

## THE IMMUNOLOGICAL RESPONSE FOR SPLEEN AS BIOMARKER AGAINST OF *Aeromonas hydrophila* BACTERIAL IN *Cyprinus carpio* FISH

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### ABSTRACT

The present investigation included a study of *Aeromonas hydrophila* during the period from May 2012 until April 2014 using 140 fishes of the common carp *Cyprinus carpio*. The study also aimed to investigate the possible role of biotic and chemical materials as an immune adjuvant like bacteria, chitin, Carboxy Methyl Cellulose (CMC) and gelatin. According to the method of antigen dependent and combined antigen independent antigen dependent immunomodulation, fish in aquarium were immune preconditioned with bacteria, chitin 1, 2, 5 %; CMC 1, 2, 5 % and gelatin 1, 2 and 5 % supplemented in dietary pellets for four weeks then injected with three live LD<sub>50</sub> doses of *A. hydrophila*. Spleen index, spleen cellular responses as lymphocyte density in white parenchyma and melanomacrophage centers MMC hyperplasia noted with various degrees in those treatments and being higher than control.

### INTRODUCTION

The pathogenic *Aeromonas hydrophila* is one of the most common bacteria in freshwater habitats throughout the world which frequently cause disease among cultured and feral fishes (12). It often referred to as a complex of pathogenic organisms that are associated with bacterial hemorrhagic septicemias and other ulcerative conditions in fishes (21). Although motile aeromonads appropriately receive much notoriety as pathogens of fish, it is important to note that these bacteria also compose a part of the normal intestinal microflora of healthy fish (3). Therefore, the presence of these bacteria, by itself, is not indicative of disease and, consequently, stress is often considered to be a contributing factor in outbreaks of diseases caused by these bacteria. Such stressors are most commonly associate with environmental and physiological factors that adversely affect fish under intensive culture (18).

Spleen in fish is composed of a system of splenic ellipsoids, melanomacrophage centers MMC and lymphoid tissue. In most species, ellipsoids are clustered together and organized around the other two components. The ellipsoids are thick-walled capillaries that open in parenchyma and formed from the division of the splenic

arterioles (10). The cells along the walls are actively involved in the macrophage phagocytosis of antigens, usually in the form of antibodies or metabolic products. Antigens may be detained for long periods of time and thus has an important role in immunological memory (3).

Histological investigation was carried out for the spleen to watch immunological protection and immunological modification as the spleen is bloody lympho body gland with immunological cycle in immunological association in fishes. This organ is affected by environmental conditions, as well as, bacterins, where the density of lymphatic cells increase inside white parenchyma along with elevation of MMC activity (20).

## MATERIALS AND METHODS

A total number of 140 common carp fish *Cyprinus carpio* were used for laboratory experiments during June 2013 until December 2013 (total weight 80-100 g) procured from Middle East Company in Babylon Province, Iraq.

All fishes which showing bacterial infection were screened by taking the samples from skin ulcers, spleen, liver and intestine. All samples were totally grinded, centrifugated, supernatant collected and cultured on macconkeys agar media to study the morphological characters and biochemical tests of *A. hydrophilla* (6).

### Preparation of natural polymers

Three natural polymers were used as immunological catalysts by supplementing into diets. Gelatin, chitin and CMC were purchased from local markets. Chitin was extracted from shrimp peels according to the method of Islam (9). Rates of 1%, 2% and 5% from each polymer were used.

### Determination of pathogenic *A. hydrophilla* lethal dose for fish

Seven ponds were used, ten fishes in each pond with weight from 80-100 g. All fishes were injected with  $1 \times 10^4$  and  $1 \times 10^{10}$  from bacterial pathogen *A. hydrophilla*. Fishes not showed any pathological symptoms after 23 days treatments with  $1 \times 10^4$  and  $1 \times 10^5$  doses of pathogen. No fish mortality was observed after treatment with  $1 \times 10^6$  dose but it was active in the first days and recuperated later. Fish samples challenged with  $1 \times 10^9$  and  $1 \times 10^{10}$  showed mortalities after 13 and 7 days respectively. All fish samples were died with  $1 \times 10^8$  at day 17 while the dose  $1 \times 10^7$  was the best between all treatments. 50% of fishes were died at day 11 and the remained samples recuperated before day 23 so this dose was selected as challenger dose according to Melnick and Adelberg's (13).

