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Immune Defenses in European Eels (*Anguilla anguilla*) Post Exposure to Formalized Killed Bacterin of *Vibrio vulnificus*

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ABSTRACT

The primary objective of this study was to assess the efficacy of the formalized killed vaccine of *Vibrio vulnificus* on European Eels (*Anguilla anguilla*) using the immersion method. The experimental fish were subdivided into four groups G1– G4 (30 fish/ group). G1 served as the control and was immersed in saline with 3% NaCl. G2 and G3 were vaccinated by immersion in the formalized killed vaccine for 1h. Fish of G3 (vaccinated) and G4 (non-vaccinated) were experimentally infected with *V. vulnificus* via immersion in bacterial suspension for 1h. The levels of antibodies in the skin mucus were quantified using the enzyme-linked immunosorbent assay (ELISA) after 7, 14, 21, and 28 days of vaccination. The study results indicated that the vaccinated group (G2) exhibited significantly higher levels of antibodies when compared to the first group and the vaccinated challenge group (G3). The relative percent survival (RPS) was estimated to be 83.38 and 70.88% for G2 and G3, respectively. However, the mortality rate was 80% in G4 infected with the *V. vulnificus* strain. Moreover, the expression of cytokines IL-1β and TNF alpha immune-related genes in the anterior kidneys of all groups increased in the vaccinated groups (G2 and G3) compared to the control group (G1). Additionally, in the renal parenchyma of G2, G3 and G4 groups, there were markedly active melanoma-macrophage centers observed as pathological changes.

INTRODUCTION

Global fish production increased by 1.2% in 2022, reaching 184.1 million tonnes, with output from aquaculture expected to grow by 2.6% **(FAO, 2023)**. According to the current consumption, aquaculture production needs to increase from 82,087 in 2018 to 129,000 kilotons in 2050 to meet global needs **(Naylor *et al.*, 2021; Boyd *et al.,* 2022)**.

European eel has a complex biological cycle involving marine, brackish, and freshwater habitats **(Elgendy *et al.*, 2016)**. Eel reproduction faces difficulties due to

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climate change, migration, pollution, and other factors that negatively impact wild populations of elvers and adults **(Aschonitis *et al*., 2017; Pirollo *et al*., 2023)**.

Vibrio is one of the most important pathogenic bacteria for fish, crustaceans, and marine animals. Due to its high abundance and biomass, it is widely found in marine and freshwater ecosystems **(Sherif & Abuliela, 2022)**. One of the most harmful *Vibrio* to fish farms is *V. vulnificus*. Unlike other vibrio speices, it is Gram-negative and has the ability to ferment lactose bacterium that can cause wound infection and septicemia. According to genetic, biochemical, and serological tests and host infection, *V. vulnificus* is classified into three biotypes. *V. vulnificus* inhabits temperate, tropical, and sub-tropical aquatic ecosystems that spread alarmingly to traditionally cold areas influenced by global warming **(Amaro *et al*., 2015; Oliver, 2015; Austin & Oliver, 2018, 2019; Austin *et al*., 2018)**.

Overuse of antibiotics and chemotherapeutics between fish aquacultures to control the bacterial pathogen and eliminate beneficial bacteria **(Kwon *et al*., 2007)** illegally increases drug-resistant bacteria **(Sherif & Kassab, 2023)**. *V. vulnificus* is antibiotic-resistant, hence it cannot be treated with antibiotics which is currently the most commonly used in fish farms **(Abutbul *et al*., 2004; Sherif *et al*., 2021)**. Therefore, researchers have conducted trials to use probiotics or vaccinations to overcome pathogen virulence **(Abdellatief *et al*., 2019)**; antibodies titer increased slightly significantly when estimated by ELISA in skin mucus of eels that were fed *Spirulina platensis* as a probiotic in eels ration as an immuno- stimulant against *V. vulnificus*.

The vaccination can mitigate the spreading of pathogens and increase the innate immunity of fish aquaculture. Immersion vaccination depends on taking the antigen (Ag) by the skin, gills, and gut, followed by Ag processing by the immune system and subsequent protecting response **(Bøgwald & Dalmo, 2019; Eldessouki *et al.,* 2023; Sherif *et al.,* 2023)**. The cells involved in Ag uptake differ according to the Ag's physical state and the Ag entry site **(Nakanishi & Ototake, 1997)**. The mechanism of Ag uptake in immersion vaccination is unknown, with several modulating factors. Immersion vaccination relies on the immune response of fish mucosa-associated lymphoid tissues; the mucosal Ab response precedes the systemic response **(Gong *et al*., 2021)**.

This research project was conducted with the primary objective of studying the effectiveness of a formalized whole-cell *V. vulnificus* vaccine. The vaccine was administered through the immersion method to *Anguilla anguilla* with the purpose of finding the best possible approach to control the spread of *vibrio* in eels aquacultures.

