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Zoonotic Bacterial Infection Induced Immune Responses in Fish and Rat: Biochemical and Molecular Studies.

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ABSTRACT

The present study investigates the expression of A2M gene in the *Oreochromis niloticus* and albino rats as immune response to their infection with Zoonotic bacteria (*Clostridium perfringens*). The infection was established in fish with IP injection of 0.3ml of 3X108 CFU /ml of *C. perfringens* which cause Food-borne disease, and in rat *rattus norvegicus* by feeding on insufficient cooked infected *O. niloticus*, Biochemical and molecular studies were established after three and six days post challenge. Liver functions (ALT, AST and ALP) were significantly (P≤0.05) highly elevated. Kidney functions (Urea, uric acid and Calcium) were not affected but the Creatinine showed significant increase in its level (P≤0.05). CRP also was highly elevated in rats and slightly elevated in *O. niloticus*. The cellulose acetate gel electrophoresis of serum protein showed significant increase in the total protein and its fractions for both *O. niloticus* and rats in both intervals of time. Expression pattern of A2M gene was investigated using a semi-quantitative RT-PCR in liver, spleen and kidney tissues, which showed high expression after the bacterial infection indicating that A2M is immune responder against *C. perfringens* infection.

Keywords: Alpha 2-Macroglobulin, Food-borne disease, Oreochromis niloticus, *Clostridium perfringens*, Rat.

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INTRODUCTION

Fish food has a nutritional profile superior to all terrestrial meat, being an excellent source of high quality animal protein and highly digestible energy, as well as an extremely rich source of omega-3 polyunsaturated fatty acid, fat-soluble vitamins (A, D and E) and water-soluble vitamins (B complex) and minerals (calcium, phosphorus, iron, iodine and selenium). Aquacultures are reducing the dependence on wild fish, help save depleted stocks and address our growing demand for fish, they creating thousands of jobs, with a reasonable cost, improve food safety and security, and address nation's trade deficit.

Tilapia is one of the world's most important warm-water cultured, it is native to Africa and Middle East, has emerged from mere obscurity to one of the most productive and internationally traded food fish in the world. The farming of tilapias in its crudest form is believed to have originated more than 4000 years ago from Egypt (Modadugu and Belen, 2004).

The strength of aquaculture lies in its disease–free status but unfortunately, infectious diseases have been a major impediment to the development and profitability of fish farms (Schreck, 1996). Stress in fish caused by physical disturbances encountered in aquaculture, such as handling and transport, evokes a variety of responses that may be adaptive or maladaptive. The overall effect of stress may be considered as a change in biological condition beyond the normal resting state that challenges homeostasis and, thus, presents a threat to the fish's health (Bruce and George, 1991). Pathogens can themselves induce transient or even chronic stress in fish predisposing the host to other opportunistic disease agents.

However, as in every form of intensive culture where single or multiple species are reared at high densities, infectious disease agents are easily transmitted between individuals, which makes aquaculture an important vector in the introduction, transfer and spread of aquatic pathogens and parasites. Traditionally, this problem has been addressed by the use of antibiotics to control bacterial diseases but in recent times the introduction of vaccines has greatly reduced the amount of antibiotics used. Nevertheless, the limited knowledge on immune systems in fish limits the development of new vaccines based on non-empirical strategies (Sommerset *et al.*, 2005).

Zoonotic disease is any infectious disease that can be transmitted from animals, both wild and domestic, to humans (Field *et al.,* 2001). Zoonoses involving transmission of disease and biotoxin producing agents from fish to humans. (I.e. bacteria, biotoxins and parasites). There are also many other infectious organisms of fish origin that have not been reported but have the potential to infect and harm man. The status of human host immune system plays a vital role in the severity of the disease. Human infections caused by pathogens transmitted from fish or aquatic environment are quite common and depend on the season, patients contact with fish and related environment, dietary habits and the immune system status of the exposed individual. They are often bacterial species facultatively pathogenic for both fish and human beings and may be isolated from fish without apparent symptoms of the disease. The infection source may be fish kept for both food and as a hobby (Acha and Szyfres, 2003). Some

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Bacterial pathogens transmissible to human beings through contact with fish living in the wild and fish in aquacultures. such as (*Mycobacterium spp*., *Streptococcus iniae*, *Photobactrium damselae*, *Vibrio alginolyticus*, *Vibrio vulnificus* and *Erysipelothrix rhusiopathiae*) and others Food-borne pathogens associated with fish and fish products such as *Vibrio parahaemolyticus* , *Vibrio cholerae, Echerichia coli, Aeromonas spp, Salmonella spp, Listeria monocytogenes, Clostridium botulinum, Clostridium perfringens, Campylobacter jejum and Staphylococcus aureus),* and other significant bacterial species (*Deftia acidovorans , Edwardsiella tarda, Legionella pneumophila, Plesiomonas shigelloiddes and Shigelloses spp.)* (Acha and Szyfres, 2003).

Most outbreaks of food poisoning associated with fish drive from the consumption of raw or insufficiently heat treated fish, which may be contaminated with bacteria from water environment (*Vibrio spp., C. botulinum*) or terrestrial source (*C. perfringens*, *Salmonella sp., Shigella spp., Staphylococcus spp., V. cholerae*), or fish products recontaminated after heat processing.

Alpha-2-Macroglobulin gene it is one of the immune genes, a non-specific protease inhibitor is present in body fluids of all metazoans ranging from coelenterate (corals) to vertebrata (humans), where it plays a major role in the inhibition and removal of potentially harmful proteinases and the delivery or clearance of cytokines and other hormones (Vaughan and Vale 1993). It is produced by the liver, and is a major component of the alpha-2 band in protein electrophoresis.

Because *C. perfringens* represents an ideal model for both pathogenic as well as saprophytic bacteria which directly / indirectly impact vulnerable fishes through potent toxin production, this work was planned to study some biochemical parameters and alpha-2-Macroglubulin gene expression as immune system response which may be caused by *C. perfringens* bacteria in fish and in rat fed on infected fish.

MATERIALS AND METHODS

Experimental animals:

Rat sampling

Total of 40 male albino Rats with 85 – 95 gm body weight were obtained from animal house in national research center (NRC), Dokki, Egypt. And housed randomly in four stable polycarbonate cages (n = 10 for each group) with normal temperature and photoperiod. Diets and water were given to rats (commercial diet and tap water) and allow acclimating for one week before assay.

Fish sampling

Total of 40 Nile Tilapia (*Oreochromis niloticus*) with 100 ± 20 gm body weight. obtained alive and healthy from fish aquarium in national research center (NRC), Egypt. Fish were maintained for acclimatization for one week in four clean disinfected glass aquaria (100*80*60 cm^3) According to the recommended biomass for each aquarium, supplied with dechlorinated tap water and also supplied with aerators and filters, Both sexes of fish were used and no attempt was made to determine gender or sexual maturity. Fish were examined for any abnormal behavioral changes, external signs and gross lesions before the onset of the experiment. Fish were fed on commercial fish ration (20% protein).

Pathogenic bacteria

Clostridium perfringens pure strains (ATCC 13124) were kindly obtained from the microbiological archive of the Microbiology, Animal Health Research Institute (AHRI), Dokki, Egypt (Isolates were originally retrieved from clinically affected *O. niloticus* during a previous microbiological survey that encountered tilapias from different earthen ponds located within the scope of Giza province). Bacterial isolates were reconstituted and aliquoted in 2ml microfuge tubes containing glycerol - phosphate buffered saline (pH 7.4 - 1: 1 vol /vol). The bacterial isolates aliquots were stored at the - 80°C freezer till the onset of the experimental challenge. In the experiment period the stored tubes were thawed at the room temperature and agitated using electric vortex to ensure equal distribution of the bacteria within the suspension. Loopfuls from *C. perfringens* aliquots were inoculated into sterile cooked meat broth then incubated at 25°C under complete anaerobic condition for 24 hours. The inoculated broths were checked for the required turbidity degree by matching against McFarland standard turbidity tubes (No.1) and when the Optical Density (OD) measured at wavelength of 600 nm it gave absorbance equal 0.257 .It came out that each ml of the inoculated broth contains 3 X 10^8 CFU⁄ml.

Chemicals and Kits

Alanine aminotransferase (ALT), Aspartate Transaminase (AST), Alkaline phosphatase (ALP), Gamma-glutamyltransferase (GGT), Uric acid (UA), Total Calcium (Ca), Creatinine (Cr.), Total protein and Urea were determined using kits purchased from Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim by using Roche/Hitachi cobas c311 system. CRP latex test kit was purchased from BIOTEC Laboratories Ltd Co. (Ipswich, Suffolk, UK).

BIOZOL BioFlux™ Reagent for RNA isolation was purchased from BioFlux (South San Francisco, U.S.A). Sodium chlorid, glycerol, bromophenol, glycerol - phosphate buffered saline and DEPC (diethyl pyro-carbonate) were purchased from sigma chemical company. Revert Aid™ First Strand cDNA synthesis Kit, Dream Taq™ Green PCR Master Mix (2X), GeneRuler™100 bp Plus DNA Ladder, ready-to-use were purchased from Fermentas life science Co. (Invitrogen Corporation) (Van Allen Way, Carlsbad, Canada). Primers for A2M gene was designed using following the web site NCBI and target genes sequence and purchased from Fermentas life

science Co. (Invitrogen Corporation) (Van Allen Way, Carlsbad, Canada). Dream Taq™Green PCR Master Mix (Invitrogen Corporation) was used in the PCR. Tris base, Boric acid, EDTA, agarose, ethidium bromide and RNase Free and Riblock™ RNase inhibitor were purchased from BioShop Canada Inc. (Burlington, Ontario, Canada). Fluka Cooked Meat Broth was purchased from Sigma-Aldrich Chemie (GmbH, Riedstrasse 2, D-89555 STEINHEIM). Cellulose acetate protein electrophoresis kit was purchased from Interlab S.r.l. via Rina Monti, 26 - 00155 Roma Italy. And by using Genio instrument. The protein fractions were performed using an automated system on cellulose acetate films.

