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A study on the biological and immunological characteristics of Listonella anguillarum and Vibrio vulnificus extracellular products

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Two fish pathogens, namely *Listonella anguillarum* (*Vibrio anguillarum*) and *Vibrio vulnificus*, which were beta-haemolytic, hemagglutinating with 1% D-mannose and hydroxamate siderophore producer were used in this study. The extracellular products (ECPs) of both species were of high enzymatic and haemolytic activities. SDS-PAGE analysis of ECPs of *L. anguillarum* and *V. vulnificus* revealed 4 band (20.7 to 47.5kDa) and 2 bands (30.1 to 46kDa) respectively. The cumulative mortalities in *O. niloticus* produced by crude ECPs of *L. anguillarum* (0.2ml/fish; 2.4mg protein /ml) and *V. vulnificus* (0.2ml/fish; 1.6mg protein /ml) were 90% and 80%. Heat treatment of ECPs of *L. anguillarum* and *V. vulnificus* at 56°C for 30 min reduced the mortalities to 30% and 20%, respectively. LD_{50} values in *O. niloticus* using crude and heat treated ECPs of *L. anguillarum* were 1.7 and 3.9 µg protein/g fish; while those of *V. vulnificus* were 1.34 and 2.8 µg protein /g fish, respectively. Injection of levamisole at a dose of 5mg/Kg fish, concurrently with ECPs of *L. anguillarum* and *V. vulnificus* resulted in subsidence of the mortality percentages from 90 to 40 and from 80 to 30, respectively. Ten laying hens were immunized with ECPs of *L. anguillarum* for preparation of specific egg immunoglobulins. The total protein content of IgY preparations collected from eggs of hens immunized with ECPs of *L. anguillarum* reached its peak 2 weeks post 3rd booster dose (3.820 ± 0.0700 g/dl). The molecular weight of separated protein bands of IgY preparations ranged from 183-191kDa. Fish injected (I/P) with specific anti ECPs IgY of *L. anguillarum* (4 mg/ fish) followed by I/P injection of ECPs of *L. anguillarum* (0.2 ml/fish; 2.4 mg protein /ml) showed a 10% mortality rate, while those challenged after receiving specific anti ECPs (100 mg/ kg B.W. orally) showed no mortalities.

Control of fish diseases is of a great concern in aquaculture because of the high risk of disease transmission, fish mortalities and economic losses (Dunn *et al*., 1990). The Nile tilapia, *Oreochromis niloticus* (*O. niloticus*) is one of the most important fresh warm-water fish in the world. Almost no serious diseases seem to affect tilapia species but under stress conditions they can easily be infected by several pathogens belonging to genera Streptococcus, Aeromonas, Yersenia, Vibrio and Edwardsiella (Fouz *et al.,* 2002*)*.Vibriosis is one of the most prevalent enzootic diseases of fish allover the world that occurs among various fish species including marine, brackish and occasionally freshwater fishes (Abd-El-Gaber *et al*., 1997). Outbreaks reported in several countries caused by bacteria belonging to the genus Vibrio are characterized by lethargy, dark skin, ascites and sometimes damaged eyes (Cai *et al*., 2006). Out of Vibrio species, *Vibrio anguillarum* is the most predominantly isolated pathogen from the fish showing vibriosis signs (Demürcan and Candan, 2006). Vibrionaceae exhibit a remarkable capacity to produce extracellular products (ECPs), which display haemolytic, proteolytic, dermatotoxic and cytotoxic activities (Santos *et al*., 1992; Amaro *et al*., 1995). These activities are mainly related to their ECPs. The ECPs are strongly lethal when injected into fish causing haemorrhagic enteritis, skin ulcer, neuroexcitatory signs, triggering, wriggling, contortive swimming and respiratory arrest coupled with increased mucous production (Farto *et al*., 2002). The in-vivo and in-vitro biological activities of ECPs are considerably reduced after heat treatment, but not totally lost, particularly in the highly virulent Vibrio strains (Fouz *et al*., 1993). Levamisole is an effective immune-

