Pathogenicity of Streptococcus pyogenes and its correlation with hematological alterations in infected Nile tilapia (Oreochromis niloticus) fingerlings

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Research Article

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Abstract

This study was realized to investigate the lethal dose fifty (LD$_{50}$) of *Streptococcus pyogenes* on Nile tilapia and to determine the effect of every dose on hematological parameters as indicator of infection. In this study, a total number of 100 healthy Nile tilapia (*Oreochromis niloticus*) fingerlings (15 ± 0.5 g) were infected with *Streptococcus pyogenes*. These fish were randomly divided into five groups, each with 20 fingerlings. Five concentrations of *Streptococcus pyogenes* (0, 10$^6$, 10$^7$, 10$^8$, and 10$^9$ CFU/ml) were used to realize the objective of this study. Group 1 was injected inter-perennial with 0.2 ml sterilized saline solution and it was considered as a control group. The other 4 groups were injected inter-perennial at a rate of 1ml per 10 g of inoculum. All experimental groups were examined for infection signs, mortality rate and hematological parameters. The results revealed that the LD$_{50}$ of *Streptococcus pyogenes* was 1×10$^7$ CFU/ml with ten days of tilapia infection. Also, it affected significantly on the hematological parameters and this concentration showed moderate value of infection and mortality rate compared with 1×10$^8$ CFU/ml which showed high value led to 100% immediate death of the fish. On the other hand, the concentration 1×10$^6$ CFU/ml presented normal value with control which led to weak infection. The concentration 1×10$^9$ CFU/ml was violent and fish body can’t resist it

1. Introduction

Although Nile tilapia (*Oreochromis niloticus*) is considered one of the most successful candidates in the aquaculture sector, it is more vulnerable to diseases than many other freshwater species (Abu-Elala. *et al.*, 2021; Gabr *et al.*, 2021; Shaaban *et al.*, 2021). During the second half of the twentieth century, there were outbreaks in tropical, subtropical, and temperate areas (Amal *et al.*, 2011), that are witnessed on such severe infections. Infection by *Streptococcus* spp. has been reported in tilapia farms caused Streptococcusis diseases (Liao *et al.*, 2020). *Streptococcus* spp. are gram-positive, non-motile bacteria. It is regarded as the primary cause of global aquaculture sector destruction. It causes clinical signs as hyperemic gills diffuse epithelial tissue proliferation, lesions, dermal hemorrhage, exophthalmia, corneal opacity, dark coloration, abscess of trunk muscles, erratic swimming, melanosis, hemorrhage around the jaws and base of pectoral and pelvic fins, ascitic fluid in the abdominal cavity are some clinical signs (Nasr-Eldahan *et al.*, 2021).

One of the most common *Streptococcus* species is *Streptococcus pyogenes* which has been isolated from Rosetta Branch, Egypt and caused approximately up to 60% mortality of total fish infected (Abou El-Gheit, 2005). They are non-motile and they have a spherical or ovoid shape and grow in pairs or chains (Amal *et al.*, 2011). They are optionally anaerobic, necessitating nutritionally rich development media, and typically attacks red heart cells, causing greenish discoloration or complete clearing on the body agar. Moreover, they are a type of bacteria that are fermentative in metabolism, producing primarily lactic acid but without the production of harmful gases or catalase (Amal *et al.*, 2011). The clinical signs of fish subjected to these bacteria are the same as described previously for other *Streptococcus* spp.). Infection with that bacteria could occur through skin injuries and abrasions. This process is generally involved in
high density cultivated fish. In addition, *Streptococcus* is likely to be transmitted within the same aquatic environment between various species of wild and cultivated fish (Nur-Nazifah et al., 2011). According to Amal et al., (2011), the most significant factor implemented by this species is the freshly introduced fish. In the fish farms, the bacteria are excreted in infected fish's faces, survive in the water, and become infectious with other healthy fish. In addition, it is thought that using the infected thrashed fish as feed is accountable for Streptococciosis outbreaks (Kim et al., 2007).

These bacteria have three types of toxins which affect the body. One type of the toxins may cause damage for the plasma membrane and lead to red skin rash. Other type may cause lysis for RBCs and LYM cells. The last type causes lysis for neutrophils and platelets. These toxins led finally to weaken the body's immunity but the pathogenic dose is still not known (Müller-Alouf et al., 1997 and Brouwer et al., 2020). Thus, this study aimed to verify the infectious dose of *streptococcus pyogenes* on Nile tilapia (*Oreochromis niloticus*) fingerlings. In addition to the estimated the LD$_{50}$ of *streptococcus pyogenes* using hematological parameters as indicators for infection.

