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The efficacy of levamisole as an immunostimulant on male and female *Oreochromis niloticus* challenged by *Vibrio alginolyticus*

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ABSTRACT

This study demonstrates levamisole effects as an immunestimulator on both gender in vivo infected *Oreochromis niloticus* fish with *Vibrio alginolyticus.* 86 naturally infected and apparently health tilapia were sourced from various fish farms in Kafr Elsheikh Governorate, the incidence of *V.alginolyticus* infection *revealed* (9.30%). The moribund fish were thoroughly examined to assess clinical, post-mortem, and bacteriological aspects of the disease, Identification of the bacterium included morphological analysis and, sequence of *rec*A gene, submitted *Vibrio alginolyticus* to the Genebank under accession No OR098883.1. Molecular detection of virulence genes as *collagenase was* 737 bp , *tlh* & *tdh* were 449 & 373 bp, respectively. Isolates were found to be 100% sensitive to Doxycycline and Gentamicin in the antimicrobial susceptibility test, while the strains were 100% highly resistant to Ampicillin.

The experimental healthy males and females *O. niloticus* were divided into four groups (duplicated). Fish G2 and G3 were fed a diet supplemented with levamisole (250mg/kg fish ration) for 60 days. Following the feeding period, groups G3 and G4 were challenged via intraperitoneal injection with a more virulent strain of *V. alginolyticus* (0.5 mL of 1×10^8 cfu/mL). Morbidity and mortality rates were detected. Lysozyme, total protein and albumin were measured in sera of all groups, and IL10 was assessed from the anterior kidney . Histopathological findings to the testes and ovaries of all groups were explained.

INTRODUCTION

Egypt consider number of important countries of global in fish aquaculture production (**FAO, 2024**) specially in tilapia aquaculture. Vibrios are omnipresent in the marine environment and are prevalent across brackish zones and coastal marine areas. . One of the most hazardous diseases in marine aquaculture, *V. alginolyticus*, is that causes highly significant losses in fish and shellfish farms **(Brian and**

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Dawn, 2012; younes et al. (2016). Humans who contract *Vibrio alginolyticus* are at risk of developing deadly conditions such septicemia, gastroenteritis, and necrotizing fasciitis **Economopoulou et al. (2017).** The disease is characterized by clinical signs such as hemorrhages on the fins, particularly around the operculum and vent, blindness or eye dropped, abdomen ascites, skin ulcers, muscle necrosis, and tail erosions **(Zhang et al. 2020; Zhou et al. 2024; Abdellatief et al. 2025)**.

Certain chemicals, such as Levamisole, an artificial anthelmintic agent employed for treating parasitic infections in mammals that also, strongly induces innate and specific immunity in fish, can modify how the fish immune system reacts **Reverter et al. (2014). Elias & Abdellatief (2009)** applied treatment trial with the immunostimulator Levamisole (by injection I/P) against *Y. ruckeri.* Fish pathogen, succeeded in decreasing mortality and repair fish fecundity, Levamisole don't act as antibacterial, but acts through stimulating cell mediated immunity to increase T-lymphocyte differentiate and innate phagocytosis against antigen bacterial pathogens (**Booth and McDonald, 1982).**

Despite the fact that levamisole is typically injected, it is still regarded as an adjuvant ingredient after vaccination because it increases the strength of the immune response. Due to their tendency to raise antibody titters, adjuvants are thought to boost antigen-specific immune responses. Levamisole provides a T-independent antigen to induce the specific immune response by regulating both the MHC receptor and enhancement of cytokines, such as IFN- and IL-6, that mediate reactions among natural killer cells in cell lysis.(**Holcombe et al. 2006).**

This research was undertaken to examine how levamisole acts against *V. alginolyticus* more virulent strains, isolated from naturally infected male and female *O. niloticus* , and injected to both gender *O.niloticus,* Immune response was measured and histopathological of both ovaries and tests were investigated.

MATERIALS AND METHODS

1- Collection of samples

86 *Oreochromis niloticus* (59 male and 27 females) including naturally infected and apparently healthy fish were sampled from different aquaculture farms across Kafr El-Sheikh Governorate, fish were gathered either alive or recently dead, transferred into Animal Health Research Institute's Kafr Elsheikh branch laboratory in sterile polythene bags, and then separated into male and female before the bacteriological analysis.

2-Bacteriological examinations:

Isolation of *V. alginolyticus*

Samples from, pectoral fin, heart, liver, kidney, spleen, were collected under aseptic conditions. The collected samples underwent incubation at 30°C for up to 24 hours after being inoculated into tryptone soy broth (TSB). After that, streaks of inoculation from this broth were placed on Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS), which was then incubated at 30°C for up to 24-48 h. Identification of the suspected isolates was performed through evaluation of their morphological and biochemical characterization **Holt et al. (1994).**

3- Molecular identification :

The *V. alginolyticus* isolate was confirmed by *rec*A gene (recombination-repair protein).

3.1-DNA extraction according to the manufacturer's instructions, using PathoGene-spin™ DNA Extraction Kit.

3.2-Genotypic confirmation by *rec*A gene (F: 5' GTC ARA TTG AAA ARC ART TYG GTA AAG G and R: ACY TTR ATR CGN GTT TCR TTR CC) **(Kim *et al.*, 2015)**. The PCR protocol according to **(Cai *et al.*, 2006).** DNA sequences were obtained by Applied Biosystems3130 genetic analyzer (HITACHI, Japan), and a BLAST® analysis (Basic Local Alignment Search Tool) **(Altschul*et al.,* 1990)** was initially performed to establish sequence identity to GenBank accessions. The MegAlign module of BioEdit created the phylogenetic tree, and Phylogenetic analyses used neighbour joining in MEGA 7 (**Tamura *et al.*, 2013).**

3.3-Detection of *collagenase* targeted primer for species-specific detection of *V. alginolyticus*, and some virulence genes (*tlh* & *tdh*) by PCR.