Methods

Experimental design

Four sets of aquaria with identical physical environments, room temperature (30-34°C) and natural photoperiod, fish were randomly divided (n=10) in each aquaria, the first two aquaria containing the fish group one (FG1) and fish group two (FG2) as a negative controls and the other two aquaria contain fish group three (FG3) and fish group four (FG4) as a test groups. Rats were distributed in four different cages each contains (n=10) with the same manner as the fish, rat group one (RG1) and rat group two (RG2) as a negative controls, and rat group three (RG3) and rat group four (RG4) as a test groups.To study the possible changes in the patterns of expression of genes involved in the immune response, we monitored changes in mRNA expression α-2 macroglubulin (A2M) after infection of *O. niloticus* with *C. perfringens* and food-borne diseased rats after feeding on the challenged tilapias by a RT-PCR approach. We followed the expression of gene coding for A2M, and some biochemical analysis.

Biochemical investigations were developed at the biochemistry Labs of Armed forces central labs, kobry al-kobba, Cairo, Egypt. And the molecular biology investigations were developed in the molecular biology labs in National Research Center (NRC), Dokki, Egypt.

One week after acclimation period we Stimulated the immune system of FG3 and FG4 (Bacterial challenged groups of *Oreochromis niloticus)* by injecting them Intra-peritoneal (IP) with 0.3 ml of the infectious zoonotic bacteria *C. perfringens* (3 X 10^8 CFU/ml). FG1 and FG2 group individuals were injected with identical volume normal saline and used as a negative control**.** 3-Days post challenge; blood samples from tilapia groups were taken using sterilized syringes to carry out the biochemical analysis and protein electrophoresis. Then they were dissected separately and the liver, spleen and kidney tissues were removed rapidly, and placed immediately in an ependorf tubes containing 1 ml Bio-ZOL reagent and stored on ice for subsequent RNA extraction. The ruminants tissue of each group were insufficiently cooked grill separately and used as a food for rats, as following, the rat control groups (RG1 and RG2) feed on the remains of the fish control group (FG1) and the rat test groups (RG3 and RG4) feed on the remains of the fish test group (FG3). Now it is supposed that the rats of (RG3 and RG4) groups were infected with *C. perfringens* through the diet.

Blood samples and liver, spleen and kidney tissues were collected from the rats and fish groups through two intervals (3&6 days) to carry out the biochemical and molecular investigation.

Serum Separation:

A total volume of 1500 µl of blood was drawn from the caudal vessels of tilapia **(Blamey and Michael, 1994)** and from the retro orbital sinus of rats, and collected into vacutainer tubes without anticoagulant (Terumo Corporation, Tokyo, Japan). Blood was allowed to clot at room temperature, and then centrifuged at 400 rpm for 15 min. The serum samples were dispensed into plastic tubes and stored at -20ºC until analysis. Serum protein electrophoresis was performed within 3 days after sampling.

Biochemical markers of liver

Determination of serum Alanine aminotransferase (ALT) and Aspartate Transaminase (AST) activity was according to the recommendations of the International Federation of Clinical Chemistry (IFCC), but was optimized for performance and stability (Bergmeyer *et al*., 1986 and ECCLS, 1989a and 1989b).

Estimation of Alkaline phosphatase (ALP) activity in serum was measured according to the method of (Tietz *et al*., 1983).

Estimation of Gamma-Glutamyltransferase (GGT) activity was according to (Szasz *et al.,* 1974). Estimation of serum Total protein: The total proteins were measured according to **(**Cannon *et al*. (1974**).**

Biochemical markers of kidney

Urea was measured in serum according to the method of **(Sampson *et al*., 1980)**
Creatinine was measured in serum according to **(Jaffé, 1886)**
The uric acid was measured in serum according to **(Kageyama, 1971)**

1- Estimation of total Calcium in serum: Method according to **Schwarzenbach** (1995).
2- Biochemical marker of inflammation: C-Reactive protein was measured by the ultrasensitive latex immunoassay CRP (BIOTEC Laboratories Ltd Co. (Ipswich, Suffolk, UK).
3- Serum Protein electrophoresis (cellulose acetate electrophoresis).

The concentration of serum total proteins was determined according to **(Weichselbaum, 1946)** using cobas c311. The Serum Protein Electrophoresis (SPE) kits are intended for the separation of proteins in serum by electrophoresis on cellulose acetate strips. Serum proteins were separated into five distinct, well-resolved zones or bands, each containing one or more different proteins. The patterns were examined visually for abnormalities,

including variations of the bands or appearance of extra bands. Quantitation of the dye color intensity associated with the bands is accomplished by scanning the slide in a measuring optical system (densitometer) inside the instrument. The graphs, obtained by Elfolab™ software elaboration of densimetric data, display the percentage value of each fraction (Fig. 5). The absolute value for each band reported in g/L is calculated by multiplying the band percent value by the sample's total protein concentration.

Molecular analyses

RNA extraction from tissue

The total RNA was extracted from the liver, spleen and kidney tissues as a pool from both *O. niloticus* and rats using Biozol reagent according to the method of **Nicholas and Marko, 2004.**

SMART cDNA synthesis

Two µg RNA was reverse transcribed with Revert Aid First Strand cDNA Synthesis Kit™ (purchased from Fermentas life science Co., Invitrogen Corporation, using oligonucleotides. The cDNA mixture was stored at −20 °C until its use in RT- PCR reaction.

Semi-quantitative Polymerase chain reaction technique (RT-PCR)

The resulting cDNA was subjected to Semi-quantitative reverse transcription PCR for 35 cycles with respective primers designed from the sequence of A2M gene. A housekeeping gene, β-actin gene was used as an internal control.

Table (1): Sequences of the 5' and 3' synthetic primers used in PCR.

Gene	Primers(forward and reverse 5'→3')
Fish A2M	forward primer: 5' GTCCTGCCAAGTGAGGATGT 3' reverse primer: 5' ACATTCACAGCCCCTAAGGC 3'
beta actin gene	forward primer: GTTGGTGATGAGGCCCAGAG reverse primer: AATGTCACGCACGATTTCCCT
Rat A2M	forward primer: 5' GCTCGACTGGTCCTCTATGC 3' reverse primer: 5' CCACCAAGTCCCAGATCCAC 3'
beta actin gene	forward primer: CGAGTACAACCTTCTTGCAGC reverse primer: ACGCACGATTTCCCTCTCAG

PCR products were analyzed on 1.5 % Agarose gel electrophoresis: according to the method of (Sambrook et al., 1989) and visualized by gel documentation system, image of the gel were analized Using Gel-Pro Analyzer (version 6.0) software.

Statistical analysis

Two- way ANOVA was used to determine differences among the treatment groups for each blood analyte. When a significant ($p \leq 0.05$) difference was detected, the means were compared by use of Duncan's multiple range test. The analyses were performed using the Statistical Package for Social Sciences Software (SPSS), verssion 10 (SPSS, Richmond, Virginia, USA) was used as described by **Dytham (1999).** A *p*-value ≤ 0.05 were considered statistically significant.

RESULTS

Fish Biochemical markers of liver and kidney

Changes in liver enzymes activity and Kidney function levels due to bacterial infections were reported in Fig. 1, 2 and 3 for *O. niloticus* groups. Fish ALT, AST and ALP (FG3 and FG4) challenged with bacteria were significantly higher than the controls (FG1 and FG2) at the both intervals of time; 3 and 6 days ($P \leq 0.05$). At 3-days post challenge, the rise in AST in the FG3 (152.175 ± 16.368 U/L) than the control group (43.825 ± 1.934 U/L) is may be due to cellular injury in liver that may increases AST level in serum. There is a rise in ALT of FG3 (49.25 ± 8.264 U/L) compared to the control (34.575 ± 2.691 U/L). ALT is principally found in the liver and is regarded as being more specific than AST for detecting liver cell (Hepatocytes) damage. Hepatocytes play a major role in absorbing and metabolizing many toxic chemicals. They are therefore liable to injury by various chemicals, including food. Also there is a rise in ALP activities of FG3 (35.75 ± 2.986 U/L) than the control group (25.5 ± 2.886 U/L), this rise may be linked with the increased osteoblastic activity and lack of bile flow. GGT levels were eliminated because in at least one treatment group, more than five individuals had undetectable level in both interval times. Moreover, in the 6-days post challenge there was a trend towards a statistically significant elevation of serum AST activities of fish group 4 (342.55 ± 58.423 U/L) than the control (45.2 ± 2.706), While the ALT of fish group 4 (40.8 ± 4.854 U/L) and ALP of FG4 (28.5 ± 3.511 U/L) activity returned to restore.

There was no significant difference between serum urea concentration measured at FG3 (15.5± 2.38 mg/dl) and control (16.5 ± 1.29 mg/dl), $P \geq 0.05$ as shown in Fig. (3), its concentration remained almost unchanged in FG4 (17 ± 1.41 mg/dl) as shown in (Fig. 3) ($P \geq 0.05$). Also there was no significant difference between serum uric acid (UA) concentration measured at fish group 3 (1.675 ± 0.095 mg/dl) and control (1.625 ± 0.095 mg/dl), $P \geq 0.05$ (Fig. 2). UA concentration remained almost unchanged in rat group 4 (1.75 ± 0.264 mg/dl) when compared with rat group (1.675 ± 0.095 mg/dl) $P \geq 0.05$.

It was noticed that there was a significant decrease in the serum total calcium concentration measured in fish group 3 (10.45 ± 0.525 mg/dl) than control (12.825 ± 0.684 mg/dl) $P \leq 0.05$ (Fig. 3), its concentration remained in decrease in fish group 4 (9.55 ± 1.096 mg/dl) when compared with the control ($P \leq 0.05$). In contrast, there was a slightly trend toward elevation of serum Creatinine levels in fish group 3 (0.275 ± 0.050 mg/dl) than the control (0.15

± 0.057 mg/dl) as shown in figure (2), and in fish group 4 (0.25 ± 0.057 mg/dl) than the control (0.15 ± 0.057 mg/dl) ($P \leq 0.05$). This indicates that the animal's kidney was in normal case.

Rats Biochemical markers of liver and kidney:

Changes in liver enzymes activity, Kidney function levels due to bacterial infections are reported in fig. 4, 5 and 6) for the Rat groups. Serum ALT, AST and ALP activity of Rats challenged with bacteria (RG3 and RG4) were significantly higher than the controls when measured after 3-days post challenge (fig. 4), $P \leq 0.05$ where ALT in RG3 detected as 45.875 ± 1.506 U/L and in control 29.875 ± 2.833 U/L. The AST detected in RG3 as 214.475 ± 19.078 U/L and in control as 175.225 ± 11.615 U/L. Also the ALP in RG3 calculated as 275.5 ± 25.566 U/L compared to the control (140.5 ±8.062 U/L).