### Preparation of vaccine from thermo-inactivated *A. hydrophilla*

Bacterial colonies were sub-cultured on Brain Heart Infusion Broth (BHIB) and incubated at  $37^\circ \text{C}$  for 24 h and the number of cells was calculated and diluted to  $1 \times 10^7$  dilution (15).

### The immunostimulant experiments

All fishes were randomly distributed into ponds with ten fishes for each pond. After adaptation, samples were weighted and fish feeding started on the specified diets for treatments and controls. Fish weights were measured weakly and feeding continued for 5 weeks. In the sixth week, fish samples in the 9 pond were injected with saline water while fishes in the pond 10 injected with the  $1 \times 10^7$  dose of thermally inactivated *A. hydrophila*.

The symptoms of infection in the pond 10 were started after one hour with fish inactivity and slow movement. The first fish was died after 3 hours and in the second day two fishes were also died, the remained 7 fishes were started to resume activity and in the fourth day it regain normal movement. After the second week, all studied fishes were injected except treatment 10 which was injected after 5 days by the first injection.

### The spleen index

All spleen samples were collected from fishes, weighted and the formula for measuring the spleen index was applied according to Goldsby *et al.* (5).

$$\text{Spleen index} = \frac{\text{Weight of experimental spleen/ total body weight}}{\text{Weight of control spleen/ total body weight}}$$

### Histological examination

For histological studies, spleen tissue specimens were fixed in 10% neutral formalin, embedded in paraffin, sectioned and stained with Hematoxyline and Eosin (H&E) according to the method described by Humason (8).

## RESULTS

The ratio of spleen weight to fish total weight in the various treatments was as follows: Chitin 1, 2, and 5% were 0.028, 0.0044 and 0.0043, respectively. The CMC 1% was 0.0024 and Gelatin 1% 0.0123. Spleen weight to the total weight of fish in control was as follows: Chitin 1, 2, and 5% were 0.0041, 0.0022 and 0.0046 respectively. The CMC 1% was 0.0026 and Gelatin 1% 0.0040.

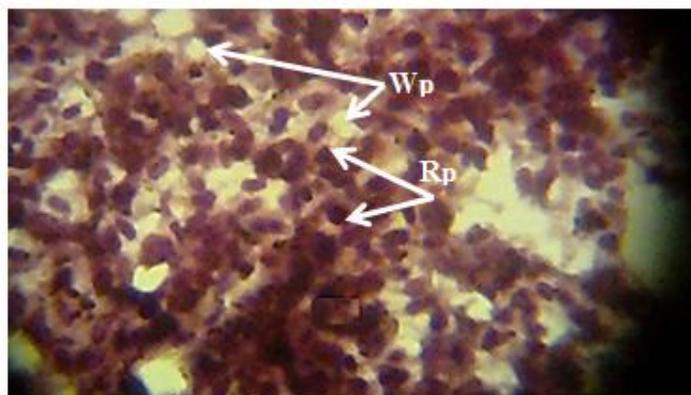
Spleen index in various treatments at the end of the experiment was, Chitin 1, 2, and 5%, 1.4704, 0.5090 and 1.0689, respectively, while CMC 1% was 1.0946 and Gelatin 1% 0.3254 as shown in table (1).

**Table (1): The spleen index of studied common carp *C. carpio*.**

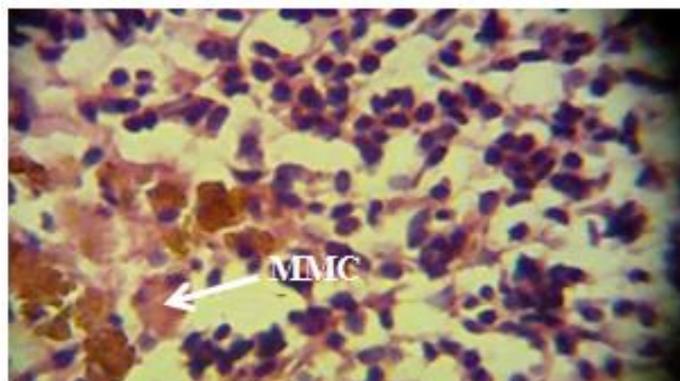
Spleen index	Spleen ratio (control)	Spleen ratio (feed)	Treatment
1.0946	0.0024	0.0026	CMC 1%
1.4704	0.0028	0.0041	Chitin1%
0.5090	0.0044	0.0022	Chitin2%
1.0689	0.0043	0.0046	Chitin5%
0.3254	0.0123	0.0040	Gelatin1%
P ≤ 0.05, Sig= 0.322			

From the spleen sections of the control samples, it was clear that the spleen consisted of red and white parenchyma. It was also obvious that the white parenchyma include lymphocyte cells with small clusters of MMC while the tissue capsule was absent. The trabeculae and the lymphocyte cells in the red parenchyma were less than in the white one.