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modulator capable of increasing specific immunity and reducing mortality in immunocompromised fish (Sahoo and Mukherjee, 2002). Passive immunization using specific antibodies has been studied extensively in animals, and is presenting an attractive approach to establish passive immunity against pathogens in both humans and animals (Carlander *et al*., 2000). The use of chickens for the production of polyclonal antibodies provides several advantages over the traditional production of antibodies in mammals. A laying hen can produce more than 40 g/year of yolk antibodies (IgY). Therefore, hens provide a hygienic, cost-efficient, convenient, humane and plentiful source of antibody production (Kovacs-Nolan and Mine, 2004). Chicken IgY have been applied successfully for scientific (Schade *et al*., 1997), diagnostic (Lonardo *et al*., 2001), prophylactic (Almeida *et al*., 1998) and therapeutic purposes (Lemamy *et al*., 1999). This investigation was conducted to evaluate the in-vivo biological impact of the ECPs of *L. anguillarum* and *V. vulnificus* in both crude and heat treated forms on *O. niloticus*. The immunopotentiating activity of levamisole in *O. niloticus* injected with crude ECPs of both *L. anguillarum* and *V. vulnificus* were studied. The prophylactic effect of yolk anti-*L. anguillarum* ECPs was also investigated.

Material and Methods

Bacterial strains and their ECPs. *L. anguillarum* and *V. vulnificus* strains (isolated and identified using API 20 E system, Bio Mereux) were used in the current study. These strains were of positive haemolytic reactions, haemagglutinating with 1% mannose and siderophore producers.

Determination of SDS- PAGE of ECPs of *L. anguillarum* and *V. vulnificus.* ECPs were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using silver staining technique as described by Laemmli (1970). Briefly, samples were boiled in the presence of SDS and 2-mercaptoethanol then 15µl of each sample with an equal volume of 2X sample buffer were left for 5 minutes to insure protein denaturation. 30 µl of each sample was applied to each well and 7.5 µl of the standard marker (broad range, Bio-Lab Company pre-stained 175-16.5) was loaded in the first well as a standard. The power supply was connected to the cell and run at 100 V. The run was terminated when the bromophenol blue tracking dye reached the bottom of the separating gel.

Extraction of ECPs (a modification of the method described by Lee *et al*., 1999).

Strains were streaked onto trypticase soy agar (+1.5% NaCl) and incubated at 37°C for 20 hours. The growth was harvested separately and inoculated into 100 ml PBS then kept in shaker incubator (60 r.p.m. at 37°C for 48 hours). The cultures were centrifuged at 16000 r.p.m. at 4°C for 30 minutes. The supernatants were filtrated via sterile-Seitz filters and concentrated by ultra-filtration to obtain the ECPs. One portion of the obtained ECPs was stored directly at -20°C till used. While the other one was subjected to heat treatment at 56°C for 30 minutes**.** The ECPs of the selected strains were of high enzymatic activities for caseinase, gelatinase, amylase, phospholipase, and lipase.

Determination of total protein content of ECPs. The protein content of ECPs of both *L. anguillarum* and *V. vulnificus* was measured using the modified method of Lowry, *et al*., (1951). Briefly 50 ml of 2% sodium carbonate in 0.1 N sodium hydroxide (1) plus 1 ml of 0.5% hydrous copper sulphate in 1% sodium potassium tartarate (2) were mixed together. The ECPs of *L. anguillarum* and *V. vulnificus* were diluted separately in 0.01 M PBS, pH7.4 to a final volume of 200 ml. One ml from the mixture (1+2) was added to the sample and the different dilutions of slandered (PBS) were allowed to stand for 20 minutes at room temperature. A 0.1 ml of folin reagent was added to each tube and mixed rapidly, then allowed to stand for 20 minutes at room temperature. The standard and the samples were read against blank in the Slorimeter at 500 nm. A standard curve was plotted using the standard protein solution. The unknown concentration of the protein was calculated from the slope of the curve.

Experimental animals.

Fish. A total of 120 apparently healthy *O. niloticus* with an average body weight of 60±10g, collected from a private farm at Beni-Suef Governorate were used in this study. Fish were kept under observation for 10 days before start of the experiment in separate aquaria (75 x 45 x 30cm) containing chlorine free tap water supplemented with air stones.