Activities of ALT, AST and ALP in RG4 were returned to restore at 6-days post challenge groups as shown in figure (4). The serum GGT level was undetectable in both intervals of times. There was no significant difference between serum urea concentration measured in challenged rat group 3 compared to the control ($P \geq 0.05$) as shown in Fig. 6, its concentration remained almost unchanged in rat group 4 ($P \geq 0.05$).

There was no significant difference between serum uric acid (UA) concentration measured in RG3 (3.275 ± 0.221 mg/dl) and RG1 (3.1 ± 1.141 mg/dl) as shown in Fig. (5). UA concentration remained almost unchanged in RG4 (3.2 ± 0.182 mg/dl), $P \geq 0.05$. There was significant decrease in serum total calcium concentration measured in challenged rat after 3 days (10.25 ± 0.369 mg/dl) compared to the control ($P \leq 0.05$) as shown in Fig. (6), but its concentration remained in decrease after 6 days challenge. In contrast, there was a slightly trend toward elevation of serum Creatinine levels as appear in rat after 3 days of infection (1.195 ± 0.129 mg/dl) than the control (0.55 ± 0.129 mg/dl) and after 6 days of infection (1.4 ± 0.258 mg/dl), Figure (5). This indicates that the Rat's kidney was not affected by the infection.

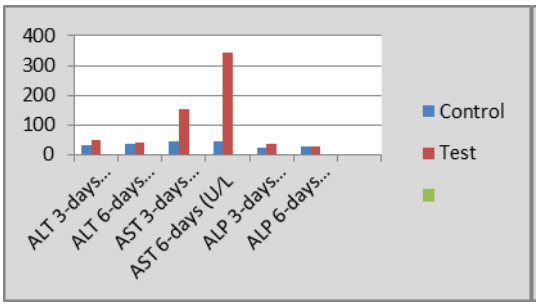

Figure (1): Serum ALT, AST and ALP of the control and challenged *O. niloticus* (mean ± S.D)

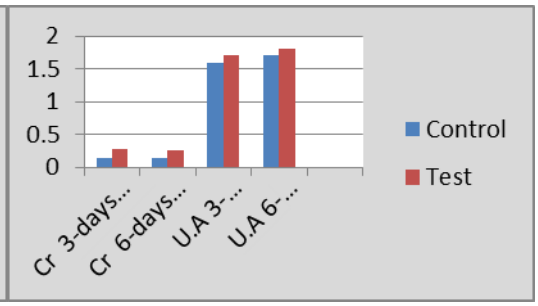

Figure (2): Serum Creatinine (Cr.) and Uric acid (U.A) of the control and challenged *O. niloticus* (mean ± S. D).

ISSN: 0975-8585

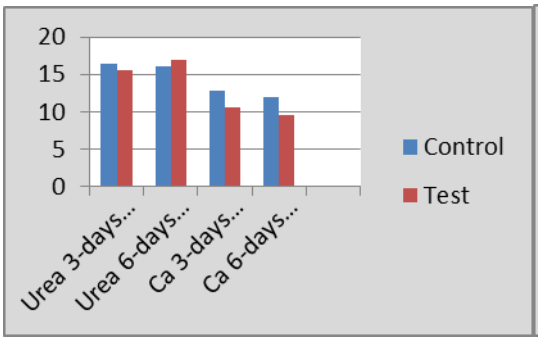

Figure (3): Serum urea and total calcium of the challenged *O. niloticus* (mean ± S.D).

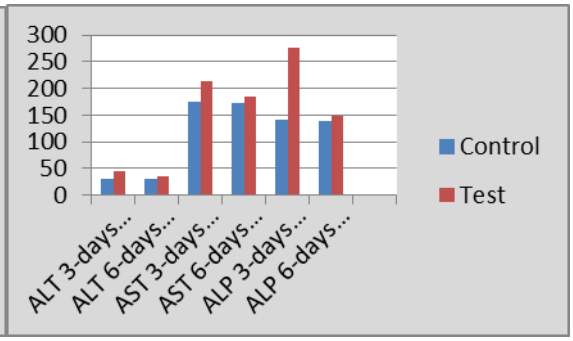

Figure (4): Serum ALT, AST and ALP of the control and challenged rat (mean ± S.D)

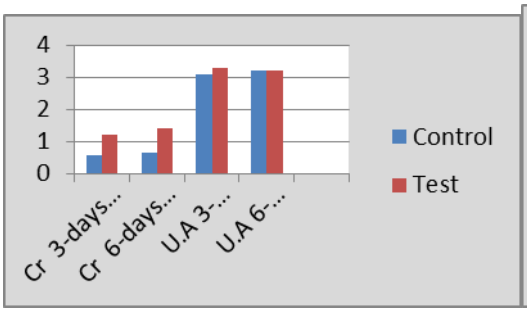

Figure (5): Serum Cr. and Uric acid of the control and challenged rat (mean ± S.D.)

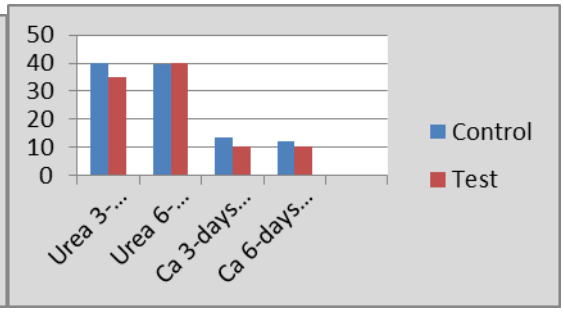

Figure (6): Serum urea and total calcium of the control and challenged rat (mean ± S.D)

Biomarkers of inflammation:

C - reactive protein:

Biochemical changes in the circulating CRP which are associated with inflammation are summarized for *O. niloticus* (Fig. 7) and for the rats (Fig. 8). *O. niloticus* CRP concentrations were slightly elevated with maximal values (10.5 ± 3.0 mg/L) 3-days after injection ($P \leq 0.05$) this corresponds to about 1.75 fold increase compared with control levels and the CRP level still increasing after 6-days post challenge. In rats, CRP concentrations were markedly elevated with maximal values (96 mg/L) ($P \leq 0.05$) when measured at 3-days after injection, this corresponds to about 16 fold increase compared with control levels and CRP level still high in the 6-day post challenge rats groups (96 mg/L).

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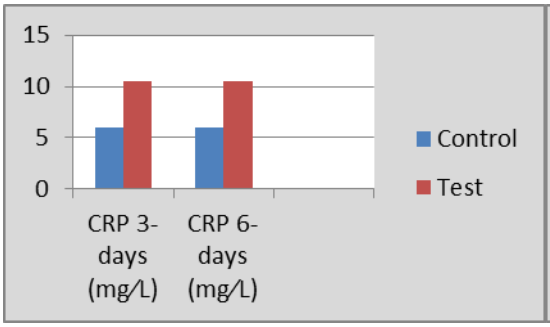

Figure (7): Changes in CRP levels of control and challenged *O. niloticus* (mean ± S.D).

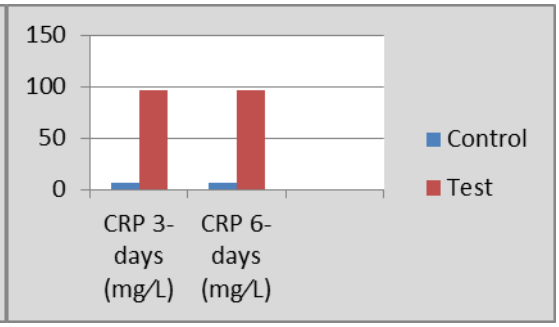

Figure (8): Changes in CRP levels of control and challenged rat (mean ± S.D).

Serum Protein Electrophoresis (SPE) (cellulose acetate electrophoresis):

In this study we used the cellulose acetate paper electrophoresis. Serum total protein and protein fraction levels in *C. perfringens* challenged groups (in both *O. niloticus* and rats) were elevated after 3rd day post challenge group and then begin to restore in the 6th day post challenge groups but still higher than the controls, as following:

***O. niloticus* Serum Protein and Electrophoresis:**

Results of *O. niloticus* serum protein electrophoretic patterns were summarized in tables (2 , 3) and figure (9; A, B, C and D) for the *O. niloticus* four groups. show that total protein was significantly highly increased in the *C. perfringens* challenged group FG3 (6.812 ± 0.592 gm/dl) than the negative controls FG1 (3.205 ± 0.262 gm/dl) and FG2 (3.45 ± 0.445 gm/dl) and then began to restore their level in FG4 (5.007± 0.895 gm/dl) but still higher than the control groups ($P \leq 0.05$) as shown in Tables (12 and 13) and (Fig. 9). Albumin concentrations in *C. perfringens* challenged *O. niloticus* groups FG3 and FG4 (3.375 ± 0.326 gm/dl) and (2.44 ± 0.407 gm/dl) were significantly increased ($P \leq 0.05$) than the negative control ones FG1 and FG2 (1.475 ± 0.085 gm/dl) and (1.597 ± 0.186 gm/dl) respectively. Alfa1 (α1) globulin concentrations in *C. perfringens* challenged *O. niloticus* groups FG3 and FG4 (0.717 ± 0.069 gm/dl) and (0.495 ± 0.077 gm/dl) were significantly increased ($P \leq 0.05$) than the negative control ones FG1 and FG2 (0.357 ± 0.029 gm/dl) and (0.367 ± 0.046 gm/dl) respectively. Alfa2 (α2) globulin concentrations in *C. perfringens* challenged *O. niloticus* groups FG3 and FG4 (0.975 ± 0.097 gm/dl) and (0.79 ± 0.142 gm/dl) were significantly increased ($P \leq 0.05$) than the negative controls FG1 and FG2 (0.49 ± 0.049 gm/dl) and (0.57 ± 0.074 gm/dl) respectively. Total Beta globulin concentrations in *C. perfringens* challenged *O. niloticus* groups FG3 and FG4(1.302 ± 0.076 gm/dl) and (1.005 ± 0.228 gm/dl) were significantly increased ($P \leq 0.05$) than the negative control ones FG1 and FG2 (0.722 ± 0.102 gm/dl) and (0.747 ± 0.106 gm/dl) respectively. Gamma globulin concentrations in *C. perfringens* challenged *O. niloticus* groups FG3 and FG4 (0.442 ± 0.049 gm/dl) and (0.277 ± 0.046 gm/dl) were significantly increased ($P \leq 0.05$) than the negative controls FG1 and FG2 (0.16 ± 0.021 gm/dl) and (0.152 ± 0.009gm/dl) respectively. The A/G ratio (Caculated) in *C. perfringens* challenged *O. niloticus* groups FG3 and FG4 (0.982 ± 0.035 gm/dl) and (0.952 ± 0.023 gm/dl) were significantly increased in a non-significant

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manner from the negative controls ($P \geq 0.05$) FG1 and FG2 (0.857 ± 0.056 gm/dl) and (0.862 ± 0.037 gm/dl) respectively.