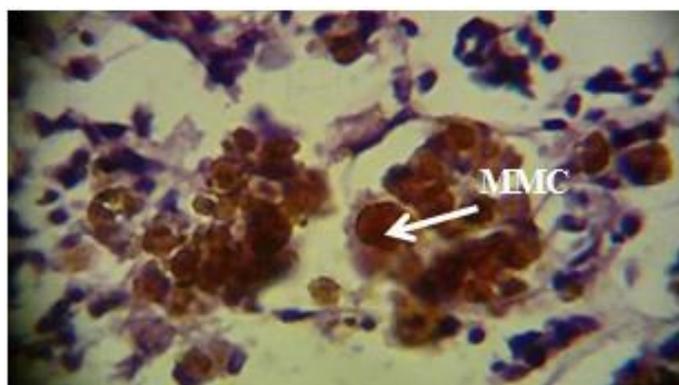
It was observed the spleen sections with immunostimulants that the lymphocytes are increasing in density in the white parenchyma in comparison with the control with proliferative responses of the melanomacrophage centers (MMC). The best results were obtained with CMC 1% and relatively lower by Chitin (2% and 5%) and Gelatin (1%) (Figs.1-6).



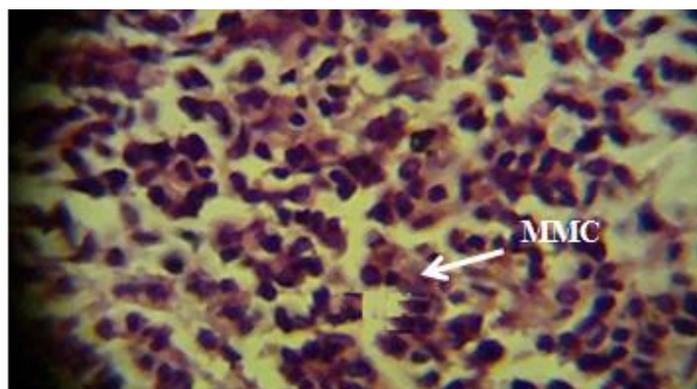
**Fig. 1. Spleen section of control *C. carpio* showing the nodular arrangement, white parenchyma (Wp) and red parenchyma Rp (H&E, x400).**



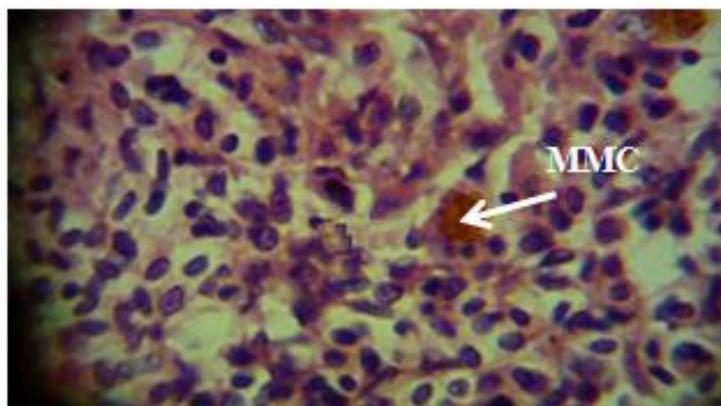
**Fig. 2.** Spleen section of *C. carpio* fed on Chitin 1%. MMC, Melano Macrophage Center (H&E, x400).



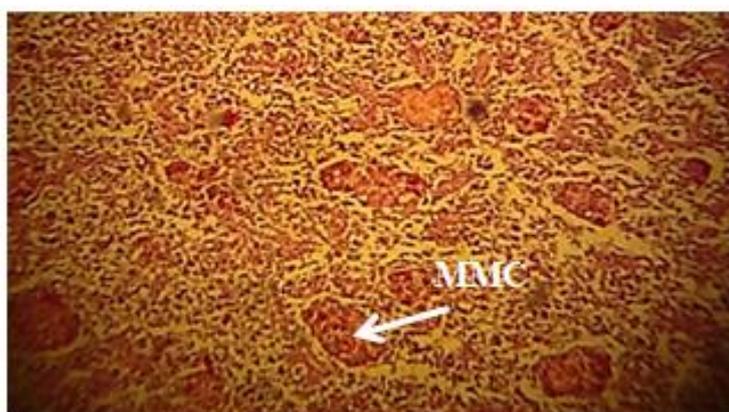
**Fig. 3.** Spleen section of *C. carpio* fed on Chitin 2%. MMC, Melano Macrophage Center. (H&E, x400).