MATERIALS AND METHODS

1. Fish sampling and accommodation

A total of 175 *A. Anguilla,* without any previous history of diseases, were collected from private fish farm. One hundred and twenty eels weighing 20± 5g were subdivided into four groups of 30 fish/group in aerated glass aquaria (100 × 50 × 60cm) (Table 1). During the experimental time, fish were fed on a commercial diet containing 35% crude protein daily at 3% body weight, according to the method implemented by **Eurell *et al.* (1978)**. Fish were observed during the acclimatization period and experimental time in the wet laboratory unit at the Fish Diseases Research Department at Animal Health Research Institute, Al-Dokki. The aquaria's water parameters were supplied with dechlorination, while water temperature and salinity were adjusted at 25± 3°C and 5g/ L, respectively, till the end of the experiment, following the method outlined by **Collado *et al.* (2000)**. After 15 days of acclimatization, ten eels were examined for the presence of a natural pathogenic infection (bacterial, fungal, and parasitic).

2. Bacterial strain identification

Vibrio vulnificus was previously isolated and identified by API*20NE, according to the method of **Salama *et al.* (2016)** from naturally infected eels. The isolate was cultured in Tryptic Soy Agar with 3% (Na Cl) for 24h at 25ºC.

3. Vaccine preparation

The formalized killed vaccine was prepared according to method of **Collado *et al.* (2000)**. The *V. vulnificus* strain was recovered from culture Tryptic Soy Agar with sterile phosphate-buffered saline (1% NaCl), followed by incubation at 25°C for 24h. The cells were inactivated by adding 1% (v/ v) formalin, centrifuged at 12000 × g for 20min at 4°C, and re-suspended in PBS-1% NaCl at a concentration of 8.0± 0.5 $\times 10^9$ cells/ ml (absorbance at 600 nm of 0.75). The final concentration of the formalin vaccine was 0.3%.

4. Sterility and toxicity tests

Before using the prepared vaccine, the sterility of the formalized killed vaccine was confirmed by the absence of bacterial growth after the inoculation at 25°C for 7 days. The lack of toxicity for eels was evaluated by intraperitoneal injection of 5 eels with 0.1ml of 1ml WCB+9ml PBS.

5. Vaccination application

The fish were directly immersed for 1h in a bath containing the vaccine diluted to a concentration of about 1 × 10^7cfu/ ml at a constant temperature of 25± 2°C, following the method outlined by **Horne and Ellis (1988)**. Two (booster effect) doses of vaccine were administered by immersion at 7 intervals days, following the guidelines of **Song *et al.***

(1982). All vaccination experiment groups were duplicates. The samples of G1 were immersed for 1h in saline with 1% salt (NaCl).

6. Vaccination and challenge process

The fish of G1 were immersed in sterile saline with 1% salt. Additionally, fish of G2 and G3 were vaccinated by immersing in 1×10^7cfu/ ml formalin vaccine. One week after the last booster vaccination, fish of G3 (vaccinated) and G4 (non-vaccinated) were immersed for 1h in a bacterial solution *V. vulnificus* suspension in PBS containing 1×10^7cfu/ ml, according to the method of **Amaro *et al.* (1995)**. Mortalities were daily recorded.

The efficacy of vaccination was evaluated by calculating the relative percent survival (RPS), following the method of **Amend (1981)**, as follows:

$$RPS = 1 - (\%\ \text{mortality in vaccinated fish} / \%\ \text{mortality in controls}) \times 100$$

To assess the impact of the challenged strain, only mortalities were taken into account if the bacteria were re-isolated as pure culture from internal organs, such as the liver and kidney. The bacterial re-isolation was carried out through morphological examination using Thiosulfate–citrate–bile salts–sucrose agar (TCBS agar), gram stain, oxidase, vibriostate O/125, and biochemical analysis using API *20NE kits (bio-Mertieux).

Table 1. Experimental fish design groups

Fish group	No. of fish	Dose	Route
G1 (control)	30	saline with 1% NaCl	Immersion/ 1h
G2 (vaccinated)	30	1×10^7 cfu/ml formalin vaccine	Immersion/ 1h
G3 (vaccinated/ challenged)	30	1×10^7 cfu/ml formalin vaccine + $1x10^7$cfu/ml of *V.vulnificus* strain	Immersion/ 1h
G4 (non-vaccinated/ challenged)	30	1×10^7cfu/ml of *V.vulnificus* strain	Immersion/ 1h
Kidney collection for IL-Iβ and TNF-α	40 5/ group/ double	Anterior kidney collection from all groups	

7. Specific immune response

Skin mucus was weekly collected after a week of administering a dose of the vaccine from all groups, additionally it was collected on days 14, 21, and 28, then centrifuged for

20min at 8000 ×g, and filtered through 0.45µm pore size and supernatants stored at −20°C following the method of **Xu *et al.* (2009)**.

8. Antibodies level in skin mucus

2.8.1- Preparation of hyperimmune sera in rabbits, following the method of **Stevenson and Daly (1982)**.

