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Use of Immunohistochemical and PCR Methods in Diagnosis of Infectious Haematopoietic Necrosis Disease in Some Rainbow Trout Hatcheries in Iran

[1]Adel Haghighi Khiabanian Asl, [2]Mehdi Soltani, [3]Bahram Kazemi, [1]Iraj Sohrabi Haghdoust and [4]Isa Sharifpour
[1]Department of Pathology, Veterinary Sciences School, Islamic Azad University of Iran,
Science and Research Campus, Tehran, Iran
[2]Department of Marine Health and Disease, Veterinary School, Tehran University, Tehran, Iran
[3]Cellular and Molecular Biology Research Center,
Shaheed Beheshti University of Medical Sciences, Tehran, Iran
[4]Iranian Fishery Research Institute, Tehran, Islamic Republic of Iran

Abstract: The aim of this research was investigation on infection in reproducing and breeding centers of Rainbow trout in Iran by infectious Necrosis haematpoietic virus. We collected 100 samples from 100 reproducing and breeding centers of Rainbow trout of the Iran that showed clinical sings or were healthy in appearance in larva stage and finger link. Samples were tested by immunohistochemistry and Nested-PCR methods. By immunohistochemistry methods 35 samples and by PCR 43 samples were positive. There were 38 samples with clinical symptoms of Infectious Haematpoietic Necrosis. The results of this study show the dispersion of Infectious hematopoietic necrosis virus in some reproducing and breeding centers of Rainbow trout in the Iran. Also the result of two methods had acceptable overlapping.

Key words: IHN, immunohistochemistry, Nested-PCR-Rainbow trout, Iran

INTRODUCTION

Infectious Haehatopoietic Necrosis is a dangerous, acute and systematic disease of salmonid fish infected by Rabdo viruses, which is epidemic in domesticated and wild populations (Fallahi, 2002).

Financial damages of this disease require more attention. Epidemiologically this disease is contagious by both vertical and horizontal ways and infects host in marine environments and the fatality of larva in temperature less than 15°C reaches its highest. Up to now studies and researches have caused the isolation of virus from some producers of Rainbow trout in Iran's fields (Soltani, 2001).

Considering the diagnosis of virus infection, using methods like Immunohistochemistry and PCR for diagnosing of the infection and detecting centers of infection and quarantine are so important (OIE, 2005).

Serious epidemic of this disease among salmonid was reported by Amend for the first time in 1970 (Amend, 1970). Other spices of salmonid like black salmonid, Rainbow trout are sensitive to IHN virus (Behtar, 1998). Cause of disease is a type of Rhabdovirus with single strand RNA with dimensions of 75-65×190-150 nano meters which is sensitive to heat (temperature), Acid (pH less than 4), ether, Ultraviolet rays and iodophores. This virus is able to grow on cell lines such as: EPC, CHSE214 and RTG-2 in temperature 4-20°C (Soltani, 2001). The first symptom of this disease is the abundant death of finger link and in most cases it spreads in temperatures less than 15°C (Fallahi, 2002). Increasing histopathologic changes with resulting complexes of reaction between viral antigens and monoclonal antibody reaction which is related to the concentration of virus and can be observed in virus structure in anterior kidney, spleen by electronic microscope (Adams *et al*., 1994). Other symptoms are thinness, swimming without balance, weakness, scoliosis, lordosis, exophthalmia and partially hard stool, darkness of back part of body, petechia in abdomen cavity and mesenteric lipids (Fallahi, 2002).

Because the infectious haematopoietic necrosis (IHN) is important disease agent in farmed rainbow trout and disease outbreaks cause major losses in fish farming worldwide and it is one of the viral fish diseases that have a considerable economic impact on Iranian aquaculture and. We decided to set up sensitive and specific methods for detection of virus in Iranian fish farmer and the aim of this research was to use of PCR and immunohistochemistry methods for diagnosing IHN disease in some rainbow trout hatcheries in Iran. This is also the first report of field trial comparing the RT-PCR and immunohistochemistry assay in trout from 100 Iranian fish farms.