**Fig. 4.** Spleen section of *C. carpio* fed on Chitin 5%. MMC, Melano Macrophage Center (H&E, x400).



**Fig. 5. Spleen section of *C. carpio* fed on Gelatin 5%.MMC, Melano Macrophage Center (H&E, x400).**



**Fig. 6. Spleen section of *C. carpio* fed on CMC 1%. MMC, Melano Macrophage Center (H&E, x100).**

## DISCUSSION

The traditionally used immune parameters are useful to determine the health status of fish and to evaluate the immunomodulatory substances for fish farming (16). Many studies investigated the changes in the non-specific immune responses of fish after administering immunostimulants in relation to infection, toxicity, stressors or pollutants (4; 16 and 11).

It is known that the immune response is either natural (innate) or adaptive or both of them. The macroscopic inspection of the studied fishes indicated that neither the internal organs nor the body cavity showed any gross signs which could be assigned to any pathological infection. In addition, no fish mortality was observed during the whole period of the experiment and the histological sections showed no any explicit pathological changes. Also, the increase in the spleen index, as indicated in the ratio of spleen weight to body weight, indicated the occurrence of an immunostimulation, a phenomenon which reflect of an immune protection. Therefore, the presence of the

cellular protection through the spleen tissue sections and the MMC distribution in spleen parenchyma could be confirmed in comparison with the control. The existence of mixed and cellular non differentiated prevention is coinciding with the results of (10); (7); (1); (14) and (21).

Immunostimulants are natural and synthetic compounds that counteract the immunosuppressive state of fish by promoting the non-specific immune response, antibody production and/or up-regulation of inflammatory response (19; 2 and 17). Natural immunostimulants are widely selected, as in the current study, because they are biocompatible, biodegradable and safe for the environment and human health (16 and 11). The importance of using natural immunostimulants in aquaculture, as shown by the results of the present investigation, can be related directly to its role in improving the immune response of fish when it supplemented into fish diets in comparison with the controls (2 and 16). However, the health of fish and enhancement of immunity in aquaculture settings are of primary concern and worth additional studies.

### الإستجابة المناعية للطحال كدليل حياتي ضد بكتيريا *Aeromonas hydrophila* في أسماك الكارب الشائع

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### الخلاصة

اشتمل البحث الحالي على دراسة تتعلق بالبكتيريا الممرضة *Aeromonas hydrophila* خلال المدة من حزيران 2012 الى نيسان 2014. جلبت 140 سمكة كارب شائع *C. carpio* من المزارع التجارية في محافظة بابل بعد أن حضرت أحواض التربية الخاصة بتجارب الدراسة الحالية. احتسبت الجرعة القاتلة لنصف المجتمع السمكي. وأعيد إنتاج المرض تجريبياً لكن بشدة أقل من المرض الطبيعي. استخدمت بعض المواد الحيوية والكيميائية مواد محورة للمناعة مثل البكتيرين المقتول بالحرارة والكايتين والتعلبية والجيلاتين. وقد استعمل أسلوب التهيئة المناعية المسبقة Immune preconditioning لأسماك التجربة بالمواد المزعومة إنها محورة للمناعة، بعدها نبه الجهاز المناعي لأسماك التجربة بثلاث جرع من البكتيريا الحية. حضر تركيز 1% و2% و5% من كل من الكايتين والتعلبية والجيلاتين وأضيف لها عليقه غذائية دنيا لمدة أربعة أسابيع، بعدها جرى حقن الأسماك بالعضلة تحت الزعنفة الظهرية أو بتجويف الاسماك البطني بثلاث جرع من الممرض *A. hydrophila*. لوحظ المؤشر والاستجابة الخلوية للطحال وبدرجات مختلفة من خلال كثافة خلايا اللمفوسايت Lymphocyte في اللب الأبيض والزيادة العددية بخلايا البلاعم الكبيرة ذات الميلايين بالمعاملات وكانت أعلى من المعاملة السيطرة.

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