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Research Article

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Isolation and characterization of MHCIIA gene from Nile tilapi a

Aml A. El-Semlawy [1],Karim S. El-Said[1], Mohammed A. El-Magd [2], Tarek M. Mohamed [1*]

[1]Department of Chemistry (Biochemistry section), Faculty of Science, Tanta University, Egypt

[2]Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt.

[*] **Corresponding Author:** Dr. Tarek M. Mohamed, E-mail:tarek96:7@hotmail.com

Keywords

Major histocompatibility complex; Genotype; Nile tilapia; SSCP-PCR

Abstract

This study was conducted to isolate and characterize the major histocompatibility complex (MHC) *class IIA* gene from Nile tilapia and to compare between different genotypes of this gene following infection with *Aeromonas hydrophila*. To achieve our goals PCR was used to isolate *MHC IIA* gene following extraction of genomic DNA from fish blood and then single strand conformational polymorphism (SSCP) was used to genotype resistant and susceptible/diseased fish. The results showed 12 nucleotides deletion polymorphisms in *MHC IIA* with a product size of 267 bp in control and resistant fish and 255 bp in susceptible/diseased fish**(Glover KA, et al.2007)** .Additionally , sequences out of 50 from susceptible/diseased fish showed a non-synonymous G23T SNP which interestingly changed the stop codon to glycin (G) amino acid with subsequent right shift to the open reading frame (ORF) . This SNP also resulted in three genotype (TT ,TG ,GG) with frequencies of 0.9,0.07 and 0.03 respectively . total protein , albumin and globulin as well as in the phagocytic. The G23T SNP was also associated with the expression level of *MHC IIA* in spleen with lowest expression in susceptible/diseased fish carrying GG genotype to resistant fish **(Xu T et al .,2008)**. Thus , SNP-disease resistance association analysis revealed that genotypes (GG and TG) and alleles (G) of the G23T SNP were high risk for infection

1. Introduction

Infection of Nile tilapia(*Oreochromis niloticus*) by *Aeromonas hydrophila* (*A. hydrophila*) causes hemorrhagic septicemia which is accompanied by high rate of mortality and economic losses **(Rahman et al.,2001 Li et al.,2006;AndAbdel-Tawwab et al.,2008)**. The current available treatment of this bacterium and other bacteria is the antimicrobial drugs which when used in large doses and for long time may lead to depression of fish immunity, growth and their residues can negatively affect consumers and the environment **(Martinez Cruz et al., 2012)**. Enhancing fish immunity in aquaculture is the main target of many fish farms stake holders. Among different immunity related molecules, the major histocompatibility complex (MHC) come on the top for their role in presenting antigens to T cell receptors **(Croisetiere et al., 2008).** The MHC molecules have two classes, class I and class II. The latter is subdivided into subclass A and B. *MHC IIA* presents in B cells, macrophages/monocytes and dendritic cells and stimulate helper $CD4^+$ T cells **(Hofmann et al., 2017)**. Its encoding gene has 4 exons and 3 introns in Nile tilapia ,**(Pang et al., 2013)** and expressed mainly in spleen, head kidney, gill, and liver **(Pang et al., 2013).** *MHC IIA* is highly polymorphic especially in its exon 2 **(Xu et al., 2011)**. By comparing the genotypes of *MHC IIA* gene in resistant/susceptible fish to diseases, brood stock having only the resistant alleles can be selected . Therefore, this study was conducted to isolate and characterize the *MHCIIA* gene from Nile tilapia and to compare between different genotypes of this gene

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following infection with Aeromonas hydropphila .

2.Materials and methods

Fish rearing and grouping

Nile tilapia fish (*n = 110*) with average size between 70 and 90 g were acclimated for 14 days before the start of the experiment. They were reared in fiberglass tanks (20 fish per tank). These tanks filled with appropriate amount of de chlorinated water at pH ranged from 6.5 -7, temperature at a range of 26-28 °C, and light cycle of 14 h light and 10 h dark with facilities for suitable aeration. Fish were fed a commercial ratio a rate of 3% body weight daily with a composition as previously described **(Abo-Al-Ela et al., 2017) .** Control (non-challenged) fish was intraperitoneally injected by PBS (n=30). Second group , Fish were challenged by intraperitoneal injection of *A. hydrophila* ($0.5x10^6$ CFU/fish) and symptoms and survival rate were daily observed. After 14 days of challenge, 10 fish were dead, 30 showed no symptoms (resistant fish), and the remaining 40 showed symptoms (diseased/susceptible fish). The specific symptoms of this disease include exophthalmia, hemorrhages, erosions and ulcers at fins base, pale gills, and desquamated scales. Internally, severe enteritis with yellow mucous in intestinal lumen, enlarged pale liver, congested inflamed spleen, and ascites were also observed. All blood samples were collected at the 15^{th} day of challenge from the caudal vein in vacutainer tubes coated by EDTA .

