https://doi.org/10.22319/rmcp.v11i3.5242

Article

Diagnosis of the infectious pancreatic necrosis virus (IPNV) by nested PCR in adult trouts

Catalina Tufiño-Loza [a]

José Juan Martínez-Maya [a]

Amaury Carrillo-González [b]

Diana Neria-Arriaga [c]

Celene Salgado-Miranda [d]

Edith Rojas-Anaya [e]

Elizabeth Loza-Rubio [e*]

[a] Universidad Nacional Autónoma de México. Facultad de Medicina Veterinaria y Zootecnia, Ciudad de México, México.

[b] Universidad del Valle de México, Campus Toluca, Estado de México, México.

[c] SENASICA, Ciudad de México, México.

[d] Universidad Autónoma del Estado de México. Centro de Investigación y Estudios Avanzados en Salud Animal, Estado de México, México.

[e] INIFAP. Centro Nacional de Investigaciones Disciplinarias en Salud Animal e Inocuidad.

Carretera México-Toluca km 15.5, Colonia Palo Alto, Ciudad de México, México.

* Corresponding author: loza.elizabeth@inifap.gob.mx, eli_rubio33@hotmail.com

Abstract:

The isolation of the infectious pancreatic necrosis virus (IPNV) in cell culture is currently the main diagnostic method. Although it is a reliable method, it is expensive, and the results take three weeks. This study aimed to establish and evaluate the use of a nested PCR (nPCR) for the rapid diagnosis of the IPNV, decreasing the diagnosis time and increasing its sensitivity. Therefore, two pairs of primers were designed based on Mexican sequences. The first pair (RT-PCR) amplified a 682 bp product, and the second pair (nPCR) 229 bp of the VP2 gene. Subsequently, 70 rainbow trout fry (*Oncorhynchus mykiss*) were infected with the virulent strain MEX3-CSM-05 at a dose of $1X10^{5.8}$ TCID50/0.02 ml. From each organism, the kidney, spleen, pyloric caeca, liver, intestine, and gills were collected. To evaluate the tests, a total of 26 clinically healthy adult trouts from commercial farms in the State of Mexico were used. The detection frequency of the IPNV using RT-PCR was 87.1 % in gills, 61.4 % in liver, 61.4 % in pyloric caeca, 58.6 % in kidney, 35.7 % in the intestine, and 32.9 % in the spleen ($P<0.05$). RT-PCR negative samples were positive in the nPCR. Similarly, samples from the wild trout organs were positive. In conclusion, the RT-PCR was less sensitive than the nPCR, which showed a sensitivity of 100 %. Therefore, nPCR is the best option for a reliable diagnosis of the IPNV in infected and sick fish.

Key words: IPNV, Nested PCR, Rainbow trout, Diagnosis.

Received: 30/01/2019

Accepted: 09/12/2019

Introduction

The constant growth of the aquaculture industry has increased the incidence and propagation of diseases, especially those generated by viruses due to their rapid dispersion and high-degree of infection. The infectious pancreatic necrosis (IPN) is an acute systemic disease that mainly affects fish from the salmonid family. It causes elevated mortality in alevins and fry, and the surviving fish become lifelong carriers of the virus[1,2]. The etiological agent of the IPN belongs to the *Birnaviridae* family, *Aquabirnavirus* genus[3,4]. It is a non-enveloped virus, with an icosahedral-shape capsid, and an approximate diameter of 60 nm[5]. Its genome consists of two segments of double-stranded ribonucleic acid (RNA)[1]. The IPN virus (IPNV) has a wide antigenic and genotypic variability. Its presence has been notified in wild and farmed salmonids in different countries around the world,

which is why it is considered a worldwide distributed disease[6,7]. IPN outbreaks are often due to imports and sales of infected eyed eggs and fry[8]. In Mexico, this disease was identified in the year 2000 in rainbow trout fry (*Oncorhynchus mykiss*) imported from the United States of America[9]. In 2009, in the main national trout-cultural stations, the national prevalence was 11.9 % and spread to 62.5 %. However, the economic impact of the virus since its identification in 2002 to date has not been calculated[10].