3.3.1 DNA extraction.

DNA was extracted from samples using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH),

3.3.2. Oligonucleotide Primer. The primers used were given by **Metabion** (Germany) Table (1).

3.3.3. PCR amplification. The primers were tested in a 25-µl reaction that contained 12.5µl of the EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer at a concentration of 20 pmol, 5.5 µl of water, and 5 µl of DNA template. The procedure was performed on an Applied Biosystems Thermal Cycler 2720.

3.4. Analysis of the PCR Products.

The PCR products were separated, at room temperature using 5V/cm gradient electrophoresis on a 1.5% agarose gel (Applichem, Germany, GmbH). The gel was photographed using a gel documentation system (Alpha Innotech, Biometra), and the findings were analyzed using software.

Table 1. Target genes, amplicon sizes, cycle conditions, and primer sequences.

Target gene	Primers sequences 5'-3'	Amplified segment (bp)	Amplification (35 cycles)					Reference
			Primary denaturation	Secondary denaturation	Annealing	Extension	Final extension	
V. alginolyticus collagenase	CGAGTACAG-TCACTTGAAAGC C CACAACAGAACT CGCGTTACC	737	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Abu-Elala *et al.* (2016)
Tdh	CCATCTGTCCCTT TTCCTGC CCAAATACATTTT ACTTGG	373	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Mustapha *et al.* (2013)
Tlh	AAAGCGGAT-TATGCAGAAGCA CTG GCTACTTTCTAG-CATTTTCTCTGC	449	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 45sec.	72˚C 10 min.	Casandra *et al.* (2013)

4.Antibiotic susceptibility testing

The disc diffusion technique was applied to examine the susceptibility of *V. alginolyticus* isolates to commercial antibiotic discs (**Jorgensen and Ferraro, 2009**). Antibiotic discs employed in this study were : Ampicillin (AM) 10ug, Trimethoprim/ Sulphamethoxazole (SXT) 25ug, Cefotaxime (CTX) 30ug, Doxycycline (DO) 30ug, Streptomycin (S) 10ug, Tylosine (TL) 15ug, Enrofloxacin (ENR) 5ug and Gentamicin (GEN) 10ug. The interpretation of zone diameters was sensitive, moderate, and resistant according to **CLSI (2024).**

5. Experimental assay:

apparently healthy *O. niloticus* of both sexes (average weight, 70 ± 5 g), were transported from a private fish farm. They were placed in aerated boxes and brought to Fish Diseases Department (wet-lab) at AHRI in Dokki. They were reared in glass aquaria (55 * 40 *100 cm3), fed on a regular commercial diet at a ratio of 3% of body weight daily. The water of the aquaria was partially renewed daily, and the temperature was maintained at 22-25 ^{o}C. Fish were observed daily throughout a two-week period in suitable environmental conditions. Before the Experimental assay twenty (10 from male and 10 from female) fish were randomly selected for bacterial isolation to be ensure that fish free from *V.alginolyticus* .

140 fish were divided into 4 groups (duplicated) for experimental and the dose administrated were summarized in (table 2), The experimental period within 60 days, G2&G3 feed on 250mg/kg fish ration by levamisole (**Li et al. 2006**). G3 and G4 after 60 days. they challenged by virulent strain of *V.alginolyticus through* i/p route by 0.5 ml 1X10^{8}cfu /ml .(**Mowafy et al. 2025)** (strain 1 for male groups, and strain 2 for females groups). G1 inject i/p by saline , any abnormal signs were recorded and any mortality between that groups .re-isolation to *V.alginolyticus* from internal organs of G3&G4.

Table 2. Experimental assay of male and female Tilapia

Groups	Dose	Male	Female
G1: negative control (feed in regular diet)	3% of body weight	10	10
G2 :regular diet with levamisole	250mg/kg	20	20
G3 : regular diet+ lavamisole+ challenge i/p injection with *Vibrio alginolyticus*	Basel diet +250mg/kg+0.5 ml of 1X10^{8} cfu /ml	20	20
G4:positive control (feed in regular diet+ challenge i/p injection with *Vibrio alginolyticus)*	0.5 ml of 1X10^{8} cfu /ml	20	20

6. Serum analysis:

6.1. Blood Sample Collection and Processing:

Fish were anaesthetized by MS222, then blood samples were withdrew from all groups for analysis. The serum was separated by centrifuging at 3000 rpm for 10 min. and stored at −80 °C until further analysis.

6.2. Serum analysis

6.2.1 Lysozyme activity

100 µl of serum was liquated in a flat-bottomed microplate and mixed with the Micrococcus lysodeikyticus (0.4mg/ml in 0.1M PBS, pH 6.2 at 25C). The microplate was read by ELISA reader at wave length 450nm.The lysozyme activity was measured as a decrease of 0.001/minute in the optical density of cell suspension (**Abu-Elala et al. 2015**).

6.2.2. Determination of serum analysis:

Serum total protein and albumin levels were measured using test kits provided by Spectrum Diagnostics, Germany, at wavelengths of 546 nm and 578 nm, respectively. Serum globulin levels and the A/G ratio were determined through mathematical calculations.

7.Gene expression IL10

RNA extraction. RNA extraction from the anterior kidney of tilapia from experimental groups were applied using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). N.B. On column DNase digestion was done to remove residual DNA, primers used were supplied from **Metabion (Germany)** are listed in table (3). The reaction was performed in a Stratagene MX3005P real time PCR machine.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 according to the "ΔΔCt" method stated by **Yuan et al. (2006).**

Table 3. Primers sequences, target genes, amplicon sizes and cycling conditions for SYBR green rt-PCR.