Serum biochemical parameters

2.1. *Total protein assay*:

Total protein was estimated according to the method of **(Yatzidis1987,Vassault et al ., 1986),** following the manufacturer protocol Kit (Biomed diagnostic medical company).Proteins reacts with copper ions (II) to produce a blue violet color compound in alkaline medium .

$$\text{Protein} + Cu^{++} \xrightarrow{\text{Alkali}} \text{Colored Complex}$$

The color intensity is proportional to the concentration of total proteins present in the sample.

2.2.Serum Albumin assay:

Serum Albumin concentration was assayed by colorimetric method using commercial Kit (Diamond –Diagnostic , Egypt) according to the method described by **(Young ,2001)**. Albumin is bound by the BCG dye to produce an increase in the blue green color in a pH 3.8 acidic medium .The color increase is

proportional to the concentration of albumin present in the sample .Albumin determination is useful in diagnosis of hepatic and renal **.**

2.3.Determination of serum globulins level

Serum total globulins concentration was calculated mathematically by subtracting the albumin values from the total proteins values for the same samples **(Coles, 1974) .**

2.4.Immunoglobulin M (IgM) Assay :

This assay employs the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with goat-anti-rabbit antibody. Standards or samples are added to the appropriate microtiter plate wells with an antibody specific for IgM and Horseradish Peroxidase (HRP) conjugated IgM. The competitive inhibition reaction is launched between with HRP labeled IgM and unlabeled IgM with the antibody. A substrate solution is added to the wells and the color develops in opposite to the amount of IgM in the sample. The color development is stopped and the intensity of the color is measured.

2.5. Serum lysozyme activity :

Serum lysozyme activity was determined through the turbidimetry described by**(Abo-Al-Ela *et al.*, 2017**)by using500 mg/L lyophilized Micrococcus lysodekticus (OD 570 nm = 0.3) as the substrate in phosphate buffer (0.1 M , PH 6.4). Fifty microlitres of fish serum was added to 3 ml of bacterial suspension. The activity was calculated by logarithmic regression with the formula Y = A + B log X, where Y is the diameter of the lysed zone and X is the lysozyme activity. The lysozyme values are expressed in micrograms per milliliter.

2.6.Phagocytosis Assay:

Phagocytic functional assays were performed in vitro, using Candida albicans according to the method of **(Kawahara et al., 1991)**.and **(Soliman et al,1997).** Phagocytosis has been traditionally assayed by measuring the engulfment of a cell "substrate". The most common substrates used in phagocytosis assays are erythrocytes (red blood cells) and zymosan (yeast) particles. When using red blood cells (RBCs) in the assay, the RBCs are first opsonized with serum or IgG; then they are incubated with phagocytes. The RBCs that are not engulfed by the phagocytes are removed, and the phagocytes are then lysed to release the engulfed RBCs.

2.7. Polymerase chain reaction (PCR)

The collected whole blood samples were used to extract the genomic DNA using Gene JET genomic DNA purification kit following the manufacturer protocol **(Fermentas, Life technology, #K0721**). Before preceding for PCR, a Nano drop was used to measure the concentration and purity of the gDNA. The PCR was performed using gDNA as template, PCR master mix **(Fermentas, Life technology, #K1071)** and specific forward 5' CATCAAAGGAAACGAGTGCA 3' and reverse 5' CTACTTTCAGCCGCTCTCCC 3' primers for *MHCIIA* gene. These primers were designed based on the published Nile tilapia sequence with accession number of JN967619.The PCR reaction mixture (25 µl) contained 12.5 µl 2x master mix, 0.5 µlof each primer (0.4 µM), 3µl gDNA (1 ng) and 8.5µl RNase, DNase free water. The condition of PCR was initial denaturation for 5m at 94°C followed by 30 cycles of denaturation for 30 sec at 94°C annealing at 60°Cfor 30 sec, extension for 1 min at 72°C, and a final elongation at 72°C for 7 min. The PCR products were fractionated on 1 % a garose gels stained by ethidium bromide and the bands were visualized under UV trans-illuminator.