Corresponding Author: Adel Haghighi Khiabanian Asl, Department of Pathology, Veterinary Sciences School, Islamic Azad University of Iran, Science and Research Campus, Tehran, Islamic Republic of Iran

MATERIALS AND METHODS

Sampling: In March, April and May 2004, 100 samples were collected from 100 reproducing and breeding centers of cold water fishes in 17 provinces of in the country (each sample contained tissues samples included: kidney, spleen, liver, gill, heart and digestive system) immunohistochemistry samples were transferred in 10% formalin buffer and PCR samples in 20% ethanol to the lab. For doing Immunohistochemistry assay, samples were processed for tissue section preparation and staining and for doing PCR, RNA was extracted from samples.

Immunohistochemistry: This method was done as previously described by brief modification (www.chemicon.com). For eliminating cellular peroxide, slides were kept in a room temperature in 10% H_2O_2 for 10 min, were washed three times with TBS (0.05 M Tris base, 0.15 M NaCl, pH 7.6). In order to block unspecific sites, incubation done with normal serum for 10 min. (Diluted in 1:10 TBS). About 50 μL of IHN monoclonal antibody (diluted by TBS 1:800) was added to slides and was kept at 37°C for one h. Slides were washed by TBS for 3 other times and then the conjugate (F (ab)' 2 Rabbit Anti Mouse IgG- HRP) was added and kept for 30 min in 37°C. Sides were washed again for 3 times by TBS buffer and the substrate containing chromosole [(DAB) Diaminobenzidine tetra hydro chloride 3'] was added, after 10 min slides were put in water in order to stop the reaction. About 3 to 5 min they were stained by hematoxiline (0.4 g sodium iodate, 100 g potassium alum, 100 g chloral hydrate, 2 g citric acid, 2000 mL ddH_2O) and were washed for 10 min by the water. To dehydration of tissues, slides were put in 70% ethanol for 3 min, in 95% ethanol for 5 min, and again twice for 5 min in 100% ethanol. In order to make a transparent tissue it was put in xylol for 5 min. For microscopic study, slides were mounted and brown and gold's structures in infected tissues were studied by magnifying lenses of 20, 40 and 100.

RNA extraction: Viral RNA extraction was done by RNX^{plus} buffer as described by manufacturer (CinnaGen, Iran). Briefly, about 1 cubic mm of fish tissue was transferred to 1.5 mL micro tube, then 200 μL RNX^{plus} buffer was added. The mixture was incubated for 5 min at room temperature and then 50 μL of chloroform was added and centrifuged at 12000 rpm for 15 min at 4°C. Total tissue RNA (include viral RNA) was precipitated by ethanol and then dissolved in 10 μL of diethyl pyrocarbonate treated water (Kazemi *et al*., 2004).

Reverse transcription reaction: Reverse transcription (RT) was performed as previously described (Miller *et al*., 1998). Briefly, template RNA (1 μL) was incubated in a 20 μL reaction mixture containing: 40 pico mol of specific antisense external primer (IHNR5'- AGT CTT GTC ACA CTT CGA G-3'), 100 unit of reverse transcriptase enzyme (RT) (Fermentas, Lithuania), 20 unit RNasine (Fermentas, Lithuania), 1x RT buffer, 0.2 mM dNTP, for 1 h at 42°C.

PCR reaction: A nested-PCR was used to amplify a 543 bp of viral glycoprotein gene. First PCR reaction mixture was contained 10 μL of synthesized cDNA, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 1X PCR buffer, 40 pico mol each forward and reverse primers (Nest I primers: IHNF5'-CAT CTG CTC AAC AGG GTT CTT C-3' and IHNR5'-AGT CTT GTC ACA CTT CGA G-3' were amplified 543 bp of viral glycoprotein gene) and 1.25 unit of Taq DNA polymerase (CinnaGen Iran) and was carried out within 30 cycles of: denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 40 sec. One microlitre of PCR product was used as template DNA for second PCR. Second PCR reaction also was done like first PCR within 30 cycles (Nest II primers: IHN2 F 5`-AGA CGA TAG AGA AGG CGC TT - 3` and IHN2 R 5`-GAT TTC TGC TCC AGA ATT GT - 3, were amplified 484 bp of viral glycoprotein gene) (Pherson and Moller, 2000).