Target gene	Primers sequences	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)		
				Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation
*EF-1α	CCTTCAAC-GCTCAGGTC ATC	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	62˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	62˚C 1 min.	94˚C 1 min.
	TGTGGGCAG-TGTGGCAAT C								
**IL10	CTGCTAGAT CAGTCCGTC GAA				60˚C 30 sec.			60˚C 1 min.	
	GCAGAACCG TGTCCAGGT AA								

*Gröner *et al.*, 2015----** Standen *et al.*, 2016

8-Histobathological analysis

At the end of the experiment, Testes and ovaries specimens were preserved in buffered formalin (10%) for histopathological analyses. Specimens were stained with Hematoxylin-Eosin (H&E) and examined microscopically **Bancroft, et al. (1999).**

9-Biosafety measures

Biosafety precautions were implemented in compliance with the Pathogen Safety Data Sheet (PSDS) for infectious substances, *Vibrio parahaemolyticus*, issued by the Pathogen Regulation Directorate, **Public Health Agency of Canada (2010).**

10. Statistical analyses

Data concerning the ameliorating role of levamisole to *V.alginolyticus* in the experimental Nile tilapia(male and females) were analyzed to determine the variance (ANOVA). Significance between the experimental groups

using one-way ANOVA test and data were expressed as mean ± standard error (SE) at the significant difference of $P \leq 0.05$ (the SPSS software for Windows, SPSS Inc., Chicago, IL, USA)

RESULTS

Clinical signs and PM examination of naturally infected fish.

Infected fish showed off food, abnormal swimming of alive fish, exophthalmia, unilateral eye cataract, darkness skin, skin ulcer, erosion of tail and abdominal dropsy. The postmortem examination showed severe congestion of internal organs (fig.1)

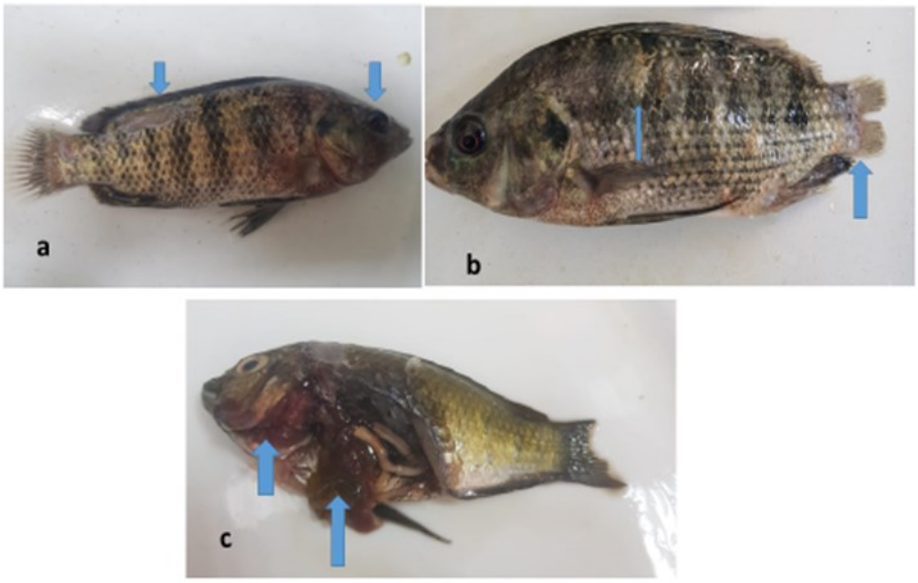


Fig (1), Naturaly infected *O. niloticus* with *V.alginolyticus* showing :
a- exophthalmia and ulcer; b- eroded tail with darkness skin; c-severe congestion of all internal organs and gills

Bacteriological and Biochemical characterization

V.alginolyticus isolates appeared as yellow colonies on TCBS agar, Phenotypic and biochemical characteristics of *V. alginolyticus* isolates were gram-negative, slightly curved rods motile, oxidase, Indole production, gelatine hydrolysis, and catalase were positive, urease, simmon citrate were negative.

Incidence of *V. alginolyticus* infection in Tilapia fish samples:

Incidence of *V. alginolyticus* total isolates from infected fish were (8/86, 9.30%); male fish (5/59, 8.47%), while female incidence were (3/27, 11.11%).

Molcular identification and virulence genes

From the sequence of *rec*A gene, the isolate was *V.alginolyticus* and was submitted to the Genebank database under accession No OR098883.1 (fig 2 & 3)

Molecular analysis to *V. alginolyticus* virulence gence revealed that all 8 strains have *Collagenase* and *tlh* 8/8, 100% for each while 2 strains only have *tdh* (table 4 & fig4.)

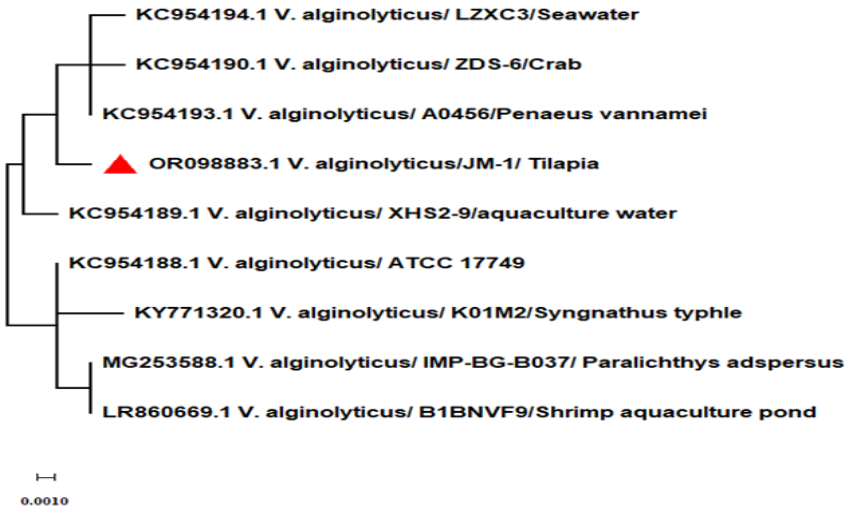


Fig 2 . Shows the phylogenetic tree of *rec*A gene of the genus *V. alginolyticus.* red triangle this study