Table (2): Assessment of the serum protein cellulose acetate electrophoresis pattern of the control and *C. perfringens* challenged *O. niloticus* at 3-days post challenge (mean ± S.D).

	O. niloticus group one (FG1) Control (n=10)		*O. niloticus* group three (FG3) 3-days post challenge (n=10)		LSD at 0.05
	Mean ± S.D	Min - Max	Mean ± S.D	Min - Max	
T. protein (g/dl)	3.205 ± 0.262 [a]	3.02 – 3.59	6.812 ± 0.592 [b]	6.22 - 7.5	0.649
Albumin (g/dl)	1.475 ± 0.085 [a]	1.41-1.6	3.375 ± 0.326 [b]	3.09-3.74	0.305
Albumin %	46.15 ± 1.613 [a]	44.7-48	49.5 ± 0.941 [b]	48.1-50.1	1.075
Alpha1 (g/dl)	0.357 ± 0.029 [a]	0.33-0.4	0.717 ± 0.069 [b]	0.65-0.80	0.064
Alpha 1 %	11.175 ± 0.411 [a]	10.7-11.7	10.525 ± 0.125 [b]	10.4-10.7	0.307
Alpha2 (g/dl)	0.49 ± 0.049 [a]	0.43-0.55	0.975 ± 0.097 [b]	0.88-1.09	0.105
Alpha2 %	15.25 ± 0.939 [a]	14.1-16.4	14.275 ± 0.170 [b]	14.1-14.5	0.547
Beta (g/dl)	0.722 ± 0.102 [a]	0.63-0.85	1.302 ± 0.076 [b]	1.19-1.36	0.153
Beta %	22.45 ± 1.519 [a]	20.9-23.9	19.15 ± 0.998 [b]	18.1-20.5	1.127
Gamma (g/dl)	0.16 ± 0.021 [a]	0.014-0.019	0.442 ± 0.049 [b]	0.039-0.051	0.039
Gamma %	4.975 ± 0.359 [a]	4.5-5.3	6.55 ± 0.351 [b]	6.2-6.9	0.426
A/G ratio	0.857 ± 0.056 [a]	0.81-0.92	0.982 ± 0.035 [a]	0.93-1.01	0.043

The data with the same superscript letters are significantly different ($P \leq 0.05$) from the control group.

Table (3): Assessment of the serum protein cellulose acetate electrophoresis pattern of the control and *C. perfringens* challenged *O. niloticus* at 6-days post challenge (mean ± S.D).

	O. niloticus group two (FG2) Control (n=10)		*O. niloticus* group four (FG4) 6-days post challenge (n=10)		LSD at 0.05
	Mean ± S.D	Min - Max	Mean ± S.D	Min - Max	
T. protein (g/dl)	3.45 ± 0.445 [a]	2.99 – 4.01	5.007± 0.895 [b]	4.24 - 6.1	0.649
Albumin (g/dl)	1.597 ± 0.186 [a]	1.39-1.8	2.44 ± 0.407 [b]	2.09-2.93	0.305
Albumin %	46.775 ± 0.275 [a]	46.5-47.1	48.85 ± 0.574 [a]	48.1-49.3	1.075
Alpha1 (g/dl)	0.367 ± 0.046 [a]	0.32-0.43	0.495 ± 0.077 [b]	0.42-0.58	0.064
Alpha 1 %	10.65 ± 0.251 [a]	10.3-10.9	9.85 ± 0.264 [b]	9.5-10.1	0.307
Alpha2 (g/dl)	0.57 ± 0.074 [a]	0.5-0.67	0.79 ± 0.142 [b]	0.66-0.95	0.105
Alpha 2 %	16.5 ± 0.244 [a]	16.2-16.7	15.85 ± 0.191 [b]	15.7-16.1	0.547
Beta (g/dl)	0.747 ± 0.106 [a]	0.64-0.87	1.005 ± 0.228 [b]	0.81-1.30	0.153
Beta %	21.65 ± 0.479 [a]	21.2-22.3	19.9 ± 0.864 [a]	19.1-21.1	1.127
Gamma (g/dl)	0.152 ± 0.009 [a]	0.14-0.16	0.277 ± 0.046 [b]	0.23-0.34	0.039
Gamma %	4.425 ± 0.442 [a]	4.0-4.9	5.55 ± 0.404 [b]	5.2-6.1	0.426
A/G ratio	0.862 ± 0.037 [a]	0.81-0.9	0.952 ± 0.023 [a]	0.92-0.97	0.043

The data with the same superscript letters are significantly different ($P \leq 0.05$) from the control group.

Rats Serum Protein and Electrophore:

Results of rats serum protein electrophoretic patterns were summarized in tables (4, 5) figure (10: A, B, C and D) for the four rats groups. The total protein was significantly highly

increased ($P \leq 0.05$) in the *C. perfringens* challenged group RG3 (10.33 ± 0.986 gm/dl) than the negative controls RG1 and RG2 (6.89 ± 0.355 gm/dl) and (7.0 ± 0.114 gm/dl) respectively and then began to restore their level but still higher than the control groups as shown in RG4 (8.33 ± 0.385 gm/dl). The albumin concentrations in *C. perfringens* challenged Rat groups RG3 and RG4 (5.245 ± 0.534 gm/dl) and (4.685 ± 0.188 gm/dl) were significantly increased ($P \leq 0.05$) than the negative controls RG1 and RG2 (3.205 ± 0.253 gm/dl) and (3.29 ± 0.103 gm/dl) respectively. Alfa1 (α1) globulin concentrations in *C. perfringens* challenged Rat groups RG3 (1.842 ± 0.157 gm/dl) was significantly increased ($P \leq 0.05$) than the negative controls RG1 and RG2 (1.132 ± 0.060 gm/dl) and (1.135 ± 0.005 gm/dl) respectively and then began to restore their level but still higher than the control groups (1.287 ± 0.043 gm/dl). Alfa2 (α2) globulin concentrations in *C. perfringens* challenged rat groups RG3 (0.735 ± 0.067 gm/dl) was significantly increased than ($P \leq 0.05$) than the negative control ones RG1 and RG2 (0.532 ± 0.017 gm/dl) and (0.517 ± 0.018 gm/dl) respectively and then began to restore their level but still higher than the control in RG4 (0.655 ± 0.074 gm/dl). Total Beta globulin concentrations in *C. perfringens* challenged Rat groups RG3 (2.31 ± 0.155 gm/dl) was significantly increased ($P \leq 0.05$) than the negative control ones RG1 and RG2 (1.747± 0.087 gm/dl) and (1.807± 0.035 gm/dl) respectively and then began to restore their level but still higher than the control in RG4 (2.025 ± 0.123 gm/dl). Gamma globulin concentrations in *C. perfringens* challenged Rat groups RG3 (0.60 ± 0.097 gm/dl) was significantly increased ($P \leq 0.05$) than the negative controls RG1 and RG2 (0.28± 0.042 gm/dl) and (0.242± 0.025 gm/dl) respectively and then began to restore their level but still higher than the controls in RG4 (0.377± 0.047 gm/dl). A/G ratio in *C. perfringens* challenged rat groups RG3 and RG4 were increased in a non-significant manner from the negative control ($P \geq 0.05$) ones RG1 and RG2 (1.032 ± 0.015 gm/dl) (0.962 ± 0.022 gm/dl) (0.867 ± 0.055 gm/dl) and (0.89 ± 0.025 gm/dl) respectively.

Table (4): Assessment of the serum protein cellulose acetate electrophoresis pattern of the control and *C. perfringens* challenged Rats at 3-days post challenge (mean ± S.D).

Rats	Rats group one (RG1) Control (n=10)		Rats group three (RG3) 3-days post challenge (n=10)		LSD at 0.05
	Mean ± S.D	Min - Max	Mean ± S.D	Min - Max	
T. protein (g/dl)	6.89 ± 0.355 [a]	6.5 – 7.36	10.33 ± 0.986 [b]	9.01 - 11.3	0.611
Albumin (g/dl)	3.205 ± 0.253 [a]	2.99-3.54	5.245 ± 0.534 [b]	4.52-5.76	0.342
Albumin %	46.45 ± 1.558 [a]	44.5-48.1	50.725 ± 0.359 [b]	50.2-51	0.991
Alpha1 (g/dl)	1.132 ± 0.060 [a]	1.05-1.19	1.842 ± 0.157 [b]	1.43-1.80	0.095
Alpha1 %	16.425 ± 0.403 [a]	16.1-17	15.95 ± 0.129 [b]	15.8-16.1	0.515
Alpha2 (g/dl)	0.532 ± 0.017 [a]	0.51-0.55	0.735 ± 0.067 [b]	0.45-0.61	0.056
Alpha2 %	7.75 ± 0.238 [a]	7.5-8.0	5.25 ± 0.129 [b]	5.1-5.4	0.439
Beta (g/dl)	1.747± 0.087 [a]	1.64-1.85	2.31 ± 0.155 [b]	2.08-2.41	0.119
Beta %	25.325 ± 0.403 [a]	25-25.9	22.45 ± 0.932 [b]	21.2-23.3	0.781
Gamma (g/dl)	0.28± 0.042 [a]	0.23-0.32	0.60 ± 0.097 [b]	0.52-0.73	0.065
Gamma %	4.05 ± 0.750 [a]	3.1 -4.7	5.875 ± 0.590 [b]	5.1-6.5	0.589
A/G ratio	0.867 ± 0.055 [a]	0.8-0.93	1.032 ± 0.015 [b]	1.01-1.04	0.036

The data with the same superscript letters are significantly different ($P \leq 0.05$) from the control group.

ISSN: 0975-8585

Table (5): Assessment of serum protein cellulose acetate electrophoresis pattern of the control and *C. perfringens* challenged Rats at 6-days post challenge (mean ± S.D).