Detection of PCR product: PCR product was electrophoresed on 2% agarose gel, stained by ethidium bromide and DNA band was observed by UV light under UV Tranilluminator (Boffey, 1984).

RESULTS

Out of 100 tested samples, 35 samples were positive by immunohistochemistry, 43 samples were positive by PCR method and 38 samples showed symptoms of the disease. Table 1 shows number of centers under observation- number of positive samples and percentage of infection by immunohistochemistry and PCR methods.

Table 2 compares immunohistochemistry and PCR methods in diagnosing IHN disease and also shows clinical symptoms in fish in 100 hatcheries.

Figure 1 shows an infected tissue of fish. IHN antigen-antibody reaction is observed in golden- brown.

Figure 2 shows PCR product as 484 bp paralleled by 100 bp DNA ladder marker.

Present results showed that the most infected centers are Ardebil (50%) and Ghom provinces (100%) by Immunohistochemistry method and Ardebil (83.33%), Ghazvin, Kerman and Ghom provinces (100%) by PCR method.

Table 1: Frequency of IHN disease in fish breeding centers of each province of Iran

	Immunohistochemistry			PCR		
Province	No. of studied centers	Positive samples	Infectious (%)	No. of studied centers	Positive sample	Infectious(%)
Mazandaran	13	4	30.76	13	4	30.76
Kohkiloyeh	10	6	60	10	7	70
Ardebil	6	3	50	6	5	83.33
Gilan	2	1	50	2	0	0
Fars	11	4	36.36	11	6	54.54
East Azarbaijan	8	3	37.5	8	3	37.5
Lorestan	12	1	8.33	12	2	16.66
Markazi	2	1	50	2	1	50
Esfahan	9	2	22.22	9	4	44.44
Charmahal	10	3	30	10	3	30
Kordestan	4	2	50	4	1	25
Ghazvin	2	1	50	2	2	100
West Azarbayjan	4	3	75	4	3	75
Ghom	1	1	100	1	1	100
Tehran	2	0	0	2	0	0
Kermansha	3	0	0	3	0	0
Kerman	1	0	0	1	1	100
100 Centers, 35 positive cases 100				Centers, 43 positive cases		

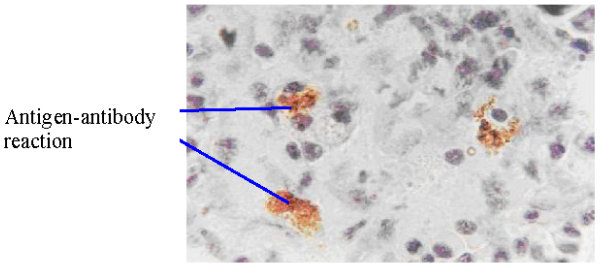

Fig. 1: Cross section of fish spleen tissue stained by immunohistochemistry and detected by monoclonal antibody. Antigen-antibody reaction was stained in golden-brown (100×12.5 magnification)

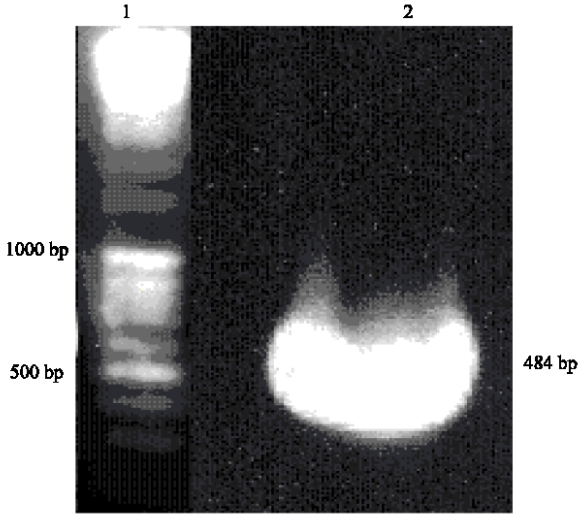

Fig. 2: Two percent agarose gel electrophoresis, Lane 1: 100 bp DNA ladder marker, Lanes 2: IHN PCR product (484 bp)