Chickens. A total of ten 23 weeks-old local breed laying hens (El-Azab project, El Fayoum Governorate) were used for production of yolk anti-ECPs specific immunoglobulin.

Table (1): Inoculation scheme of crude and heat treated ECPs of *L. anguillarum* and *V. vulnificus* in *O. niloticus*.

Fish group	No of fish	Treatment (IP route)
Crude ECPs (30 fish)	10 fish	0.2ml/fish crude ECPs of *L. anguillarum* (2.4mg protein /ml)
	10 fish	0.2ml/fish crude ECPs of *V. vulnificus* (1.6mg protein /ml)
	10 fish	0.2ml/fish physiological saline (control)
Heat treated ECPs (30 fish)	10 fish	0.2ml/fish heat treated ECPs of *L. anguillarum* (2.4mg protein /ml)
	10 fish	0.2ml/fish heat treated ECPs of *V. vulnificus* (1.6mg protein /ml)
	10 fish	0.2ml/fish sterile physiological saline (control)

Table (2): Injection of levamisole and crude ECPs of *L. anguillarum* and *V. vulnificus* in *O. niloticus*.

Groups	No of fish	Treatment
1	10	levamisole 5 mg /Kg fish (I/M) and 0.2 ml /fish of *L. anguillarum* ECPs (I/P).
2	10	levamisole 5mg/Kg fish (I/M) and 0.2 ml/fish of *V. vulnificus* ECPs (I/P)
3	10	levamisole 5 mg /Kg fish (I/M) and 0.2 ml/fish of sterile physiological saline as a control (I/P).

Experiment (I): Determination of the lethal effect of crude and heat treated ECPs of the selected strains on *O. niloticus.* Sixty fish were used for determination of lethal effect of crude and heat treated ECPs of *L. anguillarum* and *V. vulnificus* (Santos *et al*., 1991 & 1992). The inoculation scheme including routes and doses are illustrated in Table (1).

Experiment (II): Determination of median lethal dose (LD_{50}) of crude and heat treated ECPs of *L. anguillarum* and *V. vulnivicus*.

Two hundred and forty apparently healthy *O. niloticus* fish (60±10g weight) were divided into 4 equal groups and used for determination of LD_{50} of both crude and heat treated ECPs of *L. anguillarum* and *V. vulnivicus*. Five 2-fold serial dilutions of ECPs of both *L. anguillarum* and *V. vulnificus* were prepared starting with 480 and 320 µg protein/ml, respectively. Fish in each group were injected (I/P) with 0.2 ml of different ECPs concentrations; 480, 240, 120, 60, 30, 0.0 µg protein/ fish for *L. anguillarum* and 320, 160, 80, 40, 20, 0.0 µg protein/ fish for *V. vulnificus*. The other two groups of fish were inoculated (I/P) with heat treated ECPs at the same concentrations (Zhong *et al*., 2006).

Fish were kept under observation and mortalities among all groups were recorded and LD_{50} was calculated according to Reed and Muench (1938).

Experiment (III): Determination of immunopotentiating effect of levamisole (Siwicki, 1989):

Thirty fish were divided into 3 equal groups (each of 10) for determination of expected immunopotentiating effect of levamisole when simultaneously injected with crude ECPs of *L. anguillarum* and *V. vulnificus* (Table 2).

Experiment (IV): Preparation of yolk immunoglobulin (IgY) against *L. anguillarum* ECPs. Ten laying hens were immunized (I/M) at four different sites of the pectoral muscle (0.25ml per site) with 0.5ml of ECPs of *L. anguilarum* emulsified with an equal volume of Freund's complete adjuvant (FCA). Two weeks later, three booster doses of ECPs of *L. anguilarum* mixed with Freund's incomplete adjuvant (FIA) were given at two-weeks intervals. Eggs of laying hens were collected before immunization and at two-weeks intervals after immunization up to the 8th weeks (Almeida *et al*., 1998). The collected eggs were kept at 4ºC until used.