Viral isolation on fish cell lines, such as *Lepomis macrochirus* fry fibroblasts (BF-2), Chinook salmon embryo cells (CHSE-214), or rainbow trout gonad fibroblasts (RTG-2), followed by its identification through immunofluorescence in apparently healthy fish, is the suggested diagnosis by the World Organization for Animal Health[7]. However, although it is a reliable method, it is also expensive and slow; it takes at least three weeks to confirm a negative result[11,12]. Furthermore, this time is crucial in viral dissemination due to the rapid dispersion in lotic currents, which can cause important economic losses[2,13]. There are other viral identification tests for antigen detection, such as immunohistochemistry and ELISA (Enzyme-linked immunosorbent assay)[7]. However, problems like the dependence on these tests and monoclonal antibodies during importation, the autofluorescence of fish tissue, a high viral titer, the difficulty to obtain fresh samples from fish tissue, and the cross-reaction of antibodies frequently limit the universal and routine use of the techniques mentioned above[14,15].

Therefore, several protocols have been proposed for an efficient and rapid identification of the IPNV in infected cell cultures or tissues using the reverse transcription-polymerase chain reaction (RT-PCR) and some of its variants[16-24]. This study aimed to establish and evaluate the use of a nested PCR (nPCR) with primers designed based on Mexican viral sequences to diagnose the IPNV in tissues from experimentally infected trouts in aquaculture production units; this method would decrease the diagnosis time, which would allow implementing several IPN control strategies and prevent fish exposition in the production units.

Material and methods

Cell line

The BF-2 cell line from *Lepomis macrochirus* (ATCC® CCL 91) was used to propagate the viral MEX2-CSM-05 strain[25]. The BF-2 cell line was cultured at 20 °C in Leibovitz's L-15 medium (In vitro, Mexico) supplemented with 10 % fetal bovine serum (FBS) (Biowest,

Mexico), 100 IU/ml of penicillin, 100µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B (In vitro, Mexico).

Virus

The MEX2-CSM-05 viral strain reported by Salgado-Miranda *et al*[25] was propagated in confluent monolayers of the BF-2 cell line at 15 °C in Leibovitz's L-15 medium (In vitro, Mexico) supplemented with 2 % fetal bovine serum (FBS) (Biowest, Mexico). At 72 h post-infection, the monolayer showed signs of cytopathic effect (CPE), which consists of a gradual loss of the monolayer and rounding of infected cells. Hence, culture flasks were frozen at -20 °C and thawed twice. Subsequently, the cell suspension was collected and centrifuged at 1,200 xg for 15 min at 4 °C[7] to obtain the supernatant, from which the viral titer (Tissue Culture Infectious Dose, TCID) was determined by the Reed and Muench method.

Trouts

For the experimental study, 100 rainbow trout fry (*O. mykiss*) were acquired from the Aquaculture Center El Zarco of the Comisión Nacional de Acuacultura y Pesca (CONAPESCA). Fry had an average size of 3 cm, an average weight of 1 g, and 480 degrees-days (value obtained by multiplying the age in days by the average of temperature in Celsius degrees during the shelf life)[26]. To evaluate the test, a total of 26 clinically healthy adult rainbow trouts (*O. mykiss*) were acquired from commercial farms in the Estado de México [State of Mexico]. The clinical evaluation of fish consisted of studying their behavior, as well as their external appearance; the species showed normal countercurrent swimming patterns, where it responds to noises and stimuli (escape response). The color of the fish was normal, greenish-yellow; fish had a white belly and black dots on their back and fins. Their skin was soft, without bruises, and with intact fins. Before starting the experiment, the health status of the fish was evaluated using ten organisms from the aquaculture center. The presence of bacteria, parasites, and the IPNV was determined by isolation and PCR in the Aquaculture Health Laboratory of the Centro de Investigación y Estudios Avanzados en Salud Animal [Animal Health Research and Advanced Studies Center] of the Universidad Autónoma del Estado de México; results were negative for bacteria, parasites, and the IPNV. Therefore, the use of clinically healthy animals for the experiment was assured. In trouts from the commercial production units of Estado de México, only the behavior and external appearance evaluation was performed

since they were used to evaluate the nested PCR for the detection of the IPNV. The fish were kept in polypropylene tanks with 60 L of recirculating water, with a photoperiod of 12 h of light/ 12 h of darkness and temperature between 14-17 °C. Fish were fed with a commercial product, supplying 3 % of their total biomass per day.