	1	2	3	4	5	6	7	8	9		eq->
1		100%	99%	99%	99%	99%	99%	99%	99%	1	OR098883.1 V. alginolyticus/JM-1/ Tilapia
2	100%		100%	100%	99%	99%	99%	99%	99%	2	KC954193.1 V. alginolyticus/ A0456/Penaeus vannamei
3	99%	100%		100%	99%	99%	99%	99%	99%	3	KC954194.1 V. alginolyticus/ LZXC3/Seawater
4	99%	100%	100%		99%	99%	99%	99%	99%	4	KC954190.1 V. alginolyticus/ ZDS-6/Crab
5	99%	99%	99%	99%		99%	99%	99%	99%	5	KC954189.1 V. alginolyticus/ XHS2-9/aquaculture water
6	99%	99%	99%	99%	99%		100%	100%	99%	6	MG253588.1 V. alginolyticus/ IMP-BG-B037/ Paralichthys adspersus
7	99%	99%	99%	99%	99%	100%		100%	99%	7	LR860669.1 V. alginolyticus/ B1BNVF9/Shrimp aquaculture pond
8	99%	99%	99%	99%	99%	100%	100%		100%	8	KC954188.1 V. alginolyticus/ ATCC 17749
9	99%	99%	99%	99%	99%	99%	99%	100%		9	KY771320.1 V. alginolyticus/ K01M2/Syngnathus typhle
	1	2	3	4	5	6	7	8	9		

Fig 3.The identity percentage between this study isolate and other references isolates

Table 4. Prevalence of species specific targeted primer (*collagenase*)for detection of *V. alginolyticus*, and virulence genes (*tlh* & *tdh*)

Sample	*type*	*collagenase*	*tlh*	*tdh*
1	male	+	+	+
	female	+	+	+
3	male	+	+	-
4	male	+	+	-
5	male	+	+	-
6	male	+	+	-
7	female	+	+	-
8	female	+	+	-
Total%		8 (100%)	8 (100%)	2 (25%)

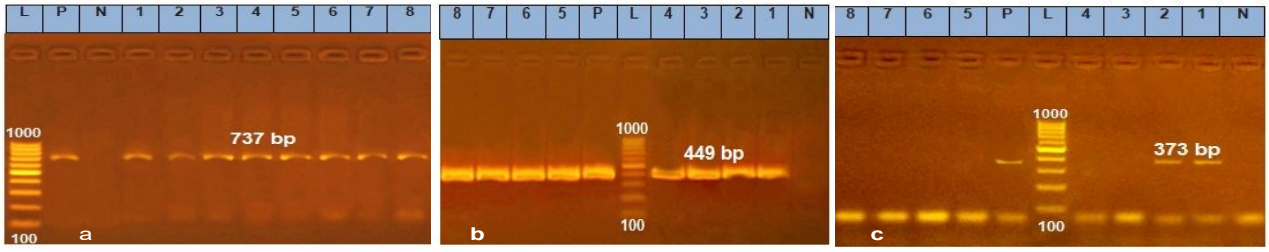


Fig (4). Agarose gel electrophoresis showing: L: marker (100-1000bp); P: positive control; N: Negative control
Lan: 1-8: *V. alginolyticus* positive strains for *collagenase* gene (737bp)
L:1-8: *V. alginolyticus* positive strains for *tlh* virulence gene (449bp)
Lan: 1 and 2: *V. alginolyticus* positive strains for *tdh* virulence gene (373bp)

Table 5. Antimicrobial susceptibility testing for *V. alginolyticus* isolates (N=8)

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Ampicillin (AM)	-	-	-	-	8	100
Trimethoprim/ Sulphamethoxazole (SXT)	5	62.5	1	12.5	2	25
Cefotaxime (CTX)	6	75	-	-	2	25
Streptomycin (S)	7	87.5	-	-	1	12.5
Tylosine (TL)	7	87.5	-	-	1	12.5
Enrofloxacin (ENR)	7	87.5	1	12.5	-	-
Gentamicin (GEN)	8	100	-	-	-	-
Doxycycline (DO)	8	100	-	-	-	-

The experimental fish :

The clinical signs of G1 & G2 were apparently healthy. G3 (levamisole fed and challenged with *V. alginolyticus)* showed mild signs of darkness skin, internal organs within normal size and color. G4 (positive control) in both male and female groups showed, general skin ulceration with heamorrhagic borders, severe congestion and heamorrhage at operculum, severe congestion of skin all over the body, severe congestion and enlarged ovaries in female group (Fig:5).

Re-isolation the pure colonies from pectoral fin, heart, Kidney, liver and spleen from challenged groups G3&G4. The isolates revealed the same morphological and biochemically character's of previously isolated *V. alginolyticus* strains.

Morbidity and mortality rates of *O.niloticus* infected experimentally by *V. alginolyticus* of feeding supplemented diet with levamisole G3 significantly lower than G4 (table.6).