2. Materials And Methods

2.1. Fish and rearing conditions

A total of 100 healthy (15 ± 0.5 g) Nile tilapia (*Oreochromis niloticus*) fingerlings were netted from El-Madina Fish Farm, Kafr Elsheikh Governorate, Egypt. Fish were transported in a plastic 1000 L container filled with aerated, controlled temperature (27 ± 1 °C) fresh water to the research animal unit of Marine Biotechnology and Natural Products Lab (MBNP) at the National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt. Fish were kept in glass aquaria with de-chlorinated tap water and continuous aeration via air pumping. The temperature (27 ± 1 °C), pH (7.5:8), ammonia (close to 0.0016 ppm) and dissolved oxygen (6 mg/L) were measured daily by Hannah instrument to keep water quality in an optimal condition. For health monitoring, the fish were kept in a separate quarantine tank for four weeks. There was no signs of pathogens. Three weeks prior to the start of the experiment, the fish were acclimatized to the experimental conditions.

2.2. *Streptococcus pyogenes* culture

*S. pyogenes* were obtained from American Type Culture Collection (ATTC), Virginia, United States of America and cultured on blood agar media, according to Gera et al. (2013). Briefly, the growth media was prepared by the addition of 15 g of pancreatic digest of casein, 5 g of peptic digest of soybean meal, 5 g sodium chloride and 15 g agar per liter of deionized water in a flask. The flask was placed on a magnetic stirrer to mix. Then, 100 ml of the aliquot were poured into in 10 flasks which were sterilized by autoclaving at 121°C for 15 minutes. The 10 flasks were left to cool and preserved in a refrigerator at 4°C. After that, 5% of sheep blood were added into Trypticase Soy Agar (TSA) to obtain blood agar media. According to Nur-Nazifah et al. (2011), bacteria were inoculated in sheep blood agar for 18 hours in 30 °C. The cultures were in an early stage of growth. Bacterial contents were measured using the plate count
method after incubation. Nearly 1 ml of broth was diluted 10-fold until 0.1 ml of each dilution was started pouring and spreading it on blood agar and incubated at 30 °C for 24 hours. Followed by incubation, the number of colonies was recorded, with special attention paid to plates containing 25 to 250 colonies, before the concentration was expressed as colony forming unit (CFU). After that, the final concentration of live *S. pyogenes* was measured. To achieve a lower concentration, the standard solutions $10^9$ was diluted ten-fold with phosphate buffered saline (PBS), and the inoculum was immediately taken. Centrifugation at 3500 g for 10 minutes at 4 °C was used to collect the bacterial cells.

### 2.3. Pathogenicity of *S. pyogenes*

Different concentrations of bacteria were investigated to determine the infected dose until reaching the lethal dose of 50 (LD50) from *Streptococcus pyogenes* according to Kizy *et al.* (2009) and El-Gheit (2005). A number of 50 individuals of Nile tilapia were divided into five duplicated groups for 10 days. They were infected by four concentrations of *Streptococcus pyogenes* (inoculation with saline water containing $1 \times 10^6$, $1 \times 10^7$, $1 \times 10^8$, $1 \times 10^9$ CFU, groups from 2 to 5, respectively) and one group was inoculated with only saline water (group 1 or control group). Inoculation was done by intra-peritoneal injection.

#### 2.3.1. Clinical examination

The clinical examination of the experimental fish was checked by observing any abnormalities on the external body surface or behavior, in addition to calculating the mortality rate.

#### 2.3.2. Hematological examination:

All individuals were anesthetized by 50 ppm MS222 (Popovic *et al*., 2012). Blood sample was collected into a tube containing sodium heparin from the tail and caudal fin. Hematological examinations were carried out on a hematology device.

### 2.4. Statistical analysis

Data were analyzed statistically using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) Qualitative data were described using number and percent. The Shapiro-Wilk test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean and standard deviation. Significance of the obtained results was judged at the 5% level. The F-test (ANOVA) for normally distributed quantitative variables was applied to compare between more than two groups, and Post Hoc test (Tukey) for pairwise comparisons.