2.8. Single strand conformational polymorphism (SSCP).

SSCP was carried out as previously described (**El-Magd et al., 2016)**. Briefly,the PCR products were mixed with equal volumes of denaturing solution (25 mM EDTA, 95% formamide, 0.025% xylene-cyanole and 0.025% bromophenol blue)and the mixture was heated for 5 min at 94°C, then loaded on a non denaturing 12% polyacrylamide gels (39:1 acrylamide to bis-acrylamide). SSCP gel was run in 1×TBE buffer at 200 V for 10 h at 4ºC. The DNA fragments in the gel were detected by silver staining (0.1% silver nitrate).

3. Statistical analysis

Allele and genotype frequencies and Hardy–Weinberg equilibrium (HWE) were calculated as previously described **(El-Magd et al., 2014).**

4. Results

4.1.Clinical signs and internal examination of A. hydrophila –challenged fish

The clinical examination of infected fish revealed the presence of typical clinical signs of *Aeromonashydrophila* infection which represented as exophthalmia, presence of haemorrhages at the base of pectoral fin with inflammation of anal opening, erosion in pectoral fin and tail fin. In severe cases scales desquamation occurs with presence of haemorrhagic erosions that turns to ulcers.

Figure (1): Showing presence of congestion and in the skin (a), scales desquamation (b) and tail erosions (c) in Aeromonas hydrophilia

4.2.Result of gross internal examination: The internal examination of infected fish revealed presence of severe enteritis with sloughing of internal mucosa and presence of yellow mucous in intestinal lumen. Liver became enlarged pale yellowish in colour and in some cases become greenish with enlarged gall bladder. Spleen appeared congested and inflamed .

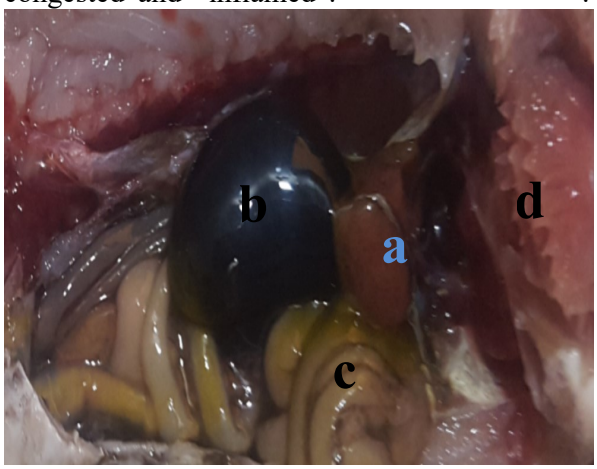


Figure (2): Showing presence of congestion in liver (a) with enlarged gall bladder (b), enteritis, with intestine filled with exudates (c) and palness in the gills (d) in *Aeromonas hydrophila* infected *Oreochromis niloticus*

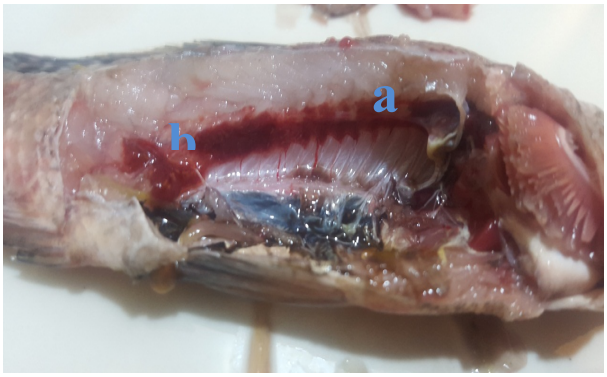


Figure (3): Showing presence of severe congestion in the anterior (a) and posterior portions of the kidney (b) in *Aeromonas hydrophila*i nfected *Oreochromis niloticus*.