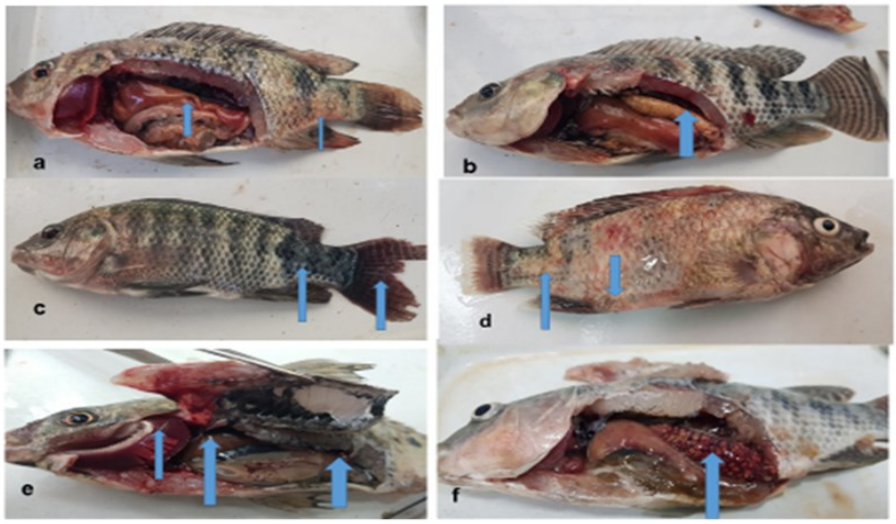


Fig 5. Experimental infected *O.niloticus* with *V. alginolyticus*
G3: Fed on levamisole then challenged with *V. alginolyticus*) showing slight redness of internal organs and mild haemorrhage noted in the caudal region (a), and normal ovaries (b).
G4: (Control positive): Showing dark skin with eroded tail and fins (c), heamorrhgic area on body surface with scale dropped (d), sloughed gill arch, congested liver with enlarged testes (e), and exophthalmia, enlarged and very congested ovaries (f).

Table 6. Morbidity and mortality of *O.niloticus* within experimental groups

Groups	Morbidity %		Mortality%	
	Male	Female	Male	Female
G1	-	-	-	-
G2	1/20 5%	2/20 10%	1/20 5%	2/20 10%
G3	7/20 35%	10/20 50%	2/20 10%	5/20 25%
G4	17/20 85%	20/20 100%	13/20 65%	15/20 75%

Males groups registered highly significant increase in G2 when levamisole added to diet compared to control G1, oppositely, its' level showed highly significant decrease in G3 when added *V.alginolyticus* challenge compared to G4 , Where as globulin highly significant decreased in both G2&3 when compared to G1&4(fig.6). P<0.005

In case of female groups , G2 showed highly significant increase among total protein and albumin levels . But when added with vibrio challenge G3 it registered decrease in total protein, highly significant, and moderate significant in albumin level while globulin level registered slightly significant decrease (fig6) Lysozyme activity showed decrease in G3 in both male and female group than G2&G4 (fig7)

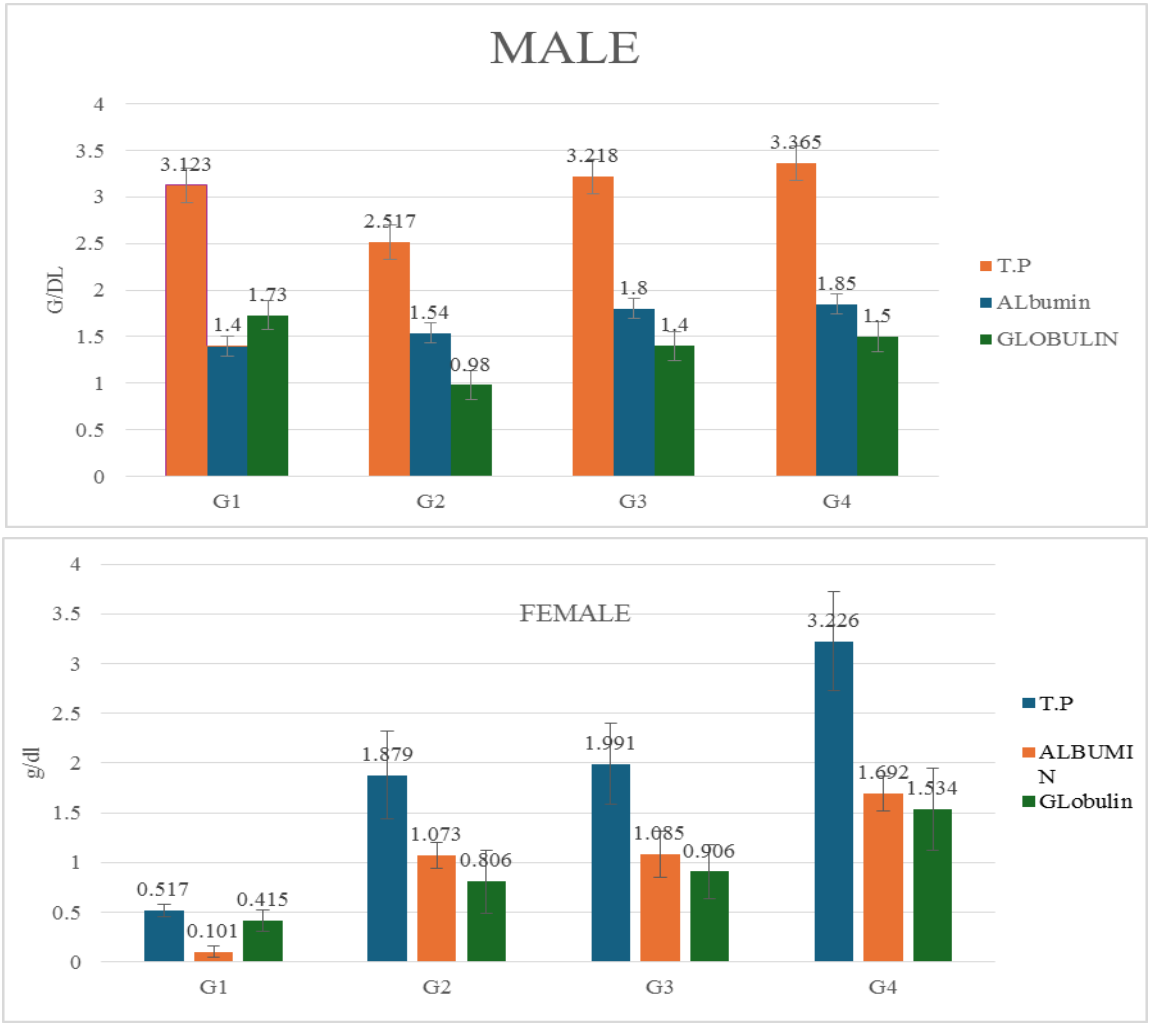


Fig 6. Total protein , albumin and globulin measures of male and female *O.niloticus* infected experimentally by *V. alginolyticus* of feeding supplemented diet with levamisole