Experimental infection with the IPNV

From the 100 rainbow trouts acquired from the Aquaculture Center, 70 were inoculated intraperitoneally with $1X10^{5.8}$ TCID 50/0.02 ml of the MEX3-CSM-05 viral strain. The 30 remaining organisms were kept in a different tank as a negative control for the tissue detection of the IPNV using the nPCR. After inoculation, daily clinical examinations were performed to identify and monitor the course of the disease with the clinical signs, which included decreased appetite, anorexia, hyperpigmentation, abdominal distension, moderate exophthalmos, and erratic spiral swimming. When the fish showed very advanced clinical signs, they were euthanized by overexposure to anesthesia with tricaine methanesulfonate (MS-222) (Sigma-Aldrich, 886-86-2, USA) at a concentration of 50 µg/ml[27]. After euthanasia, the following organs were collected and preserved at -80 °C: kidney, spleen, pyloric caeca, liver, intestine, and gills. Additionally, and on the same dates, two trouts from the negative control tank were euthanized every day to collect the organs mentioned above.

The fish from the commercial production units of Estado de México, upon their arrival to the laboratory, were euthanized to collect their organs. All the procedures that involved the handling of animals were performed according to the guidelines established by the Bioethics Committee for the care and reasonable use of experimental animals in research projects (Authorization number: CBCURAE-006) in the Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad of the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, where all procedures were performed.

RNA extraction and cDNA synthesis

Organs were macerated and homogenized with sterile phosphate-buffered saline (PBS) at a pH of 7.2, from this mixture, 250 µl were taken for total RNA extraction with Trizol (Invitrogen, 15596018, USA), following the manufacturer's instructions. The RNA was resuspended in DNase- and RNase-free water. The synthesis of the cDNA was performed using the M-MLV Reverse Transcriptase kit, oligo $(dT)_{12-18}$ (0.5µg/µl) (Invitrogen, USA).

The RNA was incubated with 1 µl of the oligo $(dT)_{12-18}$ (0.5 µg/µl) and 1µl of 10 mM dinucleotide triphosphates (dNTP) for 5 min at 65 °C. After incubation, 4 µl of 5X first strand buffer, 1 µl of 0.1 M dithiothreitol (DTT), and 1 µl of MML-V Reverse Transcriptase were added to the mixture, which was incubated for 1 h at 50 °C. Subsequently, the reaction was inactivated at 70 °C for 15 min and stored at -20 °C until use.

Primers

Table 1 shows the primers used in this study to detect a fragment of the VP2 gene of the IPNV and the EF-α constitutive gene[10,28]. The primers were synthesized at the Instituto de Biotecnología of the Universidad Nacional Autónoma de México (Cuernavaca, Morelos, Mexico).

Table 1: Sequence of primers used to detect the VP2 gene of the IPNV and the EF-α constitutive gene.

	Sequence	Position	GenBank access no.	Size (bp)	Ta
RT-PCR	For 5' CCGAATCAGGAAGTGGMMTTCTTG 3'	137-160		689	60º
	Rev 5' GTGACCACKGGGACGTCATTGTC 3'	796-818			
nPCR	For 5' TCACCGTCCTGAATCTACCAAC 3'	482-503		229	65º
	Rev 5' GTTGTGGAGTTSACGATGTCSGC 3'	688-710			
EF-α	For -5 'GATCCAGAAGGAGGTCACCA 3'	561-583	AF498320	150	55º
	Rev -5' TTACGTTCGACCTTCCATCC 3'	694-713			

Ta= annealing temperature; bp= base pairs.

PCR and nPCR

To amplify all the PCR products we used the Dream Taq DNA Polymerase kit (Thermo Scientific, USA), reactions were prepared with a final concentration of 2 mM of $MgCl_2$, 0.2 mM of dNTPs Mix, 0.2 mM of each of the previously described primers (forward and reverse), 5 µl of the cDNA of each sample, and 1.25 U of DNA polymerase. The PCR amplification was performed under the following conditions: initial denaturation at 95 °C for 1 min, followed by 35 denaturation cycles at 95 °C for 30 s, annealing at 60 °C for 30 s,

and extension at 72 °C for 30 s, with a final extension at 72 °C for 7 min. Subsequently, the template used for the nPCR was the product from the previous PCR; this amplification occurred under the same conditions, except that the annealing temperature was 65 °C. The PCR and nPCR products were analyzed by electrophoresis in a 1 % agarose gel in TAE (40 mM Tris, 20 mM acetic acid, and 2 mM EDTA) buffer, stained with GelRed (Biotium, USA), and visualized in a Quantity One 1-D Analysis System (Bio-Rad, USA).