Rats	Rat group two (FG2) Control (n=10)		Rats group four (RG4) 6-days post challenge (n=10)		LSD at 0.05
	Mean ± S.D	Min - Max	Mean ± S.D	Min - Max	
T. protein (g/dl)	7.0 ± 0.114 [a]	6.86 – 7.14	8.33 ± 0.385 [b]	7.99 - 8.73	0.611
Albumin (g/dl)	3.29 ± 0.103 [a]	3.17-3.42	4.685 ± 0.188 [b]	3.88-4.26	0.342
Albumin %	47.1 ± 0.707 [a]	46.2-47.9	49.075 ± 0.505 [a]	48.5-49.7	0.991
Alpha1 (g/dl)	1.135 ± 0.005 [a]	1.13-1.14	1.287 ± 0.043 [a]	1.25-1.35	0.095
Alpha1 %	16.2 ± 0.336 [a]	15.8-16.6	15.45 ± 0.776 [a]	14.3-16.0	0.515
Alpha2 (g/dl)	0.517 ± 0.018 [a]	0.49-0.53	0.655 ± 0.074 [a]	0.45-0.62	0.056
Alpha2 %	7.475 ± 0.287 [a]	7.1-7.8	6.65 ± 0.704 [b]	5.6-7.1	0.439
Beta (g/dl)	1.807± 0.035 [a]	1.77-1.85	2.025 ± 0.123 [a]	1.95-2.21	0.119
Beta %	25.8± 0.509 [a]	25.1-26.3	24.3 ± 0.875 [b]	23.1-25.2	0.781
Gamma (g/dl)	0.242± 0.025 [a]	0.21-0.27	0.377± 0.047 [b]	0.34-0.44	0.065
Gamma %	3.425 ± 0.309 [a]	3.0 -3.7	4.525 ± 0.403 [b]	4.2-5.1	0.589
A/G ratio	0.89 ± 0.025 [a]	0.86-0.92	0.962 ± 0.022 [a]	0.94-0.99	0.036

The data with the same superscript letters are significantly different ($P \leq 0.05$) from the control group.

The following figures show serum protein patterns on cellulose acetate. From the anodic edge of the cellulose acetate slide to the cathode, the five bands are: Albumin, Alpha 1 Globulin, Alpha 2 Globulin, Beta Globulin and Gamma Globulin. It should be specified that, with conventional serum proteins dyes, a slight or mild staining of the background between the major fractions may occur due to the presence of lipoproteins, mainly alpha and beta lipoproteins, whose protein moieties can bind to the dye. In a normal pattern, Albumin, alpha 1 and beta fractions appear to be homogeneous, well shaped bands. Alpha 2 and gamma fractions are diffuse bands with the gamma fraction showing a more intensely stained zone in its central part.

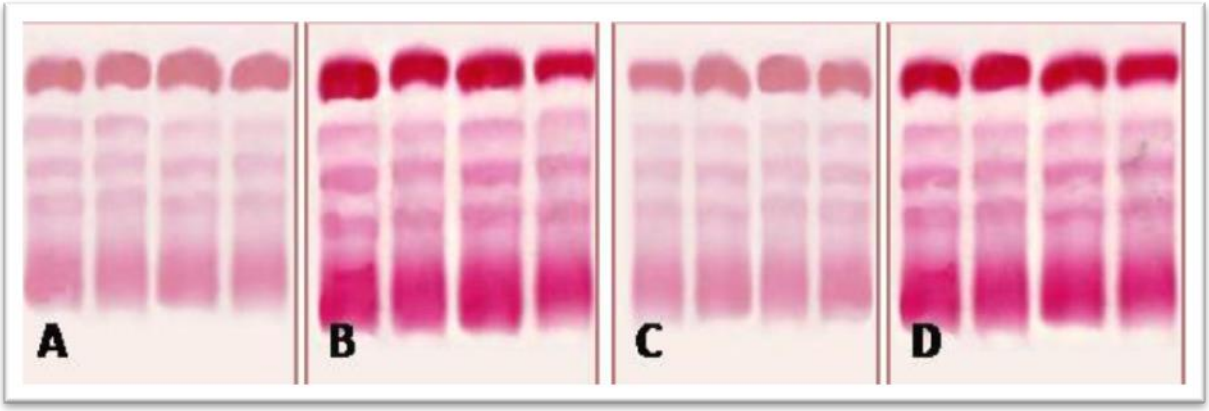

Figure (9): Cellulose acetate protein electrophoresis of the serum (A) Control *O. niloticus*, Fish group one (FG1). (B) 3-days post challenge *O. niloticus*. (C) Control *O. niloticus,* (D) 6-days post challenged *O. Niloticus*.

ISSN: 0975-8585

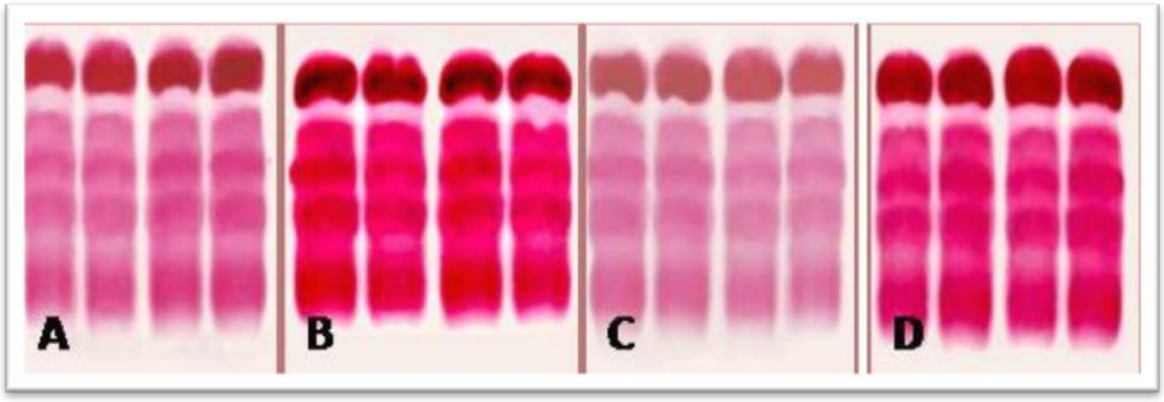

Figure (10): Cellulose acetate protein electrophoresis of the serum; (A) control rats, F: 3-days post challenged rats, G: control rats, H: 6-days post challenge Rats.

II) Molecular analyses of tissues:

Semi-quantitative RT-PCR and screening of α-2-macroglobulin gene expression

The experiment was designed to the detection of A2M gene expression, as acute phase proteins in the liver, kidney and spleen tissues of *O. niloticus* and rats challenged with *C. perfringens.* The tissues represented as a pool of tissues for the same species, the RT-PCR was utilized and PCR products were separated on agarose gel giving rise to amplified 695 bp and 986 bp for A2M of *O. niloticus* and rats respectively and 495 bp and 640 bp for β-actin of *O. niloticus* and rats respectively.

A2M gene expression:

A2M gene expression by RT-PCR in the liver, kidney and spleen tissues of infected and control groups, which represented as pool of tissues for the same group, were showed in figures (11 and 12) for *O. niloticus* groups and figures (14 and 15) for rat groups. A2M gene showed an increase in expression after 3 and 6 days of bacterial infection, while there was no expression for A2M gene in the tissues of non-infected (control) groups of both *O. niloticus* and rats. β-actin gene expression was used as a house keeping gene.

O. niloticus A2M gene/fish β-actin gene ratio (Fig. 13) and also rat A2M gene/rat β-actin gene ratio(Fig.16) were determined by densitometry which was performed by measuring the photo stimulated luminescence values using gel analyzer pro version software. After three days post challenge, The A2M gene expression of both *O. niloticus* and rats recorded the highest level compared to the control. However, after six days post challenge, A2M gene expression levels were less than the level after three days but still higher than that of the control.

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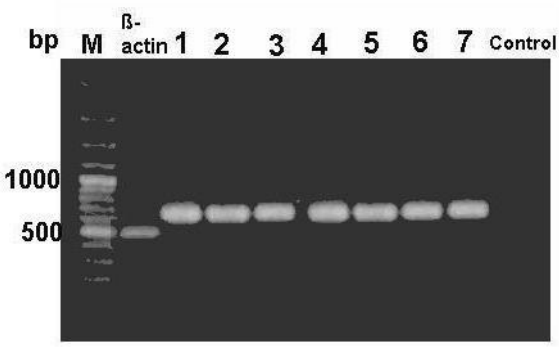

Figure (11): *O. niloticus* A2M gene expression in accordance to β-actin (2nd lane) obtained by RT-PCR 3 days post infection. *C. Perfringens* challenged individuals (1-7), control: Last lane and M:marker.

Figure (12): *O. niloticus* A2M gene expression in accordance to β-actin (2nd lane) obtained by RT-PCR 6 days post infection. *C. Perfringens* challenged individuals (1-7), control: Last lane and M:marker.

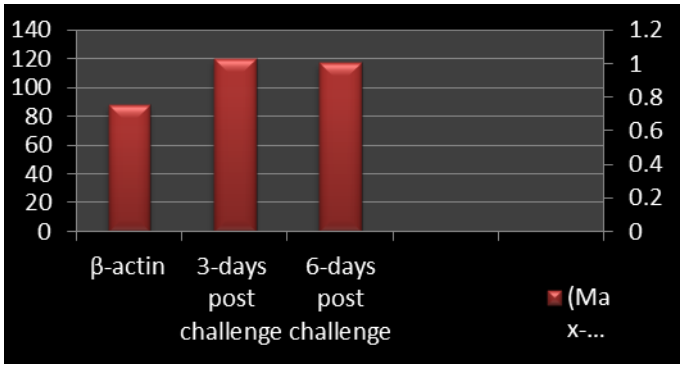

Figure (13): The relative density of expressed *O. Niloticus* A2M / β-actin gene.

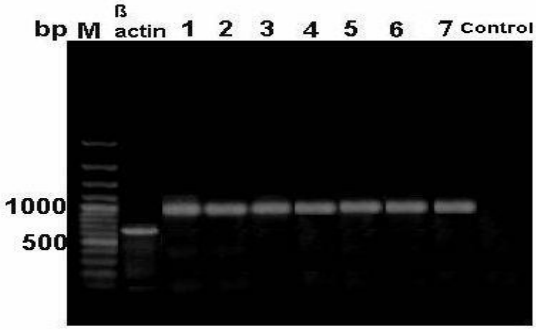

Figure (14): Rats A2M gene expression in accordance to β-actin (2nd lane) obtained by RT-PCR 3-days post challenge *C. Perfringens* challenged individuals (1-7), control: Last lane and M: marker.