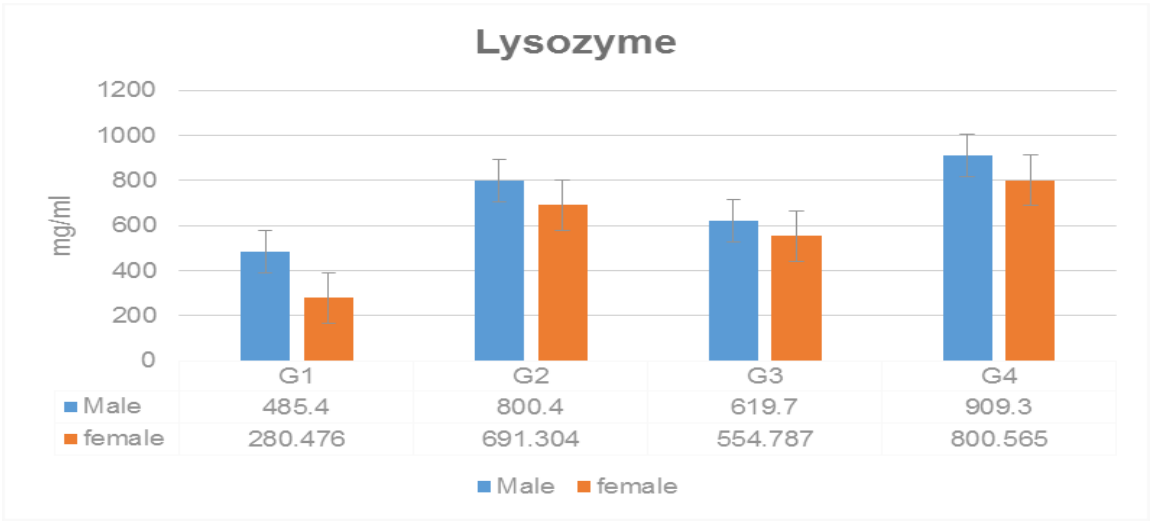


Fig 7. Lysozyme activity of male and female *O.niloticus* infected experimentally by *V. alginolyticus* of feeding supplemented diet with levamisole

Specific immune response IL10

The effect of levamisole trial on relative IL 10 gene expression related to the immune system was detected from the anterior kidney of all groups of experimental groups , Relative IL 10 gene expression was significant increase in G2(fed on levamisole) was 11.1579, G3 was 0.9659 and G4 was 0.2045 .

Pathological findings

Histopathological analysis of the ovaries and testes showed normal structures in group 2 fed on levamisole, while G3 challenged with *V.alginolyticus* revealed slight changes in both tests and ovaries. G4 showed scattered hemorrhagic patches, with many ova exhibiting signs of emptiness rather than containing vitellogenin. stage 1 and 2 recorded. Testes revealed sever vacuolation with decrease number of spermatozoa in G4 (Fig8 & 9).