2.8.2- Enzyme-linked immunosorbent assay (ELISA):

Antibody titers in mucus samples were determined by ELISA; results at 492nm were considered positive with value twice that of the control skin mucus **(Xu *et al*., 2009)**.

9. Expression of IL 1β and TNF-α immune-related gene

1- Sample collection: anterior kidneys were collected from 5 eels/ group/ double of all groups (Table 1) and stored in 2ml tubes containing RNAlater solution (Merc, Egypt), then the samples were kept overnight in the refrigerator at 4°C and frozen at −80°C for RNA extraction.

2- RNA extraction from kidney samples was performed using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). A total of 30mg of kidney sample was added to 600µl RLT buffer containing 10µl β-mercaptoethanol per 1ml. For homogenization of samples, tubes were placed into the adaptor sets, which are fixed into the clamps of the Qiagen tissueLyer. Disruption was performed in 2min high-speed (30 Hz) shaking step. One volume of 70% ethanol was added to the cleared lysate, and the steps were completed according to the purification of total RNA from animal tissues protocol of the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH).

N. B. On column DN 40ase digestion was done to remove residual DNA.

3- Oligonucleotide primers. The used primers were supplied from Metabion (Germany), as listed in Table (2).

Table 2. Oligonucleotide primers

Target gene	Primer sequence
EF-1α	CCTTCAACGCTCAGGTCATC
	TGTGGGCAGTGTGGCAATC
IL1ß	GCTGGAGAGTGCTGTGGAAGAACATATAG
	CCTGGAGCATCATGGCGTG
TNF-α	CCAGAAGCACTAAAGGCGAAGA
	CCTTGGCTTTGCTGCTGATC

Cycling conditions for SYBR green rt-PCR: Cycling conditions for SYBR green rt-PCR are listed in Table (3).

Table 3. Cycling conditions for SYBR green rt-PCR

Target gene	**Reverse transcri-ption**	**Primary denaturation**	**Amplification (40 cycles)**			**Dissociation curve (1 cycle)**			**Ref.**
			Secondary denaturation	Annealing (optics on)	Extensi on	Secondary denaturation	Annealing	Final denaturat ion	
EF-1α	50˚C 30min	94˚C 15min	94˚C 15sec	62˚C 30sec	72˚C 30sec	94˚C 1min	62˚C 1min	94˚C 1min.	*
IL1ß									**
TNF -α				60˚C 30sec			60˚C 1min		***

*(**Gröner *et al*., 2015**) ** (**Castro *et al*., 2011**) *** (**Standen *et al*., 2016**)

4- SYBR green rt-PCR. Primers were utilized in a 25µl reaction containing 12.5µl of the 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25µl of RevertAid Reverse Transcriptase (200U/ µL) (Thermo Fisher), 0.5µl of each primer of 20pmol concentration, 8.25µl of water, and 3µl of RNA template. The reaction was performed in a Stratagene MX3005P real time PCR machine.

5- Analysis of the SYBR green rt-PCR results. Amplification curves and Ct values were determined by the strata gene MX3005P software. To estimate the variation of gene expression on the RNA of the different samples, the CT of each sample was compared with that of the positive control group (G4) according to the "ΔΔCt" method stated by **Yuan *et al.* (2006)** using the following ratio: (2-DDct).

Whereas $\Delta\Delta Ct = \Delta Ct\ reference - \Delta Ct\ target$

$\Delta Ct\ target = Ct\ control - Ct\ treatment$ and $\Delta Ct\ reference = Ct\ control - Ct\ treatment$

10. Pathological examination

Tissue specimens from the skin, liver, kidney, and spleen were immediately immersed in 10% neutral buffered formalin. After proper fixation, tissues were trimmed, washed, and dehydrated in ascending grades of ethyl alcohol and then cleared in xylol and embedded in paraffin. Thin sections about 4– 6 microns thick were prepared and stained with hematoxylin and eosin "H&E", following the method outlined by **Bancroft *et al.* (2012)**.

11. Statistical analysis

Statistical analysis was performed to evaluate the significance of immune response, comparing the antibody production in mucus secretion between groups and determining the significance of variance between fish groups at $P \leq 0.05$ (using the T- student test).

12. Biosafety measures

This study applied biosafety measures in accordance with pathogen safety data sheets: Infectious substances- *Vibrio parahaemolyticus*, Pathogen Regulation Directorate, Public Health Agency of Canada (2010).

RESULTS

1. Clinical and postmortem observations of *A. anguilla*

Clinical signs of *A. anguilla* were observed during the experimental period in G3, including shallow skin ulceration and mucus secretion (Fig. 1a). In comparison, G4 showed severe septicaemia signs, such as deep haemorrhagic skin ulceration and severe mucus secretion (Fig. 1b, c). Additionally, postmortem observations showed congestion of all internal organs (Fig. 1d).

