *et al*., 2016). *Streptococcus iniae* is an opportunistic pathogen that is present in both freshwater and saltwater fishes. It is adaptable to wide range of hosts and can cause abnormal behavior, skeletal abnormalities and high mortality rate in fish (Younes *et al*., 2019). It is crucial to develop techniques for reducing the occurrence of the disease to protect both animals and humans against infections. The prolonged usage of different chemicals and antibiotics in aquaculture farms to treat bacterial diseases had led to buildup drug residues, the emergence of antibacterial resistance, environmental contamination, and harmful effects on the health of both fish and people (Heydari *et al*., 2020). The severity of disease is associated with the virulence factors of the pathogenic strain and condition of the susceptible host (Zhang, 2021), so the virulence assessment of bacteria can help to explain the potential pathogenicity of *S. iniae*. The pathogenicity of *S. iniae* was caused by complex interaction process between impairing fish immune response, avoiding phagocytosis by macrophage, and resistance of lysozyme killing mechanism (Buchanan *et al*., 2005).

Several studies were reported related to artificial challenge with various doses of *Streptococcus* in *O. niloticus*. The present study revealed that some fish died without any obvious signs but most fish were presented at bottom in the glass aquaria, eye turbidity and bilateral exophthalmia, redness in skin especially at base of fins, excess mucus secretion in the skin and detached scales, with abdominal distention and darkness in skin. Postmortem examinations revealed sever congestion in the all internal organs, musculature and gills with presence of ascetic fluid in abdomen; nearly similar manifestations were observed by Hussein (2003) and Austin and Austin (2007). These observations may be attributed to the toxic products given by *Streptococcus* sp.

Mortality rates started to occur on the first day after the challenge, and the highest MR was achieved by injecting each patient with 1.8×10^{8} cells/ml. *S. iniae* 2 was the most pathogenic isolate, with a 100% mortality rate. Different *Streptococcus* species and strains with varying mortalities produce a variety of virulence factors. These factors can be found in two forms: extracellular products such as cytotoxic, hemolytic, and enterotoxic proteins, and cell-associated structures such as flagella, pili, lipopolysaccharide, and outer membrane proteins (Handfield *et al*., 1996). Because of their virulence, the bacteria are able to infiltrate, enter, multiply, and harm the tissues of their hosts. They can also evade the host's defensive mechanism and spread, ultimately leading to the host's death (Abdullah *et al*., 2003; Pang *et al*., 2015).

Changes in reaction to different infected tissues with streptococcosis were varied according to degree of *Streptococcus* sp. virulence and bacterial cell concentrations (Samcookiyaei *et al*., 2012). The internal organs in case of experimentally infected *O. niloticus* showed some pathological change than gills because gills were noticed to be the first organ for the infection. This finding indicated that the bacteria invade primarily the tissue of gills instead of the internal organs (Miazaki and Jo, 1985). Circulatory disturbances and cellular damage were found to develop in the liver, spleen, kidneys and heart although the bacterial migration was very slight in those organs. This fact indicated that the bacterial multiplication was confined in the affected gills and the above pathological changes of visceral organs were evoked by affection of toxic substances of the bacterium instead of bacterium itself. Liver and spleen lesions showed necrotic changes with destruction in structural integrity and hemorrhage associated with hemosiderosis. Affinity of *Streptococcus* sp. to the liver was previously reported by Miyazaki *et al.* (2001) who reported that liver and kidneys are target organs of an acute septicemia. These organs are attacked by bacterial toxins where the extracellular products induced more severe tissue changes (Rey and Verján, 2009). Hemosiderosis is one of the most characteristic features demonstrated in this study. Miyazaki and Kaige (1985) attributed the lesions to the β-haemolysin secreted by the bacterium that cause haemolysis of RBCs inside the fish body followed by deposition of haemosiderin. Pathological changes in intestine were greatly similar to those reported by Roberts (1978) and these lesions could be attributed to the different bacterial toxins expressed by Aeromonads. These enzymes had a toxic effect, and the bacteria's toxic media significantly contributed to the tissues' exudation, bleeding, degeneration, and necrosis (Nahar *et al*., 2016). As a defense strategy against the dangers of the toxins, alarm substance cells and mucous cells hyperproliferate as a result of the toxic effect on the epithelial cells, (Roberts, 2001). Moreover, it damages the endothelium lining of subcutaneous blood arteries, which allows leukocytes and red blood cells to escape into the surrounding tissues. It also allows plasma proteins to escape, which results in edema, congestion, and bleeding at the infection site.

The RPS% of AKV group (*S. iniae* 2) was significantly higher (88.2 %) than the RPS% of the LAV group (*S. iniae*2) (82.4%) and higher than the RPS% of LV group (*S. iniae*1, 76.5%). The RPS% was 100% in the non-vaccinated control group. *Streptococcus iniae* attenuated vaccines seem to be quite immunogenic. Additionally, when administered through bath, these vaccines were effective and producing 86% RPS in the homologous strain (Pridgeon and klesius, 2011). From 1995 to 1997, rainbow trout farms effectively employed vaccination, which led to a reduction in *S. iniae* mortalities in fish farm from 50% to less than 5%, (Bachrach *et al*., 2001).

Our findings showed a significant increase in lysozyme activity, serum bactericidal activity, alkaline phosphatase, acid phosphatase and nitric oxide in the AKV vaccine group compared to other vaccinated groups and control groups. These findings are consistent with those reported by Pridgeon and Klesius (2011), who revealed a significant increase in serological analysis in vaccinated groups than controls, when all factors are taken into account. Similarly, Bactol *et al.* (2018) showed that autoclave-killed whole cell Aeromonas hydrophila vaccine produce significant immune response in Nile tilapia compared with formalin killed vaccine. Moreover, according to El-daim *et al.* (2023b), Nile tilapia immunized against *Streptococcus iniae* showed noticeably higher immune response after 14 days receiving autoclaved vaccine. According to Ramos-Espinoza *et al.* (2020), Nile tilapia was effectively protected from streptococcosis

caused by a similar strain thanks to the *S. iniae* vaccine provided by the AKV, as shown by the absence of any behavioral abnormalities or obvious clinical signs.

Conclusion

This investigation described the relationship between the pathogenicity of *S. iniae*, and immunogenicity of *S. iniae* isolated from the diseased fish in high mortality fish farms. To find novel antigens for developing new vaccines, various genotypes were used. The best vaccination for protecting against *O. niloticus* was autoclaved killed one.

Conflict of interest

The authors declare that they have no conflict of interest.

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