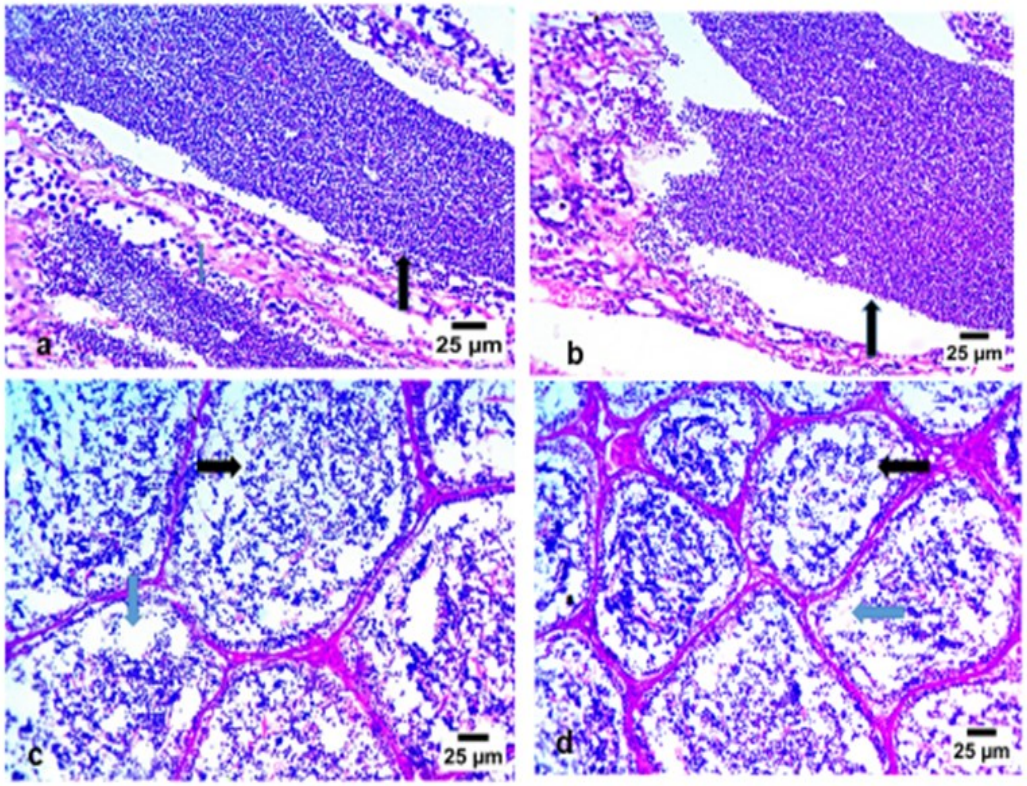
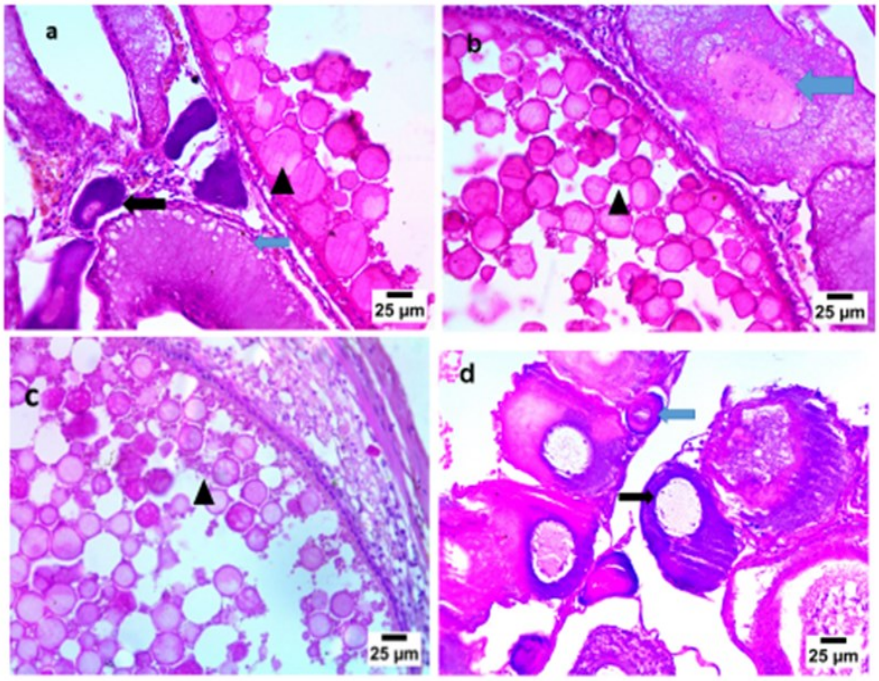


Fig 8. Pathological changes in testes of male in experimentally *O.niloticus*.
a: G1(negative control) showing normal histological structure of seminiferous tubules and spermatozoa,(H&E stain) (X400).
b:. G2(fed on levamisole) normal histological structure of seminiferous tubules and increasing spermatozoa. (H&E stain)(X400).
c: G3(fed on levamisole then challenged with *V.alginolyticus*) vacuolation (blue arrow) with moderate number of spermatozoa (black arrow) . (H&E stain) (X400).
d: G4(positive control) showing severe vacuolation (blue arrow) with decreased number of spermatozoa (black arrow). (H&E)(X400).



Fig(9) Pathological changes in experimentally *O. niloticus* females ovaries
-a:,G1: primary Oocytes at stage 5 (arrow head), few Oocytes at stage1 (black arrow) and stage2(blue arrow) (H&E stain)(X400).
-b: G2: showing primary Oocytes at stage 5(arrow head), few Oocytes at stage2 (blue arrow)(H&E stain) (X400).
–c: G3: oocytes at stage 5 (arrow head) (H&E stain)(X400).
-d: G4:most primary Oocytes a stage 2 (arrow) and few oocytes at stage 1 (blue arrow)(H&E stain)(X400).

DISCUSSION

The emergence of infections, particularly those brought on by *Vibrio* spp., is a major setback for aquaculture and is thought to have serious harmful implications for economic growth and population health.

The prevalence of *V. alginolyticus* in this study was (9.30%) among naturally infected both sexes, 8.47 % in male & 11.11 % in female tilapia fish. Similar results obtained by **Moustafa et al. (2010)** isolated *V. alginolyticus* from Suez Gulf and Qarun Lake with a percentage of (16.73%) of the cases .On the other hand, lower results were recorded by **Jaksic et al. (2002); Saad et al. (2015)** isolated *V. alginolyticus* from 3.42% ; 2% & 4% of farm water fish positive samples

Higher results reported of *V. alginolyticus* prevalence among Gilthead seabream and European seabass were (82.19%) and (87.28%) respectively from the Egyptian coastal provinces (**Abdel-Aziz et al. (2013)**. *V. alginolyticus* (32.3%)were isolated from moribund *O. niloticus and C. gariepinus* from fish farm (**Abdelsalam, et al. 2021)**

The study isolate (OR098883.1. *V. alginolyticus* /JM-1 / Tilapia) was clustered with KC954193.1 A0456/ *Penaeus vannamei* , KC954190.1 ZDS-6/Crab and KC954194.1 LZXC3/Seawater The study is late was 100% identity with KC954193.1 A0456/ *Penaeus vannamei* isolate.