Fig. 1a. Experimentally infected *A. anguilla* during the experimental period in G3 showed shallow skin ulceration and mucus secretion. **(b&c).** Experimentally infected *A. anguilla* during the experimental period in the G4 showed deep haemorrhagic skin ulceration. **(d).** Experimentally infected *A. anguilla* during the experimental period showed congestion of all internal organs

2. Phenotypic characteristics of *V.vulnificus*

V.vulnificus was re-isolated in pure strain from the kidney and liver of challenged groups on TCBS then retested by API*20NE (Table 4), and produced Gram-negative and

curved rods under microscopic examination, oxidase-positive and produced positive to vibriostat O/129.

Table 4. Phenotypic characteristics *V. vulnificus* by API*20NE

Potassium nitrate(NO_3)	+	Mannose assimilation (MNE)	-
Tryptophan production (TRP)	+	Maltose assimilation (MAL)	-
Glucose fermentation	+	Mannitol assimilation (MAN)	-
Arginine dihydrolase production (ADH)	-	N-acetyl-glucosamine(NAG)	-
		Maltose assimilation (MAL)	-
Urea	-	Potassium gluconate assimilation (GNT)	-
Esculin hydrolysis	+	Capric acid assimilation (CAP)	-
Gelatin hydrolysis	+	Adipic acid assimilation (LDI)	-
PNG	+	Malate assimilation (MLT)	-
Glucose assimilation (GLU)	-	Trisodium citrate assimilation (CIT)	-
Arabinose assimilation (ARA)	-	Phenyl acetic acid assimilation (PAC)	-

3. Result of challenge

To evaluate the degree of protection, we compared survival percentages between vaccinated eels, challenged, and controls. The RPS values are summarized in Table (5).

Table 5. RPS between vaccinated and non-vaccinated fish challenged by immersion in the formalized-killed vaccine of *V. vulnificus*

Fish group	No. of fish	Mortalities/ %	RPS
G1(control)	30	0/0	-
G2 (vaccinated)	30	4/13.3	83.38
G3 (vaccinated/challenge)	30	7/23.3	70.88
G4 (infected)	30	24/80	. -

4. Antibodies level in skin mucus

The highest level of antibodies (Fig. 2) measured by ELISA in skin mucus samples of the vaccinated group (G2) were after one week of immersion vaccination and after the second booster dose compared to the levels measured at 14, 21 and 28 days after vaccination. The immune protection after the third dose of vaccine revealed keeping high titer, significantly increasing ($P< 0.05$) in the G2 compared to other groups during the experimental period.

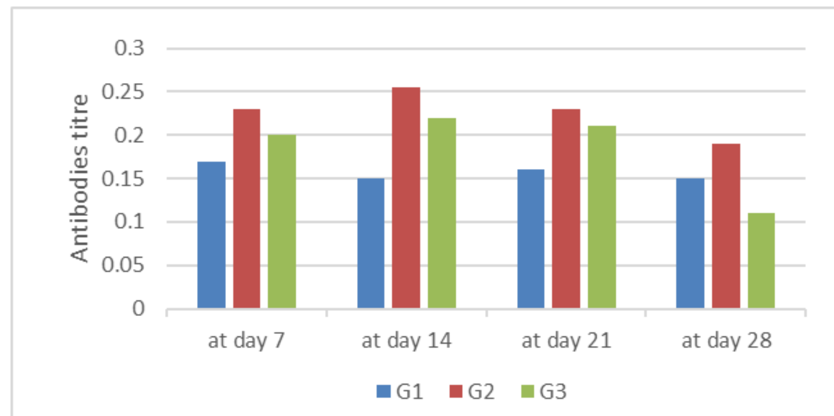

Fig. 2. Antibodies titer in skin mucus measured by ELISA

5. Specific immune response

The effect of the vaccine trial on relative IL 1β and TNF-α gene expression related to the immune system was detected from the anterior kidney of all groups of eels, as exhibited in Fig (3). Relative IL 1β gene expression in G2 was 5.27, and 2.25 in G3, while it was 10.2 in G4. Relative TNF-α gene expression in G2 was 8.16, and 3.2 in G3, while it was 12.4 in G4.

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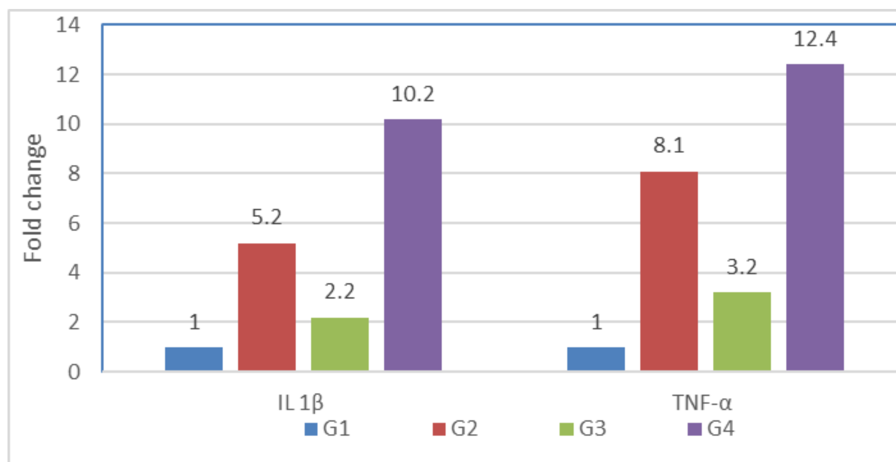

Fig. 3. Cytokines expression genes

6. Pathological findings

The current study investigated the *vibrio*-formalized vaccine's effectiveness in facing *Vibrio* challenge.