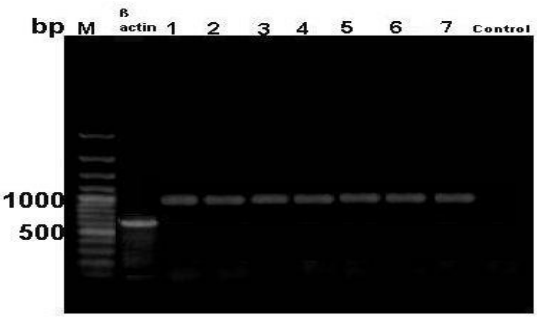

Figure (15): Rats A2M gene expression in accordance to β-actin (2nd lane) obtained by RT-PCR 6-days post challenge. *C. Perfringens* challenged individuals (1-7), control: Last lane and M: marker

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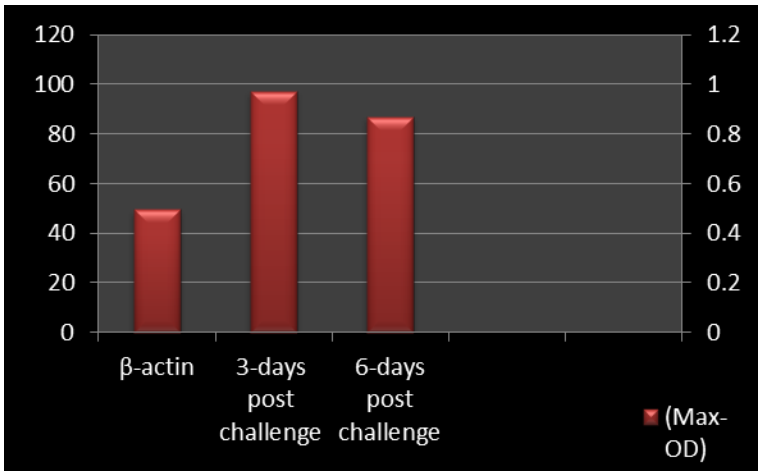

Figure (16): The relative density of expressed rats A2M / β-actin gene.

DISCUSSION

The intensification of fish farming often leads to the emergence of infectious diseases (Diab *et al.,* 2004), bacterial pathogens are among the most critical causes of mass mortalities aquaculture (Eissa *et al.,* 2010). Despite the fact that most of fish pathogens were primarily water inhabitants, yet, they can be extremely pathogenic to immune-compromised fishes. *Clostridium* infections are good examples for such normal water inhabitant that can invade fishes under certain environmental conditions. The use of animal and poultry manure as organic fertilizers in the earthen ponds based aquaculture can provide an optimal source of infection *C. perfringens* to the cohabitant fishes (Conbyo and Goss, 2001).

Dale *et al,* (1996) Stated that the infectious disease is, by definition, the consequence of infection by microorganisms, such as bacteria, viruses, or parasites. However, the manifestations of infectious disease are the result of both the action of the infecting organism and the animal's response to that infection. Hence, the study of disease requires not only attention to the characteristics of the microorganism, but also to the means by which the host responds to its presence. Every animal responds to the presence of a foreign organism by invoking a number of physiological changes, including rapid inflammatory changes collectively known as the acute phase response. The acute phase response is characterized by localized changes such as the aggregation of platelets and clot formation, dilatation and leakage of blood vessels, accumulation of leukocytes, and activation of stromal cells to release biological response modifiers. The release of mediators by resident and infiltrating cells then results in the initiation of systemic responses, including fever, leukocytosis, activation of complement and clotting systems, alterations in the plasma concentration of trace minerals, change in enzymes activity and changes in liver metabolism, including the production of a set of proteins called acute phase proteins (APP) (Heimich *et al.,* 1990). Therefore, we initiated a study to evaluate the acute phase response of *Oreochromis niloticus* and albino rats because in case of infection with *C. perfringens* , it may provide insight into the pathogenic mechanisms invoked in different diseases.

Because unlike many other types of bacteria that cause food-borne disease, *C. perfringens* not completely destroyed by ordinary cooking causing clostridial food poisoning, this is because it produces heat resistant spores (Hobbs *et al.,* 1953). But the bacteria itself are killed at cooking temperature. The heat-resistant spores they produce are able to survive and may actually be stimulated to germinate by the heat. If the food is not eaten at once but is allowed to cool slowly, the bacteria produced when the spores germinate multiply rapidly. Unless the food is reheated so that it is piping hot (at least to 60°C and preferably to 75°C), the bacteria will survive. After ingestion, if there are sufficient numbers present, the bacteria will produce toxins and the toxins will cause symptoms (Barnes *et al.,* 1963). Also this study was designed to determine if feeding on infected fish is responsible for food-borne disease or not. This work is concerned with the studying of the biochemical changes and molecular gene expression changes induced in groups of Nile tilapia (*Oreochromis niloticus*) as infected group and albino rats experimentally infected with *C. perfringens* (as a food consumer). All the rat control groups ate the remains of the fish control groups and all the rat test groups (challenged) ate the remnant of the fish test group. After the experiment times we observed that, rats of test groups (Fed on infected fish) showed some external clinical pictures like the weakness and after sacrificing, gall-bladder destination, spleenomegally and liver inflammation was noticed. All the challenged rats and *O. niloticus* groups developed inflammation this was proved after sacrificing the animals on different intervals of time (Three and six days post challenge), through a series of biochemical and molecular investigations. And this was matched with (Warrell *et al.,* 2003) which showed that poorly prepared meat and poultry, or food properly prepared but left to stand too long, the main culprits in harboring the bacterium.

As revealed from this study we investigated numerous biochemical markers for inflammation and A2M gene expression pattern. Previous studies have provided evidence that there is no replicable and consistent gold standard for diagnosis, which is typically made using a combination of clinical findings in association with the results of biochemical testing and non-invasive imaging. Currently, no single clinical or imaging finding confirms the diagnosis of food-borne disease with absolute certainty. Depending on the severity and time of testing during the course of disease and by comparing the serum biochemical markers that were investigated for the infected groups in this study with those for the control groups, we found that injection of *O. niloticus* with sterile saline and rats feeding on these non infected *O. niloticus* induced no change in serum biomarkers. The challenged *O. niloticus* and rats showed some signs of illness that can be investigated exteriorly; no mortalities were recorded along the course of the experiments in all test groups. A prominent spleenomegally was observed in rats challenged groups. The development of spleenomegally was considered to represent a common infected feature for 50% spleenomegally of (RG3) and 100% spleenomegally for (RG4). As Compared to Rats from the control groups, which showed 0.0% spleenomegally. Accordingly, spleenomegally induced by the bacterial infection with the *C. perfringens*. This phenomenon may be explained from the involvement of the stressful conditions due to bacterial infection (Brown-Bong *et al*., 1993).

Moreover, the relationship between certain diseases to changes in blood chemistry parameters has not been fully established. Those blood chemistries for which a relationship

with a disease state has been established have been primarily for major cultured fish species. Casillas *et al.* (1983) indicated that AST and ALT Activities are associated with hepatic pathology induced by carbon tetrachloride. Nelson *et al.* (1999) established the link between changes in blood urea nitrogen (BUN) to gill as opposed to kidney lesions. However, the experimental approach to reveal the link between disease state and blood chemistry is still urgently needed for many animal species. In addition to our limited knowledge of blood chemistry of fish, the issue is further compounded by deviations caused by different sampling protocols (Congleton and La Voie, 2001). All of these have contributed to a limited use of blood chemistry parameters as a tool in fish health management. Those blood chemistries important also in case of mammals. For example, the elevation of ALT may be due to hepatitis, cirrhosis, obstructive jaundice, carcinoma of the liver (Moss *et al.,* 1987). And the elevated serum levels may found in diseases involving these tissues. Hepatobiliary diseases, such as cirrhosis, metastatic carcinoma, and viral hepatitis also increase serum AST levels. Following myocardial infarction, serum AST is elevated and reaches a peak 2 days after onset (Moss *et al.,* 1987). A rise in the alkaline phosphatase may occur with all forms of cholestasis, particularly with obstructive jaundice. It is also elevated in diseases of the skeletal system, as well as with fractures and malignant tumors (Gressner, 1995). Elevated serum CRP is a risk factor for cardiovascular diseases and predicts future cardiovascular events and even mortality in apparently healthy people (Pai *et al.,* 2004) and so on.

Serum chemistry testing to look at end organ function is also imperative for bacterial infection and inflammation. Liver is a main tissue in the detoxification; Hepatocytes play a major role in absorbing and metabolizing many toxic chemicals. They are therefore liable to injury by various chemicals, including food. ALT and AST are important enzymes in liver and usually help to detect liver diseases by monitoring their concentrations (Ozer *et al.,* 2010). AST is widely distributed in tissue, principally hepatic, cardiac, muscle, and kidney. The rise in serum AST shows cellular injury in liver. ALT has been widely reported as present in a variety of tissues. The major source of ALT is the liver and is regarded as a better bio-indicator of liver cell damage and injury than AST (Mehta *et al.,* 2009) which has led to the measurement of ALT activity for the diagnosis of hepatic diseases (Sherwin, 1984 and Moss *et al.,* 1987).

In this study, the activities of serum liver enzymes were investigated and the results showed that the liver was aggressively affected with the bacterial infection in both *O. niloticus* and rat challenged groups. This was shown by the significantly elevation in the activities of AST, ALT and ALP enzymes ($P \leq 0.05$). This is may be due to Serum AST and ALT levels are common markers for hepatic toxicity: levels of these enzymes are rapidly increased when the liver is damaged by any cause, including hepatitis or hepatic cirrhosis (Sheth *et al.,* 1998). And the elevated activities of enzymes of intermediary metabolism such as ALP indicate systemic response due to bacterial infection. These results are in harmony with those of Chun-Yao *et al.* (2004) which indicate that there is increase in the *O. niloticus* ALT, AST and ALP activities when infected with *Streptococcus iniae.* While Racicot *et al.* (1975) reported that the *Aeromonas* infection in rainbow trout resulted in increases ALT and AST but not ALP. The GGT showed undetectable values in *O. niloticus* and rats (below the detection limit) this data agree with

Chun-Yao *et al.2*004) who indicate that GGT undetectable in the *O. niloticus* when infected with *Vibrio vulnificus.*

We must note also in this study that the degree of the biochemical alterations indicative of liver dysfunction was exposure time dependant. This may be explained by the difference levels in the liver enzyme with the difference of exposure time.