An effector molecule known as a virulence factor increases a microorganism's ability to infect humans. The *collagenase* gene has been used as a particular marker to distinguish *V. alginolyticus* **Cai et al. (2006)**. Every strains in our study were tested amplify a 737-pb size fragment showing the personality profile of *V. alginolyticus* **Di-Pinto et al. (2005)** and some

virulence genes as (*tlh* & *tdh*) that gave specific amplicon at 449& 373 bp, respectively, from all 8 *Vibrio alginolyticus* isolates (5males and 3females) revealed that all isolates produced *collagenase*& *tlh* genes (100%), 2 isolates (male and female) produced *tdh* gene (25%) table (4)&Fig.4).Similar results reported by **Avsever (2022)** isolated *Vibrio alginolyticus* from 17 sampling of black mussel six isolates (35.29 %) for *tdh* gene. Different results were observed by **Yen et al. (2021)** found in the 16 *V. alginolyticus* strains *tlh* was found in 11 strains (68.75%) and *tdh* was found in 1 strain (6.25%)

Regarding the results of antimicrobial susceptibility test, *V. alginolyticus* isolates were sensitive to Gentamicin & Doxycycline (100%) then Streptomycin, Tylosine & Enrofloxacin (87.5%) for each, while the isolates were resisted to Ampicillin (100%) then Trimethoprim/ Sulphamethoxazole and Cefotaxime (25%) **.** Our findings were in accordance with the findings of **Lajnef et al. (2012)** recorded that the tested strains were resistant to ampicillin (94.2 %) and **Ferrini et al. (2008)** examined 46 *V. alginolyticus* strains for resistance to ampicillin, and it was discovered that 93% of these strains were resistant. The strains were recovered from imported seafood and aquaculture facilities in Italy. In agreement with earlier reports, Vibrio spp., including *V. alginolyticus* and *V. parahaemolyticus*, exhibited high resistance rates to ampicillin (82–85%), confirming it as the most common resistance among these isolates **Vaseeharan et al. (2005).** Our results differ from those reported by **Snoussi et al. (2008)** found high rates of resistance to gentamicin (84 %) and **Lajnef et al. (2012)** reported that the isolates were resistant to gentamicin (76.8 %) Because of the widespread use of antibiotics, antibiotic-resistant bacteria are more prevalent in aquaculture systems. Additionally, the current increase in multi-antibiotic resistant bacteria are concerned, and the presence of resistance genes in bacteria has further contributed to the spread of drug resistance among microbial pathogens **Zulkifli et al. (2009).**

In this study, experimental groups infected with *V. alginolyticus* revealed internal organ congestion, scale detachment, hemorrhagic ulcers, tail and fin rust, and haemorrhages throughout the fish's body. These results are in line with earlier findings. (**Abdel-Aziz et al. 2013 ; Younes et al. 2016;El-Gohary et al. 2020).** Fish with rotten fins, irregular swimming, excessive mucus production, sluggishness, congested livers and kidneys, and enlarged spleens **Mohamad et al. (2019).**

The moralities increased in challenged group (G4) was 65%in the male and 75% in female groups. **El-Gohary et al. (2020)** reported 80% mortality within experimental trail infected group by *V. alginolyticus* in tilapia. While treated groups (G2&3) with levamisole revealed decrease in moralities compared to G4 .

In functioning fish blood physiology, Kulkarni 2021 stated that albumin being the major anionic protein in vertebrates' plasma forms from 10 – 50 % of total protein. Albumin can bind wide range of drugs reversibly. In this research albumin level ranged from 1 – 1.85 g / dl which agreed with **Kulkarni (2021)** Albumin plays a role in metabolites (as hormones or bilirubin or organic substances) transportation as well as regulating blood osmotic pressure and osmoregulation processes. **Nurfaidah et al. (2021)** had proved that albumin is influenced by fish species, sex and maturity. This would explain the increase in females' albumin than that of males in this research.

Vibrio is a hemorrhagic microbe causing destructive effects on liver cells. **Yu et al. (2021)** defined Lysozymes as antimicrobial peptides widely present in teleosts, acting as antibacterial defence, thus being highly significant in females challenged with *V. alginolyticus* (G4).

The research found that the highly significant increase in total protein and Lysozymes in groups with presence of levamisole (among both sexes) a logic explanation for its' immunomodulator function referred to **Elias & Abdellatief (2009).**

Dietary levamisole helps and regulate the inflammatory process, leading to good protec-

tion and less tissue damage during infection or stress. In this work elevates IL-10. In fed group G2 on levamisole. while reduced it in infected groups .That contrast with **Song et al. (2014**) reported elevation of anti-inflammatory IL10 cytokine in group fed on immunostimulent dietary *S. platensis* to Ctenopharyngodon idella which infected with *A.hydrophila.That incotrast with* **Awad et al. (2022)** IL10 reduced in tilapia fed *S. platensis as immunosuppression than control group .* This difference likely resulted from the timing of sample collection, which was performed right after clinical signs developed**.**

Pathological findings of ovaries in G4 appeared sever congested with enlarged size , oocyst within primary stages , While in treated groups (2&3)with levamisole revealed repaired mature oocystes. Male groups showed sever vacuolation with decrease number of spermatozoa (G4). regarded to G2) normal appeared while G3 moderate effect of levamisole against challenged with *V. alginolyticus*, **Elias & Abdellatief (2009)** reported that, treatment with levamisole repair fish fecundity as all ova were well matured filled with vitellogenin. Histopathological analysis of the ovaries of injected *O, niloticus* with *Y. ruckeri* ((ECP) revealed scattered hemorrhagic patches among the ova, with many ova exhibiting signs of emptiness rather than containing vitellogenin. Furthermore, extensive hemorrhages were noted across the entire contour of the ova, accompanied by ruptures in the ovarian theca and the subsequent oozing of vitellogenin.

CONCLUSION

This study revealed that *V. alginolyticus* pathogenic strains were isolated from fish indicated a public health problem. Doxycycline and Gentamicin could be used to treat fish infected with *V.alginolyticus*. Additionally, the molecular screening of virulence factors demonstrated that *tlh* & *collagenase* represented the dominant virulence genes in the examined isolates. Researchers recommend levamisole addition to fish ration improved health status of both *O, niloticus* gender due to antibacterial effect and positive impact on the immune system.

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