Epidermal investigation refers to normal criteria of G1 (Fig. 4a) and G4 (Fig. 4b). Unexpected severe erosion was detected in both G2 and G3 (Fig. 4c, d), respectively.

Gills showed normal histological features in G1 (Fig. 5a), and some changes in the form of tranculation of lamellae were detected in G4 (Fig. 2b). G2 showed hyperplasia of inter-lamellar tissue, with lamellar edema and separation of epithelia (Fig. 2c). In G3, swellings of lamellar tips were recorded (Fig. 2d).

Hepatic parenchyma revealed normal architecture in G1 (Fig. 3a), while G4 showed stimulation of melanoma-macrophage centers formation (Fig. 3b). The marked increase in the number of proliferated melano-macrophage centers was reported in G2 and G3 (Figs. 3c, d).

Renal tissue did not detect noticeable changes in the incidence of melanoma-macrophages in all investigated groups (Fig. 4a- d).

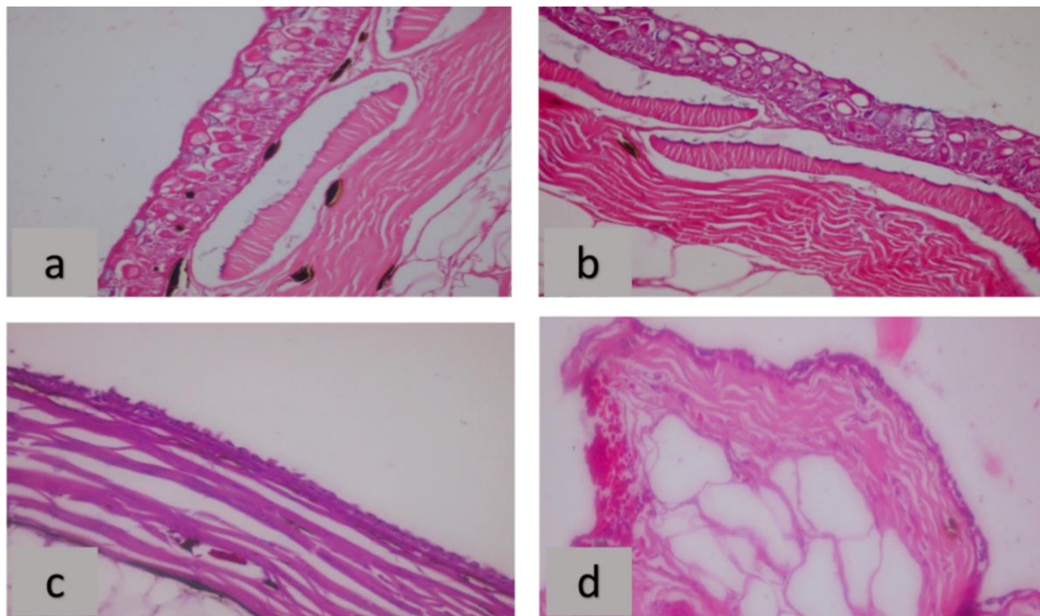

Fig. 4. Epidermis with normal histological criteria in both of G1 **(a)** and G4 **(b)**. Meanwhile severe erosion was detected in G2 **(c)** and G3 **(d)**; H&E, × 400