Serum blood urea nitrogen levels, however, may provide supplemental information in regard to renal function as renal proximal tubule cells may increase BUN re-absorption in the setting of increased neuro-hormonal activation (Shlipak *et al.,* 2002), Uric acid measurements are used in the diagnosis and treatment of numerous renal and metabolic disorders, including renal failure (Sing *et al.,* 1972 and Young *et al.,* 1972). The assay of creatinine in serum is the most commonly used test to assess renal function. Creatinine is a break-down product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body. It is freely filtered by the glomeruli, and under normal conditions, is not re-absorbed by the tubules to any appreciable extent. A small but significant amount is also actively secreted. Since a rise in blood creatinine is observed only with marked damage of the nephrons (Lamb *et al.,* 2005; Thomas and Thomas, 2005 and Lamb *et al.,* 2006). Calcium is the most abundant mineral element in the body with about 99 percent in the bones primarily as hydroxyapatite. The remaining calcium is distributed between the various tissues and the extracellular fluids where it performs a vital role for many life sustaining processes. Among the extra skeletal functions of calcium are involvement in blood coagulation, neuromuscular conduction, excitability of skeletal and cardiac muscle, enzyme activation, and the preservation of cell membrane integrity and permeability (Kozera, 1984 and Fraser *et al.,* 1987).

In the present study, kidney functions were investigated through estimation of blood urea nitrogen (BUN), serum creatinine (Finco, 1997 and Corrrréges *et al.,* 1998), serum urea and uric acid were showed non significantly different ($p \geq 0.05$) in the *O. niloticus* and rat challenged groups of both intervals (3-days and 6-days) when compared to the control groups. But serum creatinine level was found statistically significant ($p \leq 0.05$) to be slightly increased in the experimental groups after *C. perfringens* infection by 3-days and 6-days in both *O. niloticus* and rats. This change in plasma creatinine may be explained by the fact that the rise in blood creatinine is observed only with damage of the nephrons (Thomas and Thomas, 2005 and Lamb *et al.,* 2006). The serum total calcium level was found statistically significant ($p \leq 0.05$) to be decreased in the experimental groups after 3-days and 6-days post challenge with *C. perfringens* in both *O. niloticus* and rats. The hypocalcemia may be observed in nephrosis, pancreatitis, steatorrhea and hypoparathyroidism (Fraser *et al.,* 1987). Also the hypocalcemia may be due to protein binding, because the calcium levels vary with total plasma protein (McDonald and Milligan, 1992) so in our case the highly increase in the serum total protein in the challenged groups may affect on the total calcium level in the serum. The calcium data in this thesis agree with Chun-Yao *et al.* (2004) who found decrease in the serum calcium in the *O. niloticus* when infected with *Vibrio vulnificus* and *Streptococcus iniae.* Also Yildiz, (1998) indicate that *Pseudomonas fluorescens* infection in carp (*Cyprinus carpio)* caused decrease in serum calcium level.

Therefore, our results indicate that renal function is may affected by the bacterial infection. These findings are contradicted with those of Kang *et al*.,(2008) which showed increase in serum blood urea nitrogen level. The findings for serum creatinine level are in agreement with those of (Kang *et al*., 2008 & Gupta *et al*., 2008) while they are contradicted with the findings of (Theleman *et al*., 2001. The increase in serum uric acid can indicate inflammation in joint and increase joint damage. Phu *et al.* (2002) stated that *Staphylococci* and *Streptococci* cause immune complex glomerulo-nephritis in 20% or more of patient cases, presenting as proteinuria, haematuria and renal impairment. This is may be matched with our results which revealed renal impairment results from the bacterial infection with *C. perfringens*.

So we can say that this study suggests that the bacterial infection have a direct effect on the liver and kidney. And this may be probably explained by the fact that after bacterial infection the cells of several organs are damaged followed by release of a number of cytoplasmic enzymes to the blood (e.g. liver and kidney enzymes), a phenomena that provides the basis for clinical diagnosis (Sundberg *et al.,* 1994).

Also, the inflammatory response can be measured by a variety of inflammatory biomarkers. The largest database so far has been accumulated for C-reactive protein (CRP) (Kolz *et al.,* 2008). It is clear that CRP is an unspecific but sensitive marker of inflammation, regardless of the inflammatory stimuli. Despite this fact, CRP determination is a well-established laboratory test for the diagnosis and monitoring of different inflammatory processes. The determination of CRP has been used for years in the diagnosis of infection as well as to monitor the outcome of infection treatment (Lehrnbecher *et al.,* 1999). Therefore, measurements of serum CRP levels have been shown to be a powerful specification of the unidentified infections like bacterial or viral. According to Korppi *et al.* (1997) CRP measurement is recommended as the first-line method of screening of suspected bacterial inflammation.

In the present study rat serum CRP level was found statistically significant ($P \leq 0.05$) to be markedly elevated with maximal values (94.8 ± 3.79) 3-days after bacterial infection in the experimental groups. This elevation continues after 6-days post infection this may be due to CRP is an acute phase reactant with roles in innate host defense, clearance of damaged cells, and regulation of the inflammatory response. *O. niloticus* serum CRP level was found to be slightly elevated with maximal values (10.5± 3.0) at 3-days post challenge in the experimental groups. This elevation continued after 6-days from infection. The difference between the results of rats CRP and *O. niloticus* CRP may be due to that the CRP acute-phase protein in mammals but in fish is appear to be constitutively expressed and may show only a slight increase or decrease concentration during inflammatory responses (Lund and Olafsen, 1999).
Plasma proteins are synthesized predominantly in the liver, plasma cells, lymph nodes, spleen and in bone marrow. In the course of disease the total protein concentration and also the percentage represented by individual fractions can significantly deviate from normal values (Brobeck, 1973). Total protein measurements are used in the diagnosis and treatment of a variety of diseases involving the liver, kidney or bone marrow, as well as other metabolic or nutritional disorders (Brobeck, 1973). Plasma protein levels display reasonably predictable

changes in response to acute inflammation, malignancy, trauma, necrosis, infarction, burns, and chemical injury (Theodore *et al.,* 2005).

In our study there are significant increase ($P \leq 0.05$) in the serum total protein (Hyperproteinemia) this may be due to liver total protein synthesis was markedly enhanced (Breuillé *et al.,* 1998) due to the increase in the synthesis of exported proteins (acute phase proteins)in the liver (Vary and Kimball, 1992). And it has been reported that the synthesis of proteins increases in response inflammatory reaction (Gabay and Kushner, 1999). The total protein result in this study match with those of (manal *et al.,* 2011) which indicated that when *O. niloticus* injected with *C. perfringens* or *Pseudomonas florescence* the serum total proteins were elevated at 3-days post challenge and reduced in 6-days post challenge but still higher than the control groups. And also match with Voisin *et al.* (1998) which reported that when the rats injected intravenous with *Escherichia coli* (live bacteria) total liver protein synthesis was markedly enhanced. And match with Post (1966) which indicates that *Aeromonas* infection in rainbow trout resulted in increases in total protein. And our results contradicted with Barham *et al.* (1980) which indicate that the combined *Aeromonas* and *Streptococcus* infection in rainbow trout resulted in decreases in total protein.

Serum proteins electrophoresis is a common tool to determine the protein components of plasma or serum. The profile of serum proteins determined by electrophoresis is a sensitive marker for acute phase response (Rasouli *et al.,* 2005). And it is useful technique to diagnose some diseases characterized by quantitative and qualitative changes in the serum proteins. An increased synthesis of acute phase proteins has been associated with acute infection and inflammation (Maria and Maria 2006). This technique involves overlaying plasma or serum on cellulose acetate performed at pH 8.9 yields five bands: albumin and four globulins fraction (each fraction containing a number of different proteins): alpha 1(α1), alpha 2 (α2), beta (β), and gamma (γ). About sixteen of the known proteins contribute to the formation of the five bands in the electrophoretic pattern (Tietz, 1986). The electric current which applied causes migration of proteins according to their charge and size, intensity of the electric field, and characteristics of the support medium through which the protein particles migrate. The movement of the proteins creates bands in the gel which can be quantified. The protein bands are analyzed and quantified by using laser tracings from a densitometer (Kaneko, 1997).

Albumin, the major protein component of serum, and it is main protein of mammal serum, is produced by the liver under normal physiologic conditions. And it is essential for the regulation and keeping of oncotic pressure or osmotic pressure necessary for the proper distribution of body fluids in the vascular compartment and in tissues (Carapeto *et al.,* 2006). The albumin quantification in the serum is considered an indicator of an animal's proteic nutritional status (Payne and Payne, 1987). Its level is decreased under circumstances in which there is less production of the protein by the liver or in which there is increased loss or degradation of this protein, Malnutrition, significant liver disease and renal loss (e.g. in nephrotic syndrome).

Globulins, a heterogeneous group of proteins. It is much smaller fraction of the total serum protein content. And they are divided into α, β and γ globulins on the basis of their relative electrophoretic mobilities (Vanderschaeghe *et al.,* 2009 and Alberghina *et al.,* 2010). The subsets of these proteins and their relative quantity are the primary focus of the interpretation of serum protein electrophoresis (Ravel, 1995 and Jacoby and Cole, 2000). As variations in albumin and globulin concentrations are index of a pathologic condition, and their evaluation using serum protein electrophoresis is a valuable diagnostic tool. The alpha Fraction moving toward the negative portion of the gel (i.e., the negative electrode), the next peaks involve the alpha1 and alpha2 components. The alpha1-protein fraction is comprised of alpha1-antitrypsin, thyroid-binding globulin, and transcortin. Malignancy and acute inflammation (resulting from acute-phase reactants) can increase the alpha1-protein band. A decreased alpha1-protein band may occur because of alpha1-antitrypsin deficiency or decreased production of the globulin as a result of liver disease. Ceruloplasmin, alpha2-macroglobulin, and haptoglobin contribute to the alpha2-protein band. The alpha2 component is increased as an acute-phase reactant (ref). The beta fraction has two peaks labeled beta1 and beta2. Beta1 is composed mostly of transferrin, and beta2 contains beta-lipoprotein. IgA, IgM, and sometimes IgG, along with complement proteins, also can be identified in the beta fraction. (ref). The Gamma Fraction, Much of the clinical interest is focused on the gamma region of the serum protein spectrum because immunoglobulins migrate to this region. It should be noted that immunoglobulins often can be found throughout the electrophoretic spectrum. C-reactive protein (CRP) is located in the area between the beta and gamma components (Jacoby and Cole, 2000). The A/G ratio is commonly used as an index of the distribution of albumin and globulin fractions. Marked changes in this ratio can be observed in cirrhosis of the liver, glomerulonephritis, nephrotic syndrome and acute hepatitis as well as in certain acute and chronic inflammations (Brobeck, 1973).