Extraction and purification of IgY–antibodies from immunized hens' egg yolk (chloroform-PEG 6000 procedure) were performed according to Polson (1990). The yolk was collected in a sterile 50 ml screw-capped tube. The yolk volume was brought to 25 ml with sodium phosphate buffer (PBS:100 mM, pH 7.6) and

vigorously shaken. 20 ml of chloroform was added and the mixture was shaken. The obtained emulsion was distributed into 10 ml tubes and centrifuged at 1200 *xg* for 30 min at room temperature. Supernatants were pooled and mixed gradually with solid PEG 6000 to a final concentration of 12% (w/v). Further centrifugation was performed at 15700 *xg* for 10 min at 4ºC. The resulting immunoglobulin pellets were resuspended in 2ml of PBS for each egg, divided into small aliquots and stored at –80ºC.

Determination of total protein content of chicken IgY preprations. The total protein content of chicken IgY was estimated by Buiret method (Hoffmann and Richterich, 1970).

Determination of ELISA yolk immuno-globulin titers. The produced IgY antibody titers against ECPs of *L. anguillarum* were determined using ELISA according to Chart (1994). ELISA plates were coated with 100µl/well of *L. anguillrum* ECPs antigen (80 µg protein/ml) in carbonate /bicarbonate coating buffer (pH 9.6). The plates were incubated overnight at 4ºC. Plates were washed three times with PBS 0.05% Tween-20 then blocked with the blocking buffer (200µl/well) and incubated at room temperature for 2 hours. The plates were then washed three times with PBS 0.05% Tween-20. 100µl/well of different dilutions of IgY preparations from experimentally infected chickens (1:100, 1:200, 1:400 and 1:800) and negative samples (yolk collected at zero time) were added separately and the plates were incubated for 2 hours at 37ºC with shaking. The plates were then washed five times with PBS 0.05% Tween-20. 100µl/well of anti-chicken IgG alkaline phosphatase conjugate (Sigma) diluted 1:3000 in PBS, was added and the plates were incubated at 37ºC for 1 hour with shaking followed by washing five times with PBS 0.05% Tween-20. 50µl/well of substrate solution was added to all wells and the plates were incubated at 37ºC for 30 min then 50 µl/ well of 1N NaOH were added for stopping the reaction. Optical densities (OD) were measured at 405 nm. The results were considered positive when the absorbency values were more than the cut off value.

Electrophortic analysis of anti-ECPs of *L. anaguillarum* IgY. Purified IgY was analyzed by SDS-PAGE using Coomassie stain according to Lammli, 1970

Experiment (V): Determination of prophylactic effect of IgY against *L. anguillarum* ECPs:

Thirty fish were divided into three equal groups for evaluation of the prophylactic effect of anti-*L. anguillarum* ECPs immunoglobulins of yolk prepared in hens. In the 1st group, fish were injected (I/P) with 4 mg/fish anti-*L. anguillarum*-ECPs IgY (Lee *et al*., 2000). Four hours later, each fish was challenged with 0.2 ml of crude ECPs of *L. anguillarum* (2.4 mg protein/ml) via I/P route. In the 2nd group, 100 mg/kg B.W/fish of anti-L. anguillarum-ECPs IgY were added to feed pellets. Feeding trials lasted 7 days after 3 days of starvation. I/P challenge of 0.2 ml of ECPs of *L. anguillarum* (2.4 mg protein/ml) was conducted on the 8th day after the last feeding. In the 3rd group, 10 fish were injected (I/P) with sterile physiological saline and kept as negative control. The cumulative mortality and clinical symptoms were recorded for each group till the end of the experiment.

Results

SDS- PAGE of ECPs of *L. anguillarum* and *V. vulnificus.* The ECPs of *L. anguillarum* revealed 4 bands with molecular weight ranging from 20.7 to 47.5kDa. On the other hand, ECPs of *V. vulnificus* showed 2 bands with molecular weight ranging from 30.1 to 46 kDa.