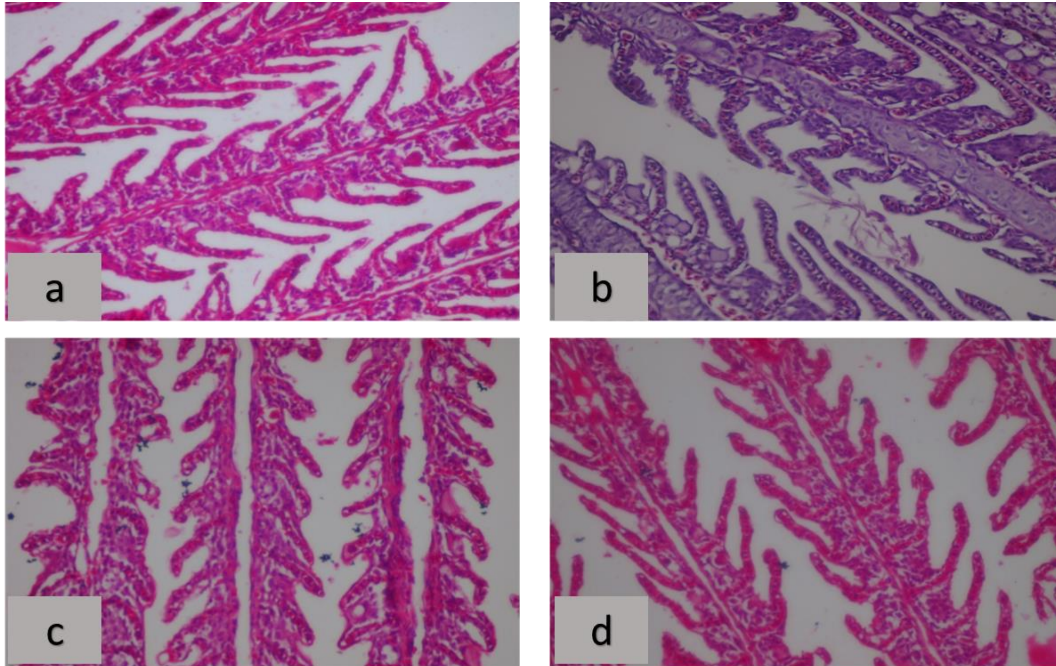

Fig. 5. Gills revealed normal histological feature in G1 **(a)**. Tranculation of gill filaments was detected in G4 **(b)**. Hyperplasia of inter-lamellar tissue, with lamellar edema and epithelial separation was demonstrated in G2 **(c)**. Swollen lamellar tip was recorded in G3; H&E400

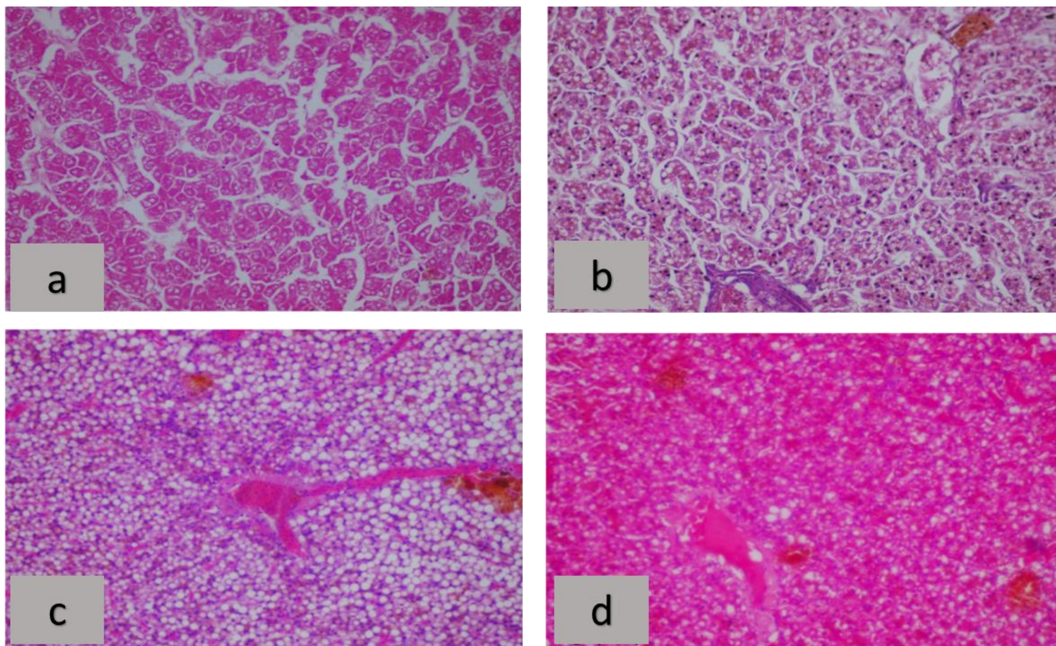

Fig. 6. Hepatic parenchyma with normal histology in G1 **(a)**. G4 exhibited some degree of melano-macrophage centers activation **(b)**. Marked increase in the number of melano-macrophage centers was reported in G2 **(c)** and G3 **(d)**; H&E, × 400