### 3. Results

#### 3.1. Toxicity of *Streptococcus pyogenes*

Pathogenicity test revealed that the LD$_{50}$ of *Streptococcus pyogenes* was $1 \times 10^7$ CFU as this dose causes 60% mortalities within 7 days after intra peritoneal inoculation (Table 1).
Table (1)

The pathogenicity test (LD$_{50}$) of *Streptococcus pyogenes*

<table>
<thead>
<tr>
<th>Group</th>
<th>$Streptococcus pyogenes$ inoculum concentration (CFU)</th>
<th>Mortality</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2 ml sterilized saline i/P</td>
<td>0%</td>
<td>control</td>
</tr>
<tr>
<td>2</td>
<td>infected by intra-peritoneal injection 1× 10$^6$ CFU/ml</td>
<td>10%</td>
<td>May be a conflict with normal death rate</td>
</tr>
<tr>
<td>3</td>
<td>infected by intra-peritoneal injection 1× 10$^7$ CFU/ml</td>
<td>60%</td>
<td>LD$_{50}$</td>
</tr>
<tr>
<td>4</td>
<td>infected by intra-peritoneal injection 1× 10$^8$ CFU/ml</td>
<td>100%</td>
<td>LD$_{100}$</td>
</tr>
<tr>
<td>5</td>
<td>infected by intra-peritoneal injection 1× 10$^9$ CFU/ml</td>
<td>100%</td>
<td>LD$_{100}$</td>
</tr>
</tbody>
</table>

In 1× 10$^6$ CFU/ml concentration, 10% mortality was shown from day 2. On contrast, 1× 10$^7$ CFU/ml concentration presented nearly 60% mortality from the first day to the 7th day. After the 7th day, no mortality was detected. In 1× 10$^8$ CFU/ml concentration, all fish died after 3 days of injection. Also, for 1× 10$^9$ CFU/ml group, all fish died in the next day of infection (Table 2).

Table (2)

Number of dead fish/day at different concentrations of *Streptococcus pyogenes*

<table>
<thead>
<tr>
<th>$S. pyogenes$ concentration</th>
<th>Death time (days)</th>
<th>Fish number</th>
<th>Total Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1×10$^6$</td>
<td></td>
<td>1 2 3 4 5 6 7 8 9 10</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1×10$^7$</td>
<td></td>
<td>2 1 3 5 1</td>
<td>60</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1×10$^8$</td>
<td></td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1×10$^9$</td>
<td></td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2. *Streptococcus pyogenes* clinical examination

The clinical examination of the infected fish subjected to 1× 10$^7$ CFU/ml revealed the presence of typical clinical signs of *S. pyogenes* infection such as skin lesion, hemorrhage in the body surface, redness in
dorsal, anal and pectoral fins and eye optic. Furthermore, the internal examination shows pale and enlarged liver and spleen and there was bloody fluid in the abdominal cavity (Fig. 1).

### 3.3. Hematological examination

Figure (2) shows white blood cells (WBCs) level and their components at different bacterial concentrations which demonstrates that 1x 10^6 group presented the same results as the control group. On contrast to 1x 10^7 which show normal value with bacterial infection in fish as LD_{50}, but in case of 1x 10^8 show high WBCs levels which refer to that bacterium was virulence. But in case of 1x 10^9 show's no value. Additionally, WBCs levels in different bacterial concentrations show a significant increase in the *S. pyogenes* group with different concentrations (10^6, 10^7 and 10^8) by (41%, 72%, and 83%) presenting a value of (45.57 ± 9.12), (96.77 ± 1.51) and (157.60 ± 22.88) respectively compared to the control group (26.70 ± 5.87). In case of LYM% levels detected no significant difference between all groups compared to the control group and similar results were observed in MID% and GRAIN%. The LYM# levels revealed a significant increase in the *S. pyogenes* group with different concentration (10^6, 10^7 and 10^8) by 28%, 74%, and 76% giving a value of 20.13 ± 16.05, 55.43 ± 1.78 and 61.27 ± 19.21, respectively compared to the control group (14.53 ± 4.88), while in the MID# levels revealed a significant increase in the *S. pyogenes* group with different concentration (10^6, 10^7 and 10^8) by 10%, 79%, and 85% presenting a value of 5.90 ± 2.60, 25.07 ± 0.25 and 36.53 ± 2.65, respectively compared to the control (5.30 ± 0.72) group. Finally in the GRAN# levels revealed a significant increase in the *S. pyogenes* group with different concentration (10^6, 10^7 and 10^8) by 65%, 57%, and 88% presenting a value of 19.53 ± 27.77, 16.27 ± 0.71 and 59.80 ± 39.80, respectively compared to the control group (5.30 ± 0.72).