Lethal effect of crude ECPs of selected bacterial species. Fish injected (I/P) with 0.2 ml/fish of crude ECPs of *L. anguillarum* (2.4 mg protein /ml) started death 24 hours post inoculation. The cumulative mortality in the first group was 90% with characteristic findings of vibriosis. On the other hand, fish injected (I/P) with 0.2 ml/fish of crude ECPs of *V. vulnificus* (1.6 mg protein/ml) started death at the second day post inoculation with 80% cumulative mortality (Table 3).

Lethal effect of heat treated ECPs of selected bacterial species. Fish injected with 0.2ml/fish of heat treated ECPs of *L. anguillarum* (2.4 mg protein/ml) started death at the 3rdday post inoculation. The cumulative mortality was 30% and mild symptoms were observed. Fish injected with 0.2 ml/fish of heat treated ECPs of *V. vulnificus* (1.6 mg protein/ml) started death at the 4th day post inoculation with 20% mortality. No clinical signs or mortalities were observed in the control group (Table 3).

LD_{50} values in *O. niloticus* of crude and heat treated ECPs of *L. anguillarum* and *V. vulnificus*. LD_{50} values for crude and heat treated ECPs of *L. anguillarum* were 1.7 and 3.9 µg protein/g fish, while those of *V. vulnificus* were 1.34 and 2.8 µg protein/g fish, respectively.

Immunopotentiating effect of levamisole against crude ECPs of *L. anguillarum* and *V. vulnificus*.

Fish injected (I/M) with levamisole at a dose of 5mg/Kg B.W. followed by I/P inoculation of 0.2 ml of crude ECPs of *L. anguillarum* (2.4 mg protein/ml) started death 48 hours post inoculation. The cumulative mortality decreased to 40 %. On the other hand, fish injected (I/M) with levamisole (5mg/Kg B.W.) then challenged with 0.2 ml/fish of crude ECPs of *V. vulnificus* (1.6 mg protein/ml) started death at the 3rd day post inoculation with a 30% cumulative mortality. Both groups that received levamisole prior to inoculation of crude ECPs revealed less

Table (3): Lethal effect of crude and heat treated ECPs of *L. anguillarum* and *V. vulnificus* injected in *O. niloticus*.

Group of fish		No. of fish injected	No. of fish deaths after:							Total deaths	Survivors	Mortality %
			24 h.	48 h.	3 d.	4 d.	1 w.	10 d.	2 w.			
Crude ECPs	ECPs of *L. anguillarum*	10	1	2	2	2	1	1		9	1	90.0
	ECPs of *V. vulnificus*	10	-	2	1	2	2	-	1	8	2	80.0
	Control	10	-	-	-	-	-	-	-	-	10	0.0
Heat treated ECPs	ECPs of *L. anguillarum*	10	-	-	2	1	-	-	-	3	7	30.0
	ECPs of *V. vulnificus*	10	-	-	-	1	1	-	-	2	8	20.0
	Control	10	-	-	-	-	-	-	-	0	10	0.0

Table (4): Immunopotentiating effect of levamisole against crude ECPs of *L. anguillarum* and *V. vulnificus* in *O. niloticus.*

Group		No. of fish injected	No. of fish deaths after:							Total deaths	Survivors	Mortality %
			24 h.	48 h.	3 d.	4 d.	1 w.	10 d.	2 w.			
Levamisole +	ECPs of *L. anguillarum*	10	-	1	1	1	-	1	-	4	6	40.0
	ECPs of *V. vulnificus*	10	-	-	1	1	-	1	-	3	7	30.0
	sterile physiological Saline	10	-	-	-	-	-	-	--	0	10	0.0

Table (5): Prophylactic effect of anti-*L. anguillarum* ECPs IgY in challenged fish.

Group	No. of inoculated fish	No. of fish deaths after:							Total deaths	Survivors	Mortality %
		24 h	48 h	3 d.	4 d.	1 w.	2w.	2 w.			
IgY (I/P) + ECPs (I/P)	10	-	-	1	-	-	-	-	1	9	10.0
IgY (oral) + ECPs (I/P)	10	-	-	-	-	-	-	-	0	10	0.0
Control	10	-	-	-	-	-	-	-	0	10	0.0

symptoms and P/M lesions than those received crude ECPs without levamisole. Control fish showed no mortalities, clinical signs or postmortem lesions (Table 4).