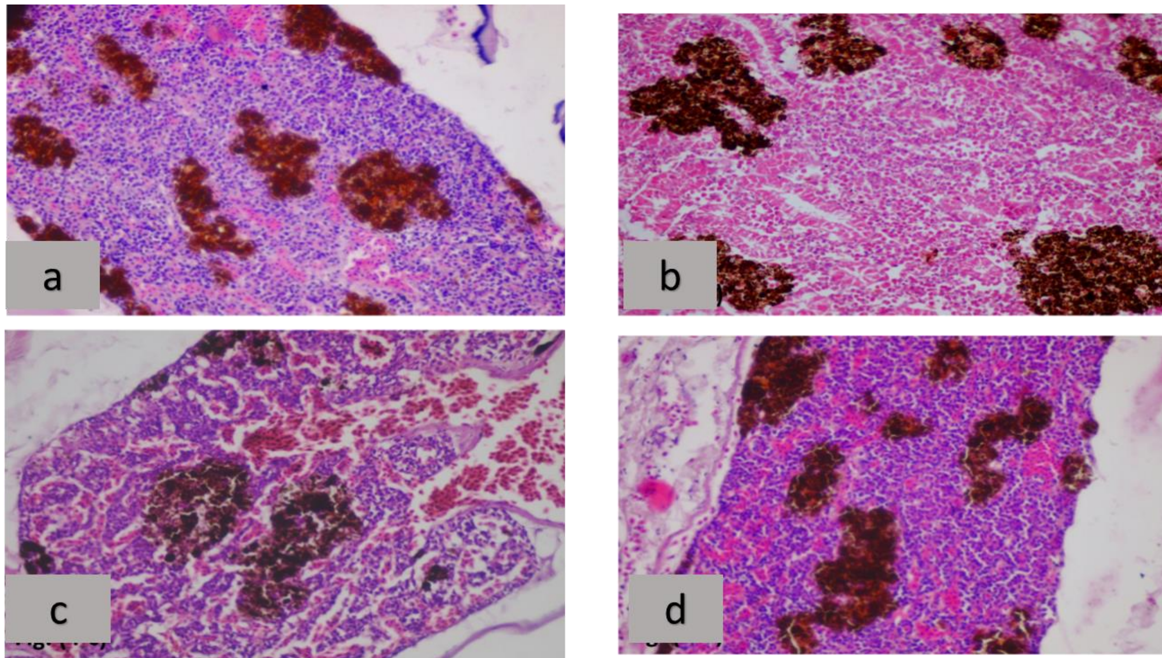

Fig. 7. Renal parenchyma with markedly active melano- macrophage centers with the same degree in different investigated group **(a, b, c & d)**; H&E, × 400

DISCUSSION

Pathogenic *Vibrio* monopolized wild eel skin mucus secretion (SMS)-microbiome from natural ecosystems, *V. anguillarum/ V. vulnificus* and *V. cholerae/ V. metoecus* being the most abundant ones in SMS **(Carda-Diéguez *et al.,* 2017)**. If pathogens are present, even at low levels, disease signs may rapidly develop when the fish become stressed. In a previous study **Salama *et al.* (2016)** indicated the role of the cutaneous route of infection in *V. vulnificus to A. anguilla.*

The results of the current study indicate that eels in the challenged group (G4) exhibited notable morphological lesions characterized by hemorrhagic skin and deep, circular reddish color ulceration. These observations align with the descriptions provided by **Haenen *et al.* (2014)**, **Salama *et al.* (2016)** and **Abdellatief *et al.* (2019)**. In addition, the study results showed that the groups that received vaccination concerning G2 and G3 exhibited a survival ratio of 83.38 and 70.88%, respectively. This ratio is slightly higher compared to the survival ratio of approximately 70%, as reported by **Collado *et al.* (2000)**. The findings suggest that vaccination might provide a certain level of protection against *V. vulnificus*; therefore, the study supports further investigation into the potential of vaccination as a preventive measure against *V. vulnificus* infections.

Immersion vaccination can be effective, depending on the ability of *V. vulnificus* capsular protein to induce an immune response in the form of shock syndrome. In a study conducted by **Esteve-Gassent *et al.* (2004)**, it was found that the efficacy of

vulnivaccine as an oral booster after prolonged immersion vaccination of glass eels at one eel farm significantly improved protection against vibriosis and eel growth, both in weight and length. The capsular polysaccharide in *V. vulnificus* is a significant surface Ag that is loosely associated with and released into the surrounding environment. It is predicted to play a significant role in *V. vulnificus* pathogenesis and immune response, as reported in the study of **Callol *et al.* (2015a)**.

V. vulnificus forms a biofilm-like structure on the eel skin **(Marco-Noales *et al.*, 2001)**. In the present study, the effect of the formalized vaccine of eel, the cutaneous layer represented by marked erosion of the epidermal layer, which could be attributed to the extreme immune reflex. The skin mucus of fish contains antibodies that act as a line defense line against pathogenic microorganisms. The results of the current study have shown that antibody titers measured by ELISA exhibited by eels immunized with the formalized-killed vaccine were moderate in mucus among G2 and G3 and antibodies detected at days 7, 14, 21, and 28. In the same sequence, **Gong *et al.* (2021)** indicated the robust mucosal immune response to immersion vaccination with peak mucosal Ab titer of inactivated *V. harveyi* vaccine earlier than serum. On the other hand, **Christopher *et al.* (2009)** postulated that this robust mucosal response peaked four days following vaccination, then declined, with the additional fact that fish mucosal immunity is relatively independent of systemic immunity.

The current study observed that two booster doses were necessary to achieve significant protection. The study's findings align with those of **Collado *et al.* (2000)**. Overall, the study highlights the importance of booster doses to attain protection against *V. vulnificus* and informs the development of future vaccination strategies.