These results of red blood cells (RBCs) indices at different bacterial concentrations are presented in Figure (3). No significant difference was shown between all groups compared to the control group. On the other hand, the concentration of 10^6 CFU/ml shows a significant increase in the RBCs level by 95% presenting a value of 7.72 ± 12.52 compared to the control group (0.38 ± 0.14). Similar to hemoglobin (HGB) levels which shows no significant difference between all groups compared to the control group. On the other hand, the concentration of 10^8 CFU/ml shows a significant increase in HGB level by 54% presenting a value of (14.53 ± 7.93) compared to the control group (6.63 ± 3.59). The hematocrit (HCT) levels showed a significant increase in the *S. pyogenes* group with different concentrations (10^6, 10^7, and 10^8) by 16%, 65% and 85% presenting a value of 4.83 ± 0.29, 11.87 ± 1.14, and 26.83 ± 17.56, respectively compared to the control group (4.07 ± 1.51). On the other hematological parameters, there is no significant difference between different concentration with each other and control, except PLT levels which showed significant increase in 1x 10^8 CFU/ml by 74% presenting a value of (1886.0 ± 1600.9) compared to the control group (483.7 ± 50.58).

### 4. Discussion
Outbreaks of *S. pyogenes* have recently been identified as among the most serious infectious diseases, with significant economic consequences in both cultured and wild populations of tilapia farms. Acute Streptococcosis infection can cause more than 50% mortality (Saleh et al., 2019). *Streptococcus* spp. are common in aquatic environments (Nasr-eldahan *et al.*, 2021). Bacterial pathogenicity, like that of other opportunistic bacteria, is associated with a sharp increase in water physicochemical parameters (ammonia, salinity, and temperature), low dissolved oxygen, and stress factors (Younes *et al.*, 2016).

The current study was an attempt to verify the pathogeny dose of *streptococcus pyogenes* into Nile tilapia infection. In addition to examining the appropriate dose for infection, estimated the LD$_{50}$ of *streptococcus pyogenes*, in which fish reinjected at four different concentrations ($1 \times 10^6$, $1 \times 10^7$, $1 \times 10^8$ and $1 \times 10^9$CFU), and found that $1 \times 10^7$ CFU showed a mortality rate of 60% compared to $1 \times 10^6$ CFU which show mortality rate by 10% and that on contract $1 \times 10^8$ and $1 \times 10^9$CFU which showing 100% mortality and this dose in contract with El-Gheit, (2005) which show that $1 \times 10^7$ CFU caused 100% mortality and $1 \times 10^5$ CFU was LD$_{50}$. Furthermore, in day of infection $1 \times 10^7$ CFU as LD$_{50}$ dose cause mortality from day 2 until day 7 and this agreement with El-Gheit, (2005) who found that *S. pyogenes* causes death and become virulence from day 2 and become normal in death after day 7 to day 10.

The clinical examination of *S. pyogenes* shows the same clinical sign of other *Streptococcus species* as in external examination show skin lesion, erratic swimming, eye optic, hemorrhagic in the body surface. Furthermore, internally show that the liver is enlarged with pale color and spleen is enlarged the abdominal cavity and intestine has fluid executed, which agreement with previous reports (Amal *et al.*, 2011 and Mishra *et al.*, 2018).

Hematological characteristics are a valuable tool that can be used as an effective and sensitive indicator for tracking physiological and pathological changes in fish (Parrino *et al.*, 2018). The next discussion show that $1 \times 10^7$ agrees with previous studies, on contract with other concentration. In this study, going to comparing hematological parameter among four different concentration of *S. pyogenes*. Where WBCs, LYM and Neutrophils (a type of GRAN) show high increase in ($1 \times 10^7$ and $1 \times 10^8$) and very low decrease in other concentration compared to the control. This increase indicates a response of the body resistance to disease-causing antigens taking into account a natural reaction to the presence of the bacterial pathogen by induction of the non-specific defense system (Alsaid *et al.*, 2014). The Increase in total leukocytes indicated an increase in the body’s immunity, as evidenced by increased activity of phagocyte cells, which act to perform phagocytosis against foreign objects entering the fish body (Alamanda *et al.*, 2007). On the other hand, the increase in lymphocytes may be due to stress of bacteria infect fish and end up causing lymphocyte proliferation (increased cell count and formation of changes in T cells and B cells) (Cano *et al.*, 2013). Furthermore, the increase in neutrophils could be due to the response to a bacterial infection, as neutrophils escape from the marginal group and join the infection area, and the thymus releases its source of reserve, resulting in increased granulopoiesis, with this increase due to the presence of many immature cells. Neutrophils entering the blood circulation, killing and digesting bacteria. And this agreement with (Lawrence *et al.*, 2018 and Afiyantithe *et al.*, 2018). On the other hand, decreasing in WBC,
lymphocytes and Neutrophils in *S. pyogenes* group, may due to the bacteria becoming virulent, and the action of bone marrow is temporarily disrupted, with the bone marrow being the factor that produces WBCs, lymphocytes, neutrophils, RBCs, and platelets (Nombela-Arrieta *et al.*, 2017 and Seo *et al.*, 2019).