Total protein content and IgY titers in yolk of immunized hens.

The mean values of the total protein content in the egg yolk of immunized chickens began to significantly increase two-weeks after the 2nd booster dose, reaching the peak two-weeks after the 3rd booster dose (3.82 g/dl). The antibody titer in IgY preparations was detectable 4 weeks post immunization (2 weeks after the 1st booster dose) with slight significant increase ($P< 0.05$) (0.511 ± 0.011) when compared with zero time (0.222 ± 0.022). It increased in a steady manner, reaching the peak two-weeks after the 3rd booster dose (0.827 ± 0.0275).

SDS-PAGE analysis of IgY preparations. The molecular weight and amount of separated protein bands ranged from 183-191kDa at different time intervals post immunization.

Prophylactic effect of anti-ECPs immuno-globulins of yolk in challenged fish.

Fish injected (I/P) with specific anti-*L. anguillarum* ECPs IgY at a dose of 4 mg/fish followed by 0.2 ml of ECPs of *L. anguillarum* (2.4 mg protein /ml) started death at the third day and the mortality was 10% while no mortalities were observed in those administrated IgY orally (100 mg/ kg B.W. fish) followed by 0.2 ml of ECPs of *L. anguillarum* (2.4 mg protein /ml) as shown in Table (5). No extensive lesions or clinical abnormalities were recorded in fish immunized orally or intraperitoneally. No mortalities, clinical signs or postmortem findings were recorded in the control group.

Discussion

Vibriosis caused by *V. anguillarum* is a devastating and major obstacle for marine, brackish and fresh water fish. Moreover, *V. vulnificus* is capable of causing outbreaks especially in freshwater fish (Plump, 1999). ECPs of Vibrio species are the main potential virulence factors. They are responsible for haemorrhagic, cytotoxic, neurotoxic and lethal effects that could lead to massive mortalities and severe pathological alterations in infected fish.

SDS-PAGE analysis of ECPs of *L. anguillarum* revealed 4 bands with molecular weight ranging from 20.7to 47.5kDa. These findings agree with Santos *et al*. (1995) revealed that the molecular masses of the protein components of *L. anguillarum* ranged from 14 to 60 kDa**.** On the other hand, ECPs of *V. vulnificus* showed 2 bands with molecular weight ranging from 30.1 to 46 kDa. These findings are in agreement with that reported by Amaro *et al*. (1992).

Generally, injection of ECPs of the selected pathogens was very toxic for fish causing severe pathological lesions and massive mortalities. Fish injected with crude ECPs of *L. anguillarum* (2.4 mg protein /ml) started death 24 hours post inoculation. Along the days of the experiment, the main external abnormalities were erratic movement, anorexia, corneal opacity with exophthalmia and necrotized muscles with haemorrhagic patches. Postmortem findings of dead fish were pale enlarged liver, severely congested spleen and kidneys, severely inflamed and congested intestinal tract that was devoid of food and filled with mucous. The cumulative mortality was 90% (Table 3). Nearly similar manifestations were described by Santos *et al*. (1992).

On the other hand, injection of crude ECPs of *V. vulnificus* (1.6 mg protein /ml) caused death in the second day post inoculation. The main clinical signs were dark colored fish with hemorrhagic patches allover fish body especially on the dorsal surface, at the base of fins and around the vent and, moreover, skin ulcers which mostly extended into musculature. Postmortem findings were severe haemorrhage and congestion in the intestine, liver, kidneys and spleen. The cumulative mortality was 80% (Table 3). These results coincide with those of Fouz *et al*. (2002) who noticed extensive haemorrhagic areas on the entire body surface of moribund *O. niloticus* especially on mouth, gills and bases of fins after I/P injection of ECPs of *V. vulnificus*.

The results shown in Table (3) clarified also that mortalities observed after injection of ECPs of *L. anguillarum* and *V. vulnificus* declined from 90 to 30 and from 80 to 20%, respectively following ECPs heat treatment. This observation is well in line with the report of Fouz *et al*. (1993) who concluded that the in-vivo biological activities of ECPs decrease considerably after heat treatment.