The head kidney and spleen are the vital immune organs of teleost fish **(Press & Evensen, 1999; Sherif *et al.,* 2022)**. These organs have been demonstrated to make essential contributions to the generation of systemic immunity and perform the vital function of removing foreign or antigenic substances from the blood of teleost fish **(Grove *et al.*, 2006; Sherif *et al.,* 2019)**. Furthermore, both surface-exposed acidic capsular polysaccharide (CPS) and lipopolysaccharide (LPS) of *V. vulnificus* can directly stimulate the expression and secretion of pro-inflammatory cytokines mediated by TNF-α expression **(Powell *et al*., 1997)**. In the current study, IL-1β and TNF-α gene expression increases in the vaccinated group in production, which is related to increased vaccine effect.

In the current study, once activated, these innate immune cells carry out several roles to eliminate an Ag, including degranulation, phagocytosis, and secretion of cytokines and chemokines to activate and/or recruit other leukocytes to the site of action **(Pandey *et al*., 2021)**.

Pathological findings revealed that the inter-lammaeller tissue of *Vibrio* and/or vaccine-treated groups showed some degree of lymphoid tissue proliferation. In the gill, the deeper epithelial of gill filament contained immune cells, while secondary lamellae with very thin epithelia had no source of immune cells **(Monteiro *et al*., 2010)**. It has been suggested that the effectiveness of immersion vaccine depends to a great extent on the active role of the gills and the presence of B cells **(Wong *et al*., 1992)**. The information on B cell and antibody responses occurring in the gills are very limited **(Salinas *et al*., 2011)**. It is anticipated that the immune response of leucocytes in the gills occurs in the earliest stage of defense reaction. **Davidson *et al*. (1997)** and **Dos Santos *et al.* (2001)** reported high number of antibody secreting cells (ASC) in the gills. **Callol *et al*. (2015b)** assumed that eels are capable of distinguishing between innocuous and harmful microorganism by their local action of their toxins rather than surface Ag, and these cells in the gills are migratory cells primarily located in the second lamellae that re-locate during infection suggesting the activation of a specific immune response to pathogen invasion in the gill.

With respect to the melano-macrophage center, one of characteristic feature of macrophages in teleost is their ability to form melano-macrophage centers in hemopoietic tissues **(Mokhtar & Abdelhafez, 2021)**. They aggregate and are frequently pigmented, either around or within chronic inflammatory lesions **(Vogelbein *et al.*, 1987)**. Melano-macrophage centers have a role in Ag trapping and presentation to lymphocytes **(Press & Evensen, 1999)**. The size of melano-macrophage centers collaret with the stress and healthy state of the fish **(Mokhtar & Abdelhafez, 2021)**.

On the other hand, liver has an immunological effect in the form of the production of antimicrobial peptides **(Press & Evesen, 1999)**. The aggregates of pigmented/melano-macrophages are seen in the adventitial layer of venous vessels, between hepatocytes and around the bile duct, and indicate clinically chronic stress **(Mokhtar & Abdelhafez, 2021)**. In the current study, peri-vascular proliferation of melano-macrophage centers was observed in G2 and G4.

Remarkably, kidneys represent one of the primary lymphoid organs of the fish **(Press & Evesen, 1999)** and the primary site for Ag uptake **(Sommerset *et al*., 2005; Bøgwald & Dalmo, 2019)**. The immune reaction primarily occurs around the blood vessel **(Grove *et al.,* 2006; Jingyun, 2007)**. In teleosts, the kidney is essential for macrophage production, and actively phagocytosing macrophages are seen in the kidney **(Meseguer *et al*., 1991)**. Less variability in melano-macrophage immune response in the renal tissues could be attributed to the minority of Ag transported to spleen and kidney **(Huising *et al*., 2003)**. This observation was previously recorded by **Dos Santos *et al.* (2001)**, suggesting that the relatively low level of antibody-secreting cells in the kidney provides an indication that immersion vaccination had a slight systemic response. Similarly, **Pettis and Mukerji (2020)** elucidated that the *V. vulnificus* capsule, capsular polysaccharide

(CPS), had a critical role in the evasion of the host innate immune system by conferring antiphagocytic ability, resistance to complement-mediated killing, and also provoking a portion of the host inflammatory cytokine response to this bacterium.

The current study revealed less degree of immune reaction in G3 than G2, which could be attributed to the fact the current hypothesis is that RtxA13 interferes with eel immune cells triggering a cytokine storm responsible for the death **(Satchell, 2007)**.

CONCLUSION

In the conducted study, it was discovered that the expressed formalized killed bacterin by *V. vulnificus* significantly affected the immune function of *A. anguilla*, enhancing its resistance to *V. vulnificus* infection. This research provides a valuable preliminary strategy to develop vaccines for *V. vulnificus* in eel farming. Moreover, the delivery method is economically viable, quick, and low-stress, making it a practical addition to the eel farming industry. Overall, the study offers a promising alternative approach to making aquaculture vaccines against *V. vulnificus* for eel farming.

RECOMMENDATION: Locally bred eel farms in Egypt require additional strategies for controlling *V. vulnificus* such as vaccination..

Acknowledgement

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