Statistical analysis

The frequency of RT-PCR and nPCR positive organs of experimentally infected trouts was analyzed in contingency tables and using a Ji-squared test[25]. Additionally, for each test, we evaluated the sensitivity and specificity (Table 2). The proportion of RT-PCR positive samples in each organ was compared with the Tukey-type multiple comparison test with angular transformation, where a statistical significance of $P<0.05$ was considered[29].

Table 2: Generation of cells with which the sensitivity and specificity calculations are performed

		Fish health status		
		Infected	**Halthy**	
Molecular assay	**Positive**	True positives (TP)	False positives (FP)	Sensibility = TP / (TP+FN) Specificity =TN / (FP+TN)
	Negative	False negatives (FN)	True negatives (TN)	

Results

The experimentally infected trouts started to show clinical signs of IPN from d 7 and until d 11 post-inoculation (pi) (Table 3). Mortality was observed from d 7 pi, reaching 100 % on d 11 pi. The main findings at necropsy in inoculated trouts were an empty stomach, pale liver, and mucus in the intestine, while the trouts from the production units were clinically healthy and without injury at necropsy.

Table 3: Kinetics of the appearance of clinical signs in rainbow trouts experimentally inoculated with the MEX2-CSM-05 strain

Post-challenge day	Description of clinical signs
1-6	No clinical signs were observed.
7	Anorexia, hyperpigmentation, decreased appetite, abdominal distension, moderate exophthalmos, erratic spiral swimming in some trouts.
8	Increased number of trouts with erratic spiral swimming, hyperpigmentation, anorexia, abdominal distension.
9	Increased number of trouts with erratic spiral swimming, hyperpigmentation, anorexia, abdominal distension. Some organisms only remained at the bottom.
10	Erratic spiral swimming, hyperpigmentation, anorexia, abdominal distension.
11	Erratic spiral swimming, hyperpigmentation, anorexia, abdominal distension.

The results obtained from the RT-PCR in the different organs of experimentally inoculated trouts allowed the detection of the IPNV in different proportions. The organs with the highest frequency of viral detection were gills (87.1 %), liver (61.4 %), pyloric caeca (61.4 %), and kidney (58.6 %) ($P<0.05$). Moreover, the organs with the lowest frequency of viral detection were the intestine (35.7 %) and spleen (32.9 %) (Table 4) ($P<0.05$). However, in these last samples, the nPCR detected a product of 229 bp, previously confirmed by sequentiation to belong to the IPNV. The organs from the trouts in the control tank were negative to the nPCR amplification.

Table 4: Detection by RT-PCR and nPCR of the VP2 gene of the IPNV in the organs of rainbow trouts inoculated with the MEX2-CSM-05 strain

Organ	RT-PCR		nPCR	
	Positive samples	%	Positive samples	%
Kidney	41/70	58.6	29/70	41.4
Liver	43/70	61.4	27/70	38.6
Pyloric caeca	43/70	61.4	27/70	38.6
Intestine	25/70	35.7	45/70	64.3
Gills	61/70	87.1	9/70	12.9
Spleen	23/70	32.9	47/70	67.1

Based on the previous results, the RT-PCR was less sensitive than the nPCR in each of the analyzed organs (Table 5); however, its specificity was of 100 %. The nPCR showed a sensitivity and specificity of 100 %. Trout organs from the commercial production units in Estado de México showed positive results only with the nPCR, the frequency of detection was 100 %.

Table 5: RT-PCR sensitivity

Organ	Sensitivity (%) (CI)	Specificity (%)
Kidney	58.6 (47.0, 70.1)	100
Liver	61.4 (50.0, 72.8)	100
Pyloric caeca	61.4 (50.0, 72.8)	100
Intestine	35.7 (24.5, 46.9)	100
Gills	87.1 (79.3, 95.0)	100
Spleen	32.9 (21.9, 43.9)	100

CI= 95 % confidence intervals.

Discussion

Currently, the diagnosis of several fish diseases uses fast, sensitive, and specific molecular techniques; this has allowed the timely detection of a large number of infectious agents. Therefore, prevention and control measures for many diseases have been developed, implemented, and improved.