Results of studying the median lethal dose of *L. anguillarum* and *V. vulnificus* ECPs in both crude and heat treated forms reveled that LD_{50} value for crude ECPs of *L. anguillarum* was 1.7 µg protein /g fish. This result is lower than that obtained by Santos *et al*. (1992) who demonstrated that the LD_{50} value of *L. anguillarum* extracellular biological products ranged from 4.72 to 6.36 µg protein/g fish. Similarly, Mo *et al*. (2002) revealed that I/M

injection of ECPs of *L. anguillarum* was toxic to flounder fish at LD_{50} value of 3.1 µg protein/g body weight. The discrepancy between the LD_{50} value of *L. anguillarum* ECPs obtained in this investigation and those of other authors may be attributed to the high virulence of the selected strains that were haemolytic, haemagglutinating and siderophore producers.

The LD_{50} value of *V. vulnificus* crude ECPs was 1.34 µg protein /g fish. This result is in agreement with that of Biosca and Amaro (1996) who investigated the lethal activities of *V. vulnificus* crude ECPs administered (I/P) using different serial dilutions. They detected LD_{50} value starting from 1 µg protein/g fish.

On the other hand, the LD_{50} values of *L. anguillarum* and *V. vulnificus* ECPs elevated to 3.9 and 2.8 µg protein/g fish after heat treatment. These results coincide with those obtained by (Fouz *et al*., 1993; Esteve *et al*., 1995).

There was a marked reduction in mortalities of *O. niloticus* when levamisole was injected (I/M) at a dose of 5 mg /Kg B.W. in combination with I/P injection of ECPs of *L. anguillarum* or *V. Vulnificus*; from 90 to 40 and from 80 to 30%, respectively (Table 4). These data throw the light on the beneficial effects of levamisole as an immunomodulator via I/M route. Modulating immune response using levamisole has been shown to posses several effects in competing diseases in animals and fish. Prost *et al*. (1992) declared that the immunostimulant effects of levamisole might arise to the level of treatment against Saprolegnia infection in carp. Other studies emphasized the role played by levamisole in enhancing the non-specific immunity against several infectious agents (Baba *et al*., 1993; Sahoo and Mukherjee, 2002). However, almost all literatures were carried out to study the immunomodulator effects of levamisole as a fish food additive, little ones used it via I/M injection. In the present study, there were positive signals for the I/M injection of levamisole at the same time of injection of tested ECPs.

IgY can be easily purified from the yolk by a simple and rapid precipitation technique (chloroform polyethylene glycol) with more than 90% homogenicity in the purified IgY (Polson, 1990, Shafiq *et al*., 1997; Ashgan-Yousef, 2001). The mean values of the total protein content in the egg yolk of immunized chickens began to significantly increase two-weeks after the 2nd booster dose, reaching the peak two-weeks after the 3rd booster dose (3.82 g/dl). The antibody titer in IgY preparations was detectable four-weeks post immunization reaching the peak two-weeks after the 3rd booster dose (0.827± 0.0275). These results are in accordance with those recorded by (Kariyawasam *et al*., 2004).

SDS-PAGE analysis of IgY preparation showed clear sharp protein bands ranged from 183-191 kDa. These findings are supported by Reschova *et al*., 2000 and Wang *et al*., 2007.

To evaluate the prophylactic effect of anti-ECPs IgY in fish, 4mg of anti-*L. anguillarum* ECPs IgY/fish was injected (I/P) in *O. niloticus*. These immunoglobulins transferred rapidly into the circulatory system in high levels enough to confer protection against vibriosis. A notable result is that the mortality in challenged fish reduced from 90% after I/P injection of crude ECPs of *L. anguillarum* alone to 10% when injected in combination with IgY preparation. These findings are consistent with the report of Aminirissehei (2001) who passively immunized coho salmon and rainbow trout intraperitoneally using *L. anguiIIarum* IgY prepared in domestic chickens. He concluded that the injected IgY provided a significant protection against *L. anguillarum*.