Table 2: Comparative study between immunohistochemistry, PCR and clinical symptoms in IHN diagnosis in 100 hatcheries of Iran

Method	Positive cases (%)	Frequency of positive cases
Immunohistochemistry	35	35
PCR	43	43
Clinical symptoms	38	38

DISCUSSION

In this research some samples were tested by two methods: 35 and 43 samples were positive by immunohistochemistry and PCR, respectively.

The results revealed infection and probably, IHN disease in reproducing and breeding fields of Rainbow trout in the country. Conducting immunohistochemistry test and PCR simultaneously is a good motivation in diagnosing this disease.

Isolation of the virus from some generations by Fallahi (2002) which affirms the result of the research showed the existence of the virus of this disease in some Rainbow trout breeding fields of the country. Critical affirmation of the disease with the isolation of IHN virus and the study of virus structure with an electronic microscope and also injecting the isolated virus to the main host and observing symptoms is going to be done.

Besides the classic virological serology methods, further tests for the identification and confirmation of the fish pathogen like i.e. PCR and DNA probe techniques are recommended by the OIE (2005). Following exact sanitary and controlling rules in reproducing and breeding centers prevents the infection from secreting through fields. Presence of careful sanitary control and quarantine problems and also separating sick fish or unusual fish from healthy fish with a good and powerful management may prevent serious disease spread in such units. In these cases positive immunohistochemical and PCR

results loan cannot be proofs of disease. Despite eliminating of these centers because of presence of pathogens is necessary, one of the results that explain lack of disease and clinical outcomes is that the species is not so strong. In these centers any transformation without testing should be avoided. Also in centers that clinical outcomes are observed elimination and disinfection of water should be done. Because of transmission of IHN causing virus by both vertical and horizontal ways (Fallahi, 2002), disinfections of all products such as gametes and fry and all instruments is necessary and serious. Since by PCR methods the viral genome is known to cause of disease and by Immunohistochemical method the pathogenic and expression of viral proteins are being known, in some cases their results are different:

- In case that virus which caused the disease reproduced in the cell and also the viral proteins are expressed, cell lysis causes abnormal shapes of the cell. (histopathological changes), till target tissue is being destroyed completely, this is possible to remained viruses to not have healthy cells for reproduction and its genome is also ruined by RNase and DNase of the environment which may not be detected in PCR test and if virus proteins are present in the environment, destroying effects of the virus is observables by immunohistochemistry.
- If the virus has past the first stages of reproducing and cell destroying has been done, the detection by PCR is possible but diagnosing of specific virus protein in target tissues get weaker.
- If the virus in target tissue is reproduced and specific proteins which were expressed, removed by immune system, the positive result in immunohistochemistry and negative result in PCR test is possible.
- In tissue preparation cases in immunohistochemistry method and also in RNA extraction with PCR method, not to find (reaching) the injured cells and virus of the disease is possible.

This is the first report of a field trial comparing the RT-PCR and immunohistochemistry assays in trout from 100 Iranian fish farms. RT-PCR followed by nested PCR using RNA directly extracted from fish tissue turned out to be the most sensitive method. By directly examining the tissue by means of a PCR test it was possible to detect viral RNA in acutely and sub acutely to chronically diseased fish as well as in asymptomatic IHN-carrier fish. Therefore, this effective and powerful assay for detecting IHN by means of PCR has great advantages compared with the presently used procedures. Bergmann *et al.* (2002) compared IFA, ELSA and PCR methods for diagnosis of infectious hematopoietic necrosis virus in rainbow trout and proposed that RT-PCR is the most sensitive method.

CONCLUSIONS

Considering the symptoms and obtained results with two methods of PCR and immunohistochemistry in breeding Rain bow trout in the country, the probability of the disease in these fields is so high and can be an alarm for responsible organizations.

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