4.3. Association between G23T SNP and immunity-related parameters

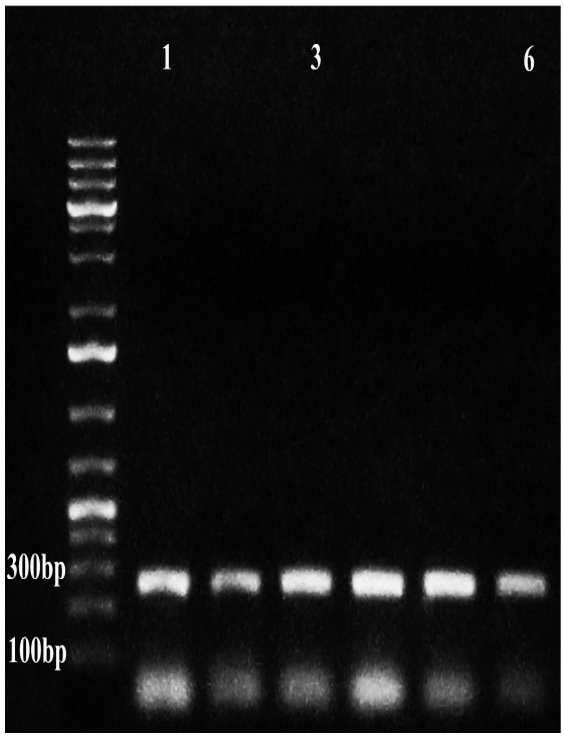
All immunity parameters (total protein, globulin, IgM, PA, PI, lysozyme activity) were significantly increased in the resistant fish than the control and susceptible fish,except albumin which showed insignificant increase (Table 1). On the other hand, susceptible fish exhibited no significant changes in these parameters as compared to control fish, except for PA, PI, lysozyme activity which was significantly elevated in susceptible than control fish. Among the three genotypes in all fish, TT fish had a significant higher levels of these parameters than heterozygous (TG) fish then homozygous GG fish .

4.4. Analysis of the detected polymorphisms

SSCP-PCR was used to screen the *MHC IIA* gene.To perform SSCP-PCR, gDNA was first isolated from fish blood.The isolated gDNA in all samples yielded the same bands size near the wells which means a successful isolation

Fig.4 Ethidium bromide stained gel showing representative DNA extracted from 2 csusceptible/diseased fish and 3 (last three lanes) resistant fish .

The isolated gDNA was used as a template for PCR which gave PCR products with sizes ranged from 255-267bp (Fig.4).



	CT (n= 30)	RT (n=30)	ST (n=40)
Totalprotein (g/dl)	3.90±0.24	5.72±0.31**	4.01±0.23
Albumin (g/dl)	2.14±0.15	2.04±0.18	1.92±0.15
Globulin (g/dl)	1.93±0.07	3.68±0.13**	2.09±0.09
IgM (µg/ml)	0.55 ± 0.02	0.97 ± 0.04**	0.61±0.02
Phagocytic activity (%)	27.8±1.42	45.5±1.19**	32±1.54*
Phagocytic index	1.30±0.02	2.04±0.06**	1.63±0.05*
Lysozomal activity	8.09±0.21	12.75±0.35**	9.12±0.12*

Fig 5.: MHC class IIA gene (255-267bp) amplified from resistant (lanes 1-3) and susceptible/diseased (lanes 4-6) *O. niloticus* fish.

To check for presence of polymorphisms, SSCP was performed on the amplified PCR products from all groups. This technique depends on change in pattern of migratory bands depending on their conformational structure. This means that changes in nucleotide sequences results in change in number and pattern of the SSCP bands. Interestingly, three different SSCP banding patterns were detected, indicating presence of polymorphisms and suggesting presence of three different genotypes, which were presented here as TT, TG, and GG (Fig.8). This nomenclature depends on the pattern of the bands where the genotype containing the larger number of bands (the sum of other bands) should be the heterozygous genotype. Subsequently, the heterozygous TG genotype contained 6 bands corresponding to the 4 bands of TT genotype and the two bands of GG genotype.

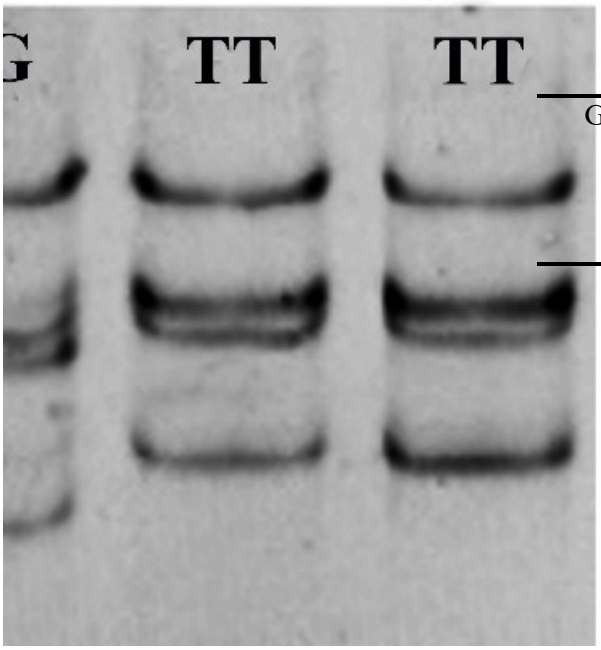


Fig.6 Identification of polymorphisms in *O. niloticus MHC IIA* gene. PCR-SSCP patterns of *MHC IIA* show the three genotypes (GG, TG, and TT) from 4 different samples.