Since its first detection in the *Salvenilus fontinalis* trout, the IPNV has been identified in a wide variety of fish and invertebrate species, but with significant impact in the salmonids distributed worldwide[30,31]. Although evidence shows that the virus is currently present in

almost all of the trout productive States in the country[32], the reported clinical disease cases are few; this may be because the Mexican isolates of the IPNV are related to the VR-299 strain from the USA, initially reported by Ortega *et al*[9], which is considered of low virulence[33]. Therefore, for this study, two pairs of specific primers were designed to detect the Mexican strains of the IPNV that circulate in the country and the reference strain Sp[10]; since, due to the antigenic variability of this virus, it has been shown that the primers that recognize strains from other parts of the world do not recognize the strains currently circulating in the country[21,34].

Recently, molecular techniques, such as the PCR, have been widely used to detect fish viruses[9,30]. The RT-PCR has been applied to detect the IPNV due to its precision, speed, and high sensitivity[18,19,21,34]. This technique can specifically detect the viral genome without previous isolation of the virus; this was conducted in Iran, where a RT-PCR confirmed the presence of the IPNV for the first time in rainbow trout farms in the Fars province, this isolate is similar to the Ab strain[35].

The use of the RT-PCR has been described for the detection of the IPNV genome in cell cultures, experimentally inoculated fish, and naturally infected fish, crustaceans, and mollusks[35]. The IPNV infection is lethal in young salmonids, although this virus can be isolated in different organs of infected fish at all ages[13]. In this study, the nPCR efficiently detected a fragment of the genome of the IPNV in rainbow trout fry and in the trouts from commercial farms, which are heavier and older.

Although the isolation of aquatic birnaviruses in apparently healthy species is common, it has been shown that the IPNV infection may not be detected even when the samples have been examined by cell culture[36,37]. Several studies showed that the RT-PCR was more sensitive than cell culture isolation for the detection of the IPNV[38]. The real-time RT-PCR is slightly more sensitive than viral isolation in cell culture; the OIE recommends the latter to detect the IPNV in carrier fish, which have low viral concentrations in kidneys, which can limit its detection by viral isolation[36,39].

According to Milne *et al*[13], fish with advanced signs of IPNV infection have relatively high viral titers. Therefore, the use of an end-point RT-PCR as a qualitative molecular technique could facilitate viral genome detection. However, in this study, the RT-PCR assay detected a fragment of the IPNV genome in a percentage of the experimentally infected fish, depending on the evaluated organ. However, the RT-PCR negative samples turned out to be positive with the nPCR assay, which had an increased sensitivity of 100 %. The same was observed in the samples from apparently healthy juvenile trouts from commercial production units; these samples were positive using the nPCR; this could be explained by the frequently low concentration of viral particles in asymptomatic carriers, which difficult viral detection by RT-PCR[13].

In this study, the results obtained in adult fish determined that the studied animals could be healthy carriers; this happens when the fish survive the infection by the IPNV and continue with their productive cycle, contributing to the vertical transmission of the virus through ova or semen. These asymptomatic carriers may show no apparent clinical signs or pathological changes[40,41]; and, depending on the progression of the disease over time and the immune response of each specimen, macro or microscopic changes may not be observed in the different fish organs. However, the persistently IPNV infected salmonids are a potential source of disease spread and potentially detectable by PCR. It is important to emphasize that it is very likely that the detected IPNV cases detected using the nPCR are related to the low virulence strains, and, therefore, cause no significant harm to the trout production. However, the potential risk of introducing and spreading other IPNV strains could also result from the eyed egg importation from the USA and other European, African, and South American countries[32]. For this reason, the proposed nPCR in this study is of great importance for early, rapid, and reliable detection.

The high sensitivity of this type of PCR has been demonstrated in numerous studies. A sensitivity of up to 10 pg has been detected in purified RNA samples from salmonid isolates[34]. Lopez-Lastra *et al*[18] developed a nPCR to detect up to 1 pg of the IPNV in asymptomatic carriers from field samples. Suzuki *et al*[37] developed a nPCR using a pair of primers based in the detection of the VP2/NS gene junction region of aquatic birnaviruses with a sensitivity of 1 fg (femtogram) of viral genome in the sample.

The nPCR increases the specificity and reduces the detection of false positives when the second pair of primers amplifies only if the first pair generated the expected DNA fragment[21,34,42]. Another advantage of the proposed nPCR is the viral detection in the infected tissues without the need to isolate the virus in cell culture, as, although it is considered a reference test for diagnosis, it is a technique that is limited to laboratories with professionals trained in the use of cell lines, the necessary equipment for their maintenance and incubation, and the identification of the cytopathic effect[7].