On the other hand, the decrease in RBCs, HGB, and HCT other hematological parameters count in the different concentration except (1×10⁸ in HGB and 1×10⁶ in RBCs) could be due to the deterioration of the hematopoietic organs located in the spleen and pronephros, where the bacteria may cause pathologies in the hemopoietin organs, particularly in the kidneys, spleen, and liver of fish, which leads to a decrease in the production of RBCs, HGB, and HCT (Alsaid *et al.*, 2014). And this agreement with Yu *et al.*, (2010) who demonstrated that there was a significant decrease in RBCs in peripheral blood, HCT percentage, and HGB rate, all of which are indicators of anemia. Furthermore Alsaid *et al.*, (2014) show same results when red hybrid tilapia injection with *Streptococcus agalactiae*. Our most recent discovery also noted that sharp decrease in these values that could have hampered oxygen and nutrient transport to tissues may be due to hypochromic microcytic anemia caused by bacteria (Harikrishnan *et al.*, 2009) and that agree well with the results of Řehulka *et al.*, (2007). The same results were obtained when fish infected with *S. agalactiae* bacteria (McNulty *et al.*, 2003). MCHC

In most animals, the investigated hematological indices, MCHC (mean corpuscular hemoglobin concentration), MCH (mean corpuscular hemoglobin), and MPV (mean platelet volume), play a critical role in the diagnosis of anemia (Alsaid *et al.*, 2014). In this study, the declines in these blood indictors (MCH and MCHC) may be linked to the declines in RBCs, HGB, and PCV caused by disruptions in hematopoietic organs of fish challenged with *S. pyogenes*. Similarly, to our findings Ranzani-Paiva *et al.*, (2004) noticed that MCH and MCHC levels in blue tilapia infected with *Corynebacterium* sp. were found to be lower, due to lower RBCs. Furthermore, Haniffa and Mydeen, (2011) notice that there are decline in MCH and MCHC in infected catfish with *Aeromonas hydrophila*.

In elevated number of platelets may be due to innate immune cells, platelets contain PRRs (pattern recognition receptors), which recognize different components that are increased during infection in *S. pyogenes* group which show increasing in platelet count after infection. Bacteria release toxins called estreptolysin O, which is capable of activating platelets, increasing platelet production, and causing platelet aggregation (Portier *et al.*, 2021). Also, during invasive infection, *S. pyogenes* bacteria may use a cloak of fibrinogen and activated platelets to evade the immune response and spread through the bloodstream. *S. pyogenes* bacteria have the ability to bind with fibrinogen to increase bacteria livelihood in the bloodstream (Carlsson *et al.*, 2005), and this in agreement with fibrinogen (Svensson *et al.*, 2014).

**Declarations**

- **Ethical Approval:**

Ethics required are approved, by the Ethical Committee of Alexandria University.

- **Human and Animal Ethics:**
The sample collection was conducted in accordance with all applicable laws, guidelines, and regulations from the government in the present study area.

- **Consent for Publication:**

The authors certify that the publisher is permitted to publish this work.

- **Availability of supporting data:**

Some of the data generated or analyzed during this study are included in this article. The other datasets used and/or analyzed during the current study are available from the author on reasonable request.

- **Competing Interests:**

The authors declare that they have no conflict of interest to declare.

- **Funding:**

Not applicable.

- **Authors’ contributions:**

The authors are all participated in the work.

- **Acknowledgments:**

The authors acknowledge all members from Alexandria University and NIOF who helped in this work.

- **Authors’ information:**

Not applicable.

**References**


**Figures**

![Figure 1](image1)

**Figure 1**
Clinical examination of Nile tilapia subjected to $1 \times 10^7$ CFU/ml, showing skin lesion, hemorrhage in the body surface (A), redness in dorsal, anal and pectoral fin (B) and eye optic (C), (1-a); enlarged pale colored liver and spleen is also enlarged (A), (1-b & 1-c, respectively); bloody fluid in abdominal cavity (B), (1-c).

Figure 2

White blood cells (WBCs) level and their components at different bacterial concentrations.
Figure 3

Red blood cells (RBCs), Hemoglobin (HGB), and platelets indices at different bacterial concentrations: HCT (hematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), RDW (red cell distribution width), PLT (platelet count) and MPV (mean platelet volume).