To address the relation between the frequencies of these three genotypes and the susceptibility to *A. hydrophila* infection, genotypic and allelic frequencies were calculated in all fish groups depending on SSCP patterns and then the Chi-square (χ^2) was estimated .The frequencies of T and G alleles in addition to the TT, GT, and GG genotypes in both resistant and susceptible fish were shown in Table 2. The three genotypes were in Hardy–Weinberg disequilibrium (HWE) because χ^2value equals 0.13(P> 0.05).

Table 2. Genotypic and allelic frequencies and χ^2valuedepending on SSCP patterns.

Genotype and allele frequencies (number)	Resistant fish (n=30)	Susceptible fish (n=40)	HWE (χ2)	P-value
TT	0.40 (8)	0.17 (5)		0.7×10^{-03}
GT	0.45 (9)	0.33 (10)	0.13	2.2×10^{-02}
GG	0.15 (3)	0.50 (15)		1.3×10^{-04}
T	0.625 (12.5)	0.335 (10)		
G	0.375 (7.5)	0.665 (20)		

5. Discussion

Enhancing the immunity of fish against diseases, especially bacterial diseases, is essential not only to improve their health and subsequently decrease their losses but also to increase their body weight gain and profitability .To achieve this goal we first should isolate and characterize immune related genes in fish and subsequently studied the potential polymorphisms between resistant and diseased fish. Here we chose MHCIIA gene as a candidate gene due to its potential role in adaptive immunity and their multiple polymorphic characters **(Rakus et al., 2008).**In Nile tilapia, **Pang et al., (2013)** found three different classes for *MHC IIA*. Herein, we first isolated *MHCIIA* gene from *A. hydrophila*-challenged Nile tilapia and then searched for presence of polymorphisms using SSCP-PCR. Notably, we found three different genotypes, TT, TG, and GG in all fish groups. The heterozygous TG genotype contained 6 bands corresponding to the 4 bands of TT genotype and the two bands of GG genotype. The frequencies of T allele and TT genotype were higher in resistant fish than susceptible fish. On the other hand, the frequencies of G allele and GG genotype were higher in susceptible fish than resistant fish. This suggests that fish carrying GG genotype and G allele may be highly susceptible to *A. hydrophila* infection.

However, fish carrying TT genotypes and alleles (T) may be considered as protected (resistant).Increase number of fish carrying GG genotypes in this study may also explain the increased prevalence of *A. hydrophila* infection among Egyptian Nile tilapia as compared to foreign breeds (**Aly et al., 2015)**.Moreover, all genotypes were in HWE (P> 0.05). This suggests presence of non-overlapping generations with random mating and absence of mutation, migration, and selection among the studied fish population .

Although, the present study may provide a new value regarding immune response against *A. hydrophila* in Nile tilapia, it is crucial to determine the actual polymorphism causing this association. To achieve this, we recommend application of gene sequences for not only the partial *MHC IIA*PCR product used in this study but also to the entire *MHCIIA* gene and regulatory regions in independent large populations along with functional genomic analyses. It is also crucial to check immune related parameters, such as globulin, IgM, phagocytic activity, phagocytic index, and lysosome activity, to check whether TT fish had higher level of immunity than GG fish.

6.Conclusion

This is the first study to demonstrate that the G risky allele in the detected G23T SNP of *MHC IIA* gene was significantly associated with decreased resistance to *A. hydrophila* infection, as revealed by reduction in total protein, globulin, IgM, phagocytic activity, phagocytic index, lysosome activity, and MRNA levels of *MHC IIA*. The results of this study may enable breeders to select Nile tilapia with protective alleles (T) for this SNP to improve the disease resistant of the fish but before doing this further studies are needed on a large population to elucidate the precise mechanism by which this SNP and other linked genetic markers can affect defense response in fish.

7 .References

Abdel-Tawwab, M., M. A. A. Mousa and F. E. Abbass. (2008)a .

Growth performance and physiological response of African catfish, Clarias gariepinus (B.) fed organic selenium prior to the exposure to environmental copper toxicity. Aquaculture, 272(1-4): 335-345.