Other variants of PCR and testing have been proposed, but their adoption depends on the equipment available in the laboratories. For example, Rodríguez *et al*[21] compared six diagnostic methods for the IPNV and found that the RT-PCR and flow cytometry were the most adequate and sensitive methods for routine detection of the IPNV. A different study[23] reported a low sensitivity (43 %) using RT-PCR; therefore, they used a short cell culture incubation protocol as a complementary technique, in addition to a multiplex PCR with three pairs of primers in one reaction to increase the probability to identify all the serotypes of the IPNV serogroup A and prevent a false negative result. Even though other molecular techniques have been proposed for a rapid and high sensitivity diagnosis, like the real-time RT-PCR and the RT-LAMP (Loop-mediated isothermal amplification), these require specialized equipment and training in designing primers and interpreting results[22,42]. In

Mexico, the diagnosis of the IPNV is mainly made by viral isolation in cell culture and the detection of the viral genome by end-point RT-PCR, this technique is performed in most of the laboratories conventionally.

Moreover, the detection of the IPNV in the different organs analyzed in this study confirms the wide spread of the virus. Usually, the organs recommended for the detection of the IPNV in cell culture are kidney, liver, spleen, and the ovarian fluid of broodfish or the entire alevin[7]. However, the main target organ for propagation is the kidney, where the virus persists[39]. Unlike the kidney, other target organs for viral detection are pancreas, intestine, liver, and gills. The pancreas is anatomically diffuse between the pyloric caeca, and it develops severe necrosis. The intestine develops an acute enteritis characterized by the necrosis of cells and glands in the digestive tract, which is responsible for the elimination of the virus in the feces and mucus that it produces[6]. In the liver, the IPNV induces apoptosis markers, also found in the intestine and pancreas, that correspond to the viral accumulation and pathological changes in the tissue[43,44]. Finally, the gills, which are responsible for oxygen exchange, may appear pale due to the degenerative and necrotic damage to the epithelium. In this study, the organs with the highest viral frequency of detection were the pyloric caeca, intestine, and gills, which, as mentioned before, have been identified as target organs for the early detection of the disease, since they are sites of early viral propagation[45].

Conclusions and implications

The nPCR with the primers developed for the identification of Mexican isolates is useful for the diagnosis of the IPNV, not only in clinically infected fish but also to detect infected fish without clinical signs. Furthermore, this study suggests the use of this method to confirm an outbreak of IPN in a production unit. If necessary, viral isolation, histopathological, or immunohistochemical studies are recommended in suspected or nPCR positive cases.

Acknowledgments

This research was carried out with the financial support of the Consejo Nacional de Ciencia y Tecnología (CONACYT) of project CB -2009-01-134099. Catalina Tufiño Loza received support from the CONACYT. The authors also thank A. Mejía, A. Fabián, and J.C. Gómez of the Universidad del Valle de México (UVM) for their excellent technical assistance.

Literature cited:

1. Rodríguez SJS, Borrego JJ, Perez PSI. Infectious pancreatic necrosis virus: biology, pathogenesis, and diagnostic methods. Adv Virus Res 2003;62(1):113-165.

2. Salgado-Miranda C. Necrosis pancreática infecciosa: enfermedad emergente en la truticultura de México. Vet Méx 2006;37(4):467-477.

3. Dobos P, Roberts TE. The molecular biology of infectious pancreatic necrosis virus: a review. Can J Microbiol 1983;29(4):377-384.

4. King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, King AMQ, *et al*, editors. Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses. New York, USA: Academic Press; 2012:499-507.

5. Dobos P. Birnaviruses. Encyclopedia of life sciences. Nature Publishing Group 2001, London.

6. Dhar A, LaPatra S, Orry A, Allnutt T. Infectious pancreatic necrosis virus. In: Woo PTK, Cipriano RC editors. Fish viruses and bacteria: Pathobiology and protection. 1rst ed. Wallingford, Oxfordshire, UK: CAB International; 2017: 1-12.

7. OIE. Manual of diagnostic tests for aquatic animals. 5th ed. Paris, France: World Organization for Animal Health. 2006.

8. Munro ES, Midtlyng PJ. Infectious pancreatic necrosis and associated aquatic birnaviruses. In: Woo PT, Bruno DW, editors. Fish diseases and disorders. Volume 3: viral, bacterial and fungal infections, 2nd ed. Oxfordshire, UK: CABI; 2011:1-65.