In the present study the cellulose acetate gel electrophoresis of the serum total protein for (*O. niloticus*) and albino rats were monitored in serum samples collected 3-days and 6-days post challenge and expressed. Serum total proteins were calculated, and the serum electrophoresis from rats and *O. niloticus* were performed. The overall data of serum total protein measuring and protein electrophoresis are showed in tables (12 and 13) for *O. niloticus* and in tables (14 and 15) for rats, and the results showed that the mean significant value of albumin and globulin fractions were significantly increased ($P \leq 0.05$) in infected rats and *O. niloticus* all over the experiment when compared with the control groups. The increase in albumin (Hyperalbuminemia) recorded in our study may be due to hepatic disturbance as a result of tissue damage by the inflammation (ref). this result match with those of manal *et al.* (2011) who indicated that when *O. niloticus* injected with *C. perfringens* or *Pseudomonas florescence* the serum albumin was elevated at 3-days post challenge and reduced in 6-days post challenge but still higher than the control groups. And contraindicate with Voisin *et al.* (1998) reported that when that rats injected intravenous injection with *Escherichia coli* (live bacteria), the Infection decreased albumin levels in the infected rats. Also, there are significant increase recorded in our result in Alpha1, Alpha2, Beta and Gama globulins in serum collected in 3-days post challenge and the value returned restore in 6-days post challenge, but still higher than the control groups, is Clearly; due to *C. perfringens* infection induced an acute

inflammatory response as evidenced by the rise in globulin. These acute inflammatory disorders usually produce an increase of some of the proteins from the α1-globulin fraction such as α1-antitrypsin, α2-macroglobulin, ceruloplasmin, haptoglobin, and α1-acid glycoprotein (Maria and Maria, 2006). The acute phase response is considered to be a dynamic process involving systemic and metabolic changes providing an early nonspecific defense mechanism against insult before specific immunity is achieved (Suffredini *et al.,* 1999). A⁄G ratio was showed increased in a non-significant manner from the negative control ones ($P \geq 0.05$); this could be due to the increase in the albumin and total globulin. The serum albumin/globulin ratio allows the detection of changes in these proteins fractions, which according to Kaneko *et al.* (1997), is the first indicator of liver and kidney diseases. This result match with those of manal *et al.* (2011) which indicated that when *O. niloticus* injected with *C. perfringens* or *P. florescence* the serum globulin and A⁄G ratio were elevated 3-days post challenge and reduced in 6-days post challenge but still higher than the control groups.

In summary, ALT, AST, ALP, Cr., Total Ca, Total protein and CRP are good indicators of deteriorating health in tilapia and rats, because there are significant changes in these parameters were observed in all infected groups.

It is well known that the ability of both cold- and warm-blooded vertebrates to resist infection by a wide range of pathogens is influenced by genetic factors (Phelps and Neely, 2005) and it is evident that innate mechanisms of defense play a vital role in preventing bacterial diseases. A significant correlation between the activity of the non-specific immune system and actual disease resistance has been difficult to establish. Progress in the characterization of the natural pathways conferring increased disease resistance could facilitate the incorporation of these sources of resistance into breeding programs to alleviate the use of antibiotics and vaccines.

In this study, we aimed at the characterization of the expression of Alpha 2-macroglobulin immune gene involved in the *O. niloticus* and albino rat innate immune response against *C. perfringens* infection. This work revealed up-regulation of A2M gene expression, which is related to innate immunity cell-mediated immunity and inflammation. This indicates that these genes are involved in the acute phase response. When pathogens invade the host, it may secrete protease (toxin) to get nutrients or damage host immune system. In mammals, the molecular signals leading to the induction of the acute phase response start from the initial recognition of PAMPs, resulting in a signaling cascade that ultimately leads to the activation of pro-inflammatory cytokines (Pandey and Agrawal, 2006). In fish, antiproteases are important factors in non-specific humoral immune defense mechanism, which contain alpha 1-antiprotease, alpha 2- antiplasmin and alpha2-macroglobulin (A2M). When fish encounter pathogen infection, antiproteases can be used as inhibitors against the protease secreted by the pathogen (Ellis, 2001).

The A2M is described as a broad spectrum protease inhibitor in the plasma of vertebrates and invertebrates (Gollas-Galván *et al.,* 2003). A2M is a member of the A2-macroglobulin family, which includes several closely related proteinase inhibitors, and C3, C4

and C5 of the complement system (Armstrong and Quigley, 1999). The molecule, which has been isolated in all class of vertebrates and several invertebrates, is a broad-spectrum proteinase-binding protein, which leads to elimination of circulating proteinases in the plasma (Melchior *et al*., 1995). The protease inhibition mechanism of A2M in human was induced by the attack of A2M bait region by protease secreted by the pathogen. The structural distortions of A2M will lead to "entrapment" and this phenomenon will limit the trapped protease in cage-shape of A2M. However, the enzyme activity of the trapped protease was not lost (Feldman *et al.,* 1985). Therefore, A2M is thought to play an important role in innate immunity (Armstrong, 2001).

In the current study, the infection of *O. niloticus* and the albino rats by *C. perfringes* induces a “priming” of the host immune response characterized by induction in expression of A2M genes in liver, spleen and kidney tissues which represented as a pool for each individual when the images analyzed to show the peak (fig. 18 for *O. niloticus* and fig. 21 for rats) and compared with the uninfected control *O. niloticus* and rats that showed no expression for the A2M gene which involved in the inflammatory and immune response against *C. perfringens* infection. As an internal control, we had chosed the β-actin gene. Because β-actin are good reference gene for normalization of RT-PCR experiments (Muncke and Eggen, 2006). The up-regulation of A2M gene may be due to the A2M play a role in restricting the ability of bacteria to invade and grow in vivo (Ellis, 2001). Therefore, the A2M gene expression pattern during bacterial infection is a necessary first step in studying and identifying innate immune response that respond to bacterial challenge. The result in this work match with Voisin *et al.* (1998) who reported that when the rats injected intravenous injection with *Escherichia coli*, the Infection increased hepatic A1-acid glycoprotein (AGP), A2-macroglobulin (A2M), and fibrinogen plasma concentrations. And also This result match with Irune *et al.* (2007) who indicate that there are up-regulation of the genes related to the immune response (including A2M gene) in zebra fish *(Danio rerio)* when injected intraperitoneally with (10^6 CFU/ml) *Listonella anguillarum*.

Many studies stated that the pathogen infections lead to change in the composition of the serum proteins. Due to the change in the genes expression of host cells, as an immune response to this infection. Achara *et al.* (2003) stated that A2M purified from some crustaceans was found to have an activity against infection of invading pathogens (Dieguez-uribeondo and Cerenius, 1998) and was necessary for activation of the prophenoloxidase activating system (proPO system) (Lee and Soderhall, 2002). The *Marsupenaeus japonicus* A2M sequence contains putative functional domains including a bait region, an internal thiol ester site, and a receptor-binding domain, which are present in mammalian A2Ms. In a healthy shrimp, the mRNA of A2M was mainly expressed in haemocytes. In addition, the expression level of A2M mRNA was dramatically increased by through time upon oral administration of peptidoglycan (PG), which is an immune stimulant,the highest expression of A2M mRNA was observed 7-days after feeding with PG, these results suggest that the shrimp A2M is an important molecule in immune system and A2M is considered to be an important element of the innate immune system in crustaceans. Irune *et al.* (2007) were study the expression of interleukin 1β, transferrin, myeloid-specific peroxidase, lysozyme, ceruloplasmin, α-2-macroglobulin-1, complement factor C3, tumour necrosis factor alpha, Toll-like receptor 22, novel immune-type

receptor 9, heat shock protein cognate 70 and CCAAT/enhancer binding protein beta subunit genes related to the immune response in Zebrafish were intraperitoneally injected with 10^6 CFU (LD_{50}) *Listonella anguillarum* and stated that the results show an induction in gene expression of genes involved in the inflammatory and immune response upon *L. anguillarum* infection. Ping-Yueh *et al.* (2009) found that, A2M is involved in the immune response of prawn and its expression strongly increased in *Lactococcus garvieae* infection.

In summary, our data demonstrated that, during the infection with *C. perfringens* the expression of A2M gene was suggested to be up-regulated gene during acute phase response in both in *O. niloticus* and albino rats. These results augment the notion that *C. perfringens* has an immunostimulator effect this is due to the toxins which produce by *C. perfringens* target different animal tissues such as skin, muscles, intestine and some internal organs (Sakurai *et al.,* 2004). The Enteropathogenic (CPE) toxin Perfringolysin O (PFO) is the most critical one among the list of *C. perfringens* toxins (Sakurai *et al.,* 2009). PFO modulates expression of Intercellular Adhesion Molecule-1 (ICAM-1) located on endothelial cells and leukocytes and up-regulated by cytokine stimulation. Once activated, leukocytes undergo trans-endothelial migration into infected tissue. PFO has been shown to significantly decrease the mobility of the leukocytes, preventing them from migrating from the bloodstream to the infected tissue to fight the disease (Sakurai *et al.,* 2009). Further, CPE toxin could directly destroyed the innate as well as humoral components of the intestinal mucosa with an ultimate end result of pathogen progression, which was the ideal clinic-pathological condition in our *C. perfringens* experimental challenge. However, it remains to be proven that an increase in the levels of expression of genes of the immune system results in an enhanced resistance against infection. If that is the case, the discovery of molecular markers associated with increased basal expression of genes related to immunity could be a relevant issue if breeding for naturally immunoactive animal is considered in future selection programs.

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