Abo-Al-Ela, H.G., El-Nahas, A.F., Mahmoud, S., Ibrahim, E.M., 2017. Vitamin C Modulates the Immunotoxic Effect of 17alpha-Methyltestosterone in Nile Tilapia. Biochemistry 56, 2042-2050.

Aly, S.M., Albutti, A.S., Rahmani, A.H., Atti, N.M., 2015. The response of New-season Nile tilapia to Aeromonas hydrophila vaccine. Int J Clin Exp Med 8, 4508-4514.

Coles, D,S,et al , Clin , Chem , 1974) .

Croisetiere, S., Tarte, P.D., Bernatchez, L., Belhumeur, P., 2008. Identification of MHC class IIbeta resistance/susceptibility alleles to Aeromonas salmonicida in brook charr (Salvelinus fontinalis). Mol Immunol 45, 3107-3116.

El-Magd, M.A., Abo-Al-Ela, H.G., El-Nahas, A., Saleh, A.A., Mansour, A.A., 2014. Effects of a novel SNP of IGF2R gene on growth traits and expression rate of IGF2R and IGF2 genes in gluteus medius muscle of Egyptian buffalo. Gene 540, 133-139.

El-Magd, M.A., Saleh, A.A., Abdel-Hamid, T.M., Saleh, R.M., Afifi, M.A., 2016. Is really endogenous ghrelin a hunger signal in chickens?: Association of GHSR SNPs with increase appetite, growth traits, expression and serum level of GHRL, and GH. General and Comparative Endocrinology 237, 131-139.

Glover KA, Grimholt U, Bakke HG, **Nilsen F, Storset A, Skaala (2007) .**

Major histocompatibility complex (MHC) variation and susceptibility to the sea louse Lepeophtheirus salmonis in Atlantic salmon Salmo salar. Dis Aquat Organ 2007, 76:57-65 .

Hofmann, M.J., Bracamonte, S.E., Eizaguirre, C., Barluenga, M., 2017. Molecular characterization of MHC class IIB

genes of sympatric Neotropical cichlids. BMC Genet 18, 1.

Kawahara, E., Ueda, T., and Nomura, S. (1991).
In vitro phagocytic activity of white spotted shark cells after injection with Aermonas salmonicida extracellular products. Gyobyo Kenkyu 26, 213−214.

Li, G., Guo, Y., Zhao, D., Qian, P., Sun, J., Xiao, C., Liang, L. and Wang, **H**. **2006**. Effects of levamisole on the immune response and disease resistance of *Clarias fuscus*. Aquaculture **253**, 212-217.

Martinez Cruz, P., Ibanez, A.L., MonroyHermosillo, O.A., Ramirez Saad, H.C., 2012. Use of probiotics in aquaculture. ISRN Microbiol 2012, 916845.

Pang, J.C., Gao, F.Y., Lu, M.X., Ye, X., Zhu, H.P., Ke, X.L., 2013. Major histocompatibility complex class IIA and IIB genes of Nile tilapia Oreochromis niloticus: genomic structure, molecular polymorphism and expression patterns. Fish Shellfish Immunol 34, 486-496.

Rahmani , C., A. Devaux, M. Lafaurie, J. P. Girard, B. Bailly and Risso-de Faverney(2001) . Cadmium induces apoptosis and genotoxicity in rainbow trout hepatocyte through generation of reactive oxygen species. Aquatic Toxicology, 53: **65–76**

Rakus, K.L., Wiegertjes, G.F., Adamek, M., Bekh, V., Stet, R.J., Irnazarow, I., 2008. Application of PCR-RF-SSCP to study major histocompatibility class II B polymorphism in common carp (Cyprinus carpio L.). Fish Shellfish Immunol 24, 734-744

Soliman, M. K. (1997).
Depression of phagocytosis in grass carp Ctenopharyngodon idella following chemical stress. Alexandria Journal of Veterinary Sciences 13, 81−86.

Vassault, A et al , Ann , BiolClin , 44, 686 , (1986) .

Xu T, Chen S, Ji X et al (2008) .MHC polymorphism and disease resistance to Vibrio anguillarum in 12 selective Japanese flounder (Paralichthys olivaceus) families. Fish Shellfish Immunol 25:213–221.

Xu, T., Sun, Y., Shi, G., Cheng, Y., Wang, R., 2011. Characterization of the major histocompatibility complex class II genes in miiuy croaker. PLoS One 6, -23823.

Young , D,S,et al , Clin , Chem , 21(10).1975

Yatzidis , H , L : J , Clin , Chem, 23/908 (1987) .