9. Ortega SC, Montes de Oca R, Groman D, Yason C, Nicholson B, Blake S. Case report: viral infectious pancreatic necrosis in farmed Rainbow Trout from Mexico. J Aquat Anim Health 2002;14(4):305-310.

10. Salgado MC. Virulencia del virus de la necrosis pancreática infecciosa en trucha arcoíris (*Oncorhynchus mykiss*) a partir de aislamientos mexicanos [tesis doctorado]. México, DF: Universidad Nacional Autónoma de México; 2014.

11. Amos KH. Procedures for the detection and identification of certain fish pathogens, 3rd ed. Fish Health Section, American Fisheries Society, Corvallis; 1985.

12. Ahne W, Thomsen I. 1986. Infectious pancreatic necrosis: detection of virus and antibodies in rainbow trout IPNV-carrier (Salmo gairdneri). J Vet Med 1986;33(7):552-554.

13. Milne SA, Galachacher S, Cash P, Porter AJR. A reliable RTPCR-ELISA method for the detection of infectious pancreatic necrosis virus (IPNV) in farmed rainbow trout. J Virol Methods 2006;132(1-2):92-96.

14. Sanz F, Coll J. Techniques for diagnosing viral diseases of salmonid fish. Dis Aquat Org 1992;13:211-223.

15. LaPatra SE. The use of serological techniques for virus surveillance and certification of finfish. Annu Rev Fish Dis 1997;6:1-28.

16. Way-Shyan W, Yea-Ling W, Jainn-Shyan L. Single-tube, non-interrupted reverse transcription PCR for detection of IPNV. Dis Aquat Org 1997;28(3):229-233.

17. Pryde A, Melvin WT, Munro ALS. Nucleotide sequence analysis of the serotype-specific epitope of infectious pancreatic necrosis virus. Arch Virol 1993;129(1-4):287-293.

18. Lopez-Lastra M, Gonzalez M, Jashes M, Sandino AM. A detection method for infectious pancreatic necrosis virus (IPNV) based on reverse transcription (RT)-polymerase chain-reaction (PCR). J Fish Dis 1994;17(3):269-282.

19. Blake S, Lee MK, Singer J, McAllister PE, Nicholson BL. Detection and identification of aquatic birnavirus by polymerase chain reaction assay. J Clin Microbiol 1995;33(4):835-839.

20. Alonso M, Rodríguez S, Pérez-Prieto SI. Viral coinfection in salmonids: infectious pancreatic necrosis virus interferes with infectious hematopoietic necrosis virus. Arch Virol 1999;144(4):657-673.

21. Rodriguez SJ, Borrego JJ, Perez-Prieto SI. Comparative evaluation of five serological methods and RT-PCR assay for the detection of IPNV in fish. J Virol Methods 2001;97(1-2):23-31.

22. Bowers RM, Lapatra SE, Dhar AK. Detection and quantitation of infectious pancreatic necrosis virus by real-time reverse transcriptase-polymerase chain reaction using lethal and non-lethal tissue sampling. J Virol Methods 2008;147(2):226-34.

23. Barrera-Mejía M, Simón-Martínez J, Salgado-Miranda C, Vega F, Ortega C, Aragón A. Development and validation of a short-time cell culture and multiplex reverse transcriptase polymerase chain reaction assay for infectious pancreatic necrosis virus in Mexican farm-sampled rainbow trout. J Aquat Anim Health 2009;21(3):167-72.

24. Tapia D, Eissler Y, Torres P, Jorquera E, Espinoza JC, Kuznar J. Detection and phylogenetic analysis of infectious pancreatic necrosis virus in Chile. Dis Aquat Org 2015;116(3):173-84.

25. Salgado-Miranda C, Rojas-Anaya E, García-Espinoza G, Loza-Rubio E. Molecular characterization of the VP2 gene of Infectious Pancreatic Necrosis Virus (IPNV) isolates from Mexico. J Aquat Anim Health 2014;26(1):43-51.

26. Dorson M, Touchy C. The influence of fish age and water temperature on mortalities of rainbow trout, Salmo gairdneri Richardson, caused by a European strain of infectious pancreatic necrosis virus. J Fish Dis 1981;4(3):213–221.

27. de las Heras A, Perez-Prieto S, Rodríguez Saint-Jean S. In vitro and in vivo immune responses induced by a DNA vaccine encoding the VP2 gene of the infectious pancreatic necrosis virus. Fish Shellfish Immunol 2009;27(2):120-129.