Yolk antibodies do not activate the mammalian complement system or interact with mammalian FC receptors that could mediate inflammatory response in the gastrointestinal tract (Carlander *et al*., 2000). No systemic effects would be expected following the oral administration of IgY. Moreover, IgY antibodies are not affected by gastric acidity or gastric enzymes; therefore, oral administration of IgY has proved to be a successful treatment of a variety of gastrointestinal infections, such as *Yersinia ruckeri*, enterotoxigenic *Escherichia coli*, Salmonella species, *Edwardsiella tarda*, *L. anguillarum*, Staphylococcus, and Pseudomonas species.

Regarding the oral administration of *L. anguillarum* anti-ECPs IgY, fish that received specific IgY (100 mg/kg B.W.), followed by I/P injection of 0.2 ml of *L. anguillarum* ECPs (2.4 mg protein/ml) showed no mortalities. The present data correlates with previous reports indicating that feeding of fish with anti-Vibrio IgY resulted in different protection levels against vibriosis which were occasionally comparable to the protection afforded by I/P injection (Mine and Kovacs-Nolan, 2002; Arasteh *et al*., 2004).

Finally it could be concluded that treatment with IgY provides a safe, efficient and economic method for managing diseases in fish. This work

also provides a basis for deeper understanding of ECPs lethality and their biological characterization in vivo.

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دراسة عن الخواص البيولوجية والمناعية للنواتج الخارجية لميكروبي الليستونيللا أنجويليرم والفبريو فلنيفيكس

تم اختيار اثنين من الميكروبات الممرضة للأسماك وهما ميكروبى الليستونيللا أنجويليرم (الفبريو أنجويليرم) وميكروب الفبريو فالنيفكس ، لها قدرة على تحليل وأيضا تلذن خلايا الدم الحمراء (فى وجود ١% سكر المانوز) وقدرة على انتاج بروتين السيديروفور (البروتين المرتبط بالحديد) كما أن النواتج الخارجية لكل من الميكروبين ذات نشاط انزيمى واضح ولها قدرة على تحليل خلايا الدم الحمراء. أظهرت نتائج الفصل الكهربائى لبروتينات ميكروبى الليستونيللا أنجويليرم والفبريو فالنيفكس أوزان جزيئية تراوحت بين ٢٠,٧ إلى ٤٧,٥ و ٣٠,١ إلى ٤٦ ك دالتون على التوالى . عند حقن النواتج الخارجية الخام لاالليستونيللا انجويليرم والفبريو فالنيفكس وجد ان نسبة النفوق فى أسماك البلطى وصلت الى ٩٠% و ٨٠% على التوالى بينما انخفضت هذه النسبة لتكون ٣٠ ، ٢٠ % بعد المعالجة الحرارية لهذه النواتج عند درجة ٥٥٦ لمدة ٣٠ دقيقة. لتحديد الجرعة المميتة لـ٥٠% من الاسماك المحقونة بالنواتج الخارجية وجد انها ١,٧ و ١,٤٣ ميكرو جرام بروتين/جرام من السمكة لميكروبي الفبريو انجويليرم والفبريو فالنيفكس على التوالى وعند المعاملة حراريا للنواتج الخارجة وجد ان الجرعة المميتة قد زادت لتصل الى ٣,٩ و ٢,٨ ميكروجرام بروتين/جرام من السمكة للميكروبين على التوالى. الفصل الكهربائى للاجسام المناعيةالمتخصصة IgY المحضرة من النواتج الخارجية لميكروب الليستونيلا انجويليرم أظهر أوزان جزيئية تراوحت بين ١٨٣ إلى ١٩١ ك دالتون. عند تقييم التاثير الوقائى لاجسام المناعيةالمتخصصة IgY المحضرة من النواتج الخارجية لميكروب اللستونيلا انجويليرم تم تقسيم أسماك البلطى النيلى قسمين: الاول تم حقنها بريتونيا بـ ٤ ملى جرام IgY/سمكة حيث تضائلت نسبة النفوق لتصل الى ١٠% بينما القسم الثانى والتى تم تجريعها عن طريق الفم بالاجسام المناعية المتخصصة IgY بجرعة ١٠٠ ملى جرام/جرام حى ، وصلت نسبة النفوق الى صفر % .