28. Ingerslev HC, Fausa EP, Jakobsen RA, Petersen CB, Wergeland HI. Expression profiling and validation of reference gene candidates in immune relevant tissues and cells from Atlantic salmon (*Salmo salar* L.). Mol Immunology 2006;43(8):1194-1201.

29. Zar JH. Biostatistical analysis. 5th ed. New Jersey, USA: Prentice Hall; 2010.

30. Wolf K. Fish viruses and fish viral diseases. Ithaca, New York: Cornell University Press; 1988.

31. Smail DA, Bain N, Bruno DW, King JA, Thompson F, Pendrey DJ, Morrice S, Cunningham CO. Infectious pancreatic necrosis virus in Atlantic Salmon, *Salmo salar* L., post-smolts in the Shetland Isles, Scotland: virus identification, histopathology, immunohistochemistry and genetic comparison with Scottish mainland isolates. J Fish Dis 2006;29(1):31–41.

32. Ortega C, Valladares B, Arguedas D, Vega F, Montes de Oca R, Murray A. Distribution of infectious pancreatic necrosis virus (IPNV) based on surveillance programs in freshwater trout farms of Mexico. J Aquat Anim Health 2016;28(1):21-26.

33. Barrera-Mejía M, Martínez S, Ortega C, Ulloa-Arvizu R. Genotyping of infectious pancreatic necrosis virus isolates from Mexico state. J Aquat Anim Health 2011;23(4):200-206.

34. Alonso MC, Cano I, Castro D, Perez-Prieto SI, Borrego JJ. Development of an in situ hybridization procedure for the detection of sole Aquabirnavirus in infected fish cell cultures. J Virol Meth 2004;116(2):133-138.

35. Akhlagi M, Hosseini A. First report on the detection of infectious pancreatic necrosis virus (IPNV) by RT-PCR in rainbow trout fry cultured in Iran. Bull Eur Ass Fish Pathol 2007;27(5):205-210.

36. Taksdal T, Ramstad A, Stangeland K, Dannevig BH. Induction of infectious pancreatic necrosis (IPN) in covertly infected Atlantic salmon *Salmo salar* L. post-smolts by stress exposure, by injection of IPN virus (IPNV) and by cohabitation. J Fish Dis 1998;21(3):193-204.

37. Suzuki S, Hosono N, Kusuda R. Detection of aquatic birnavirus gene from marine fish using a combination of reverse transcription and nested PCR. Mar Biotechnol 1997;5(4):205-209.

38. Taksdal T, Dannevig BH, Rimstad E. Detection of infectious pancreatic necrosis (IPN)-virus in experimentally infected Atlantic salmon parr by RT-PCR and cell culture isolation. Bull Eur Ass Fish Pathol 2001;21(5):214-219.

39. Ørpetveit I, Mikalsen AB, Sindre H, Evensen Ø, Dannevig BH, Midtlyng PJ. Detection of infectious pancreatic necrosis virus in subclinically infected Atlantic salmon by virus isolation in cell culture or real-time reverse transcriptase polymerase chain reaction: influence of sample preservation and storage. J Vet Diagn Invest 2010;22(6):886-895.

40. Gahlawat SK, Munro ES, Ellis AE. A non-destructive test for the detection of IPNV carriers in Atlantic halibut *Hippoglossus hippoglossus* (L.). J Fish Dis 2004;27(4):233-239.

41. Rimstad E, Hornes E, Olsvik O, Hyllseth B. Identification of a double-stranded RNA virus by using polymerase chain reaction and magnetic separation of the synthesized DNA segments. J Clin Microbiol 1990;28(10):2275-2278.

42. Soliman H, Midtlyng PJ, El-Matbouli M. Sensitive and rapid detection of infectious pancreatic necrosis virus by reverse transcription loop mediated isothermal amplification. J Virol Methods 2009;158(1-2):77-83.

43. Imajoh M, Hirayama T, Oshima S. Frequent occurrence of apoptosis is not associated with pathogenic infectious pancreatic necrosis virus (IPNV) during persistent infection. Fish Shellfish Immunol 2005;18(2):163-177.

44. Santi N, Sandtrø A, Sindre H, Song H, Hong JR et al. Infectious pancreatic necrosis virus induces apoptosis *in vitro* and *in vivo* independent of VP5 expression. Virology 2005;342(1):13-25.

45. Shankar KM, Yamamoto T. Prevalence and pathogenicity of infectious pancreatic necrosis virus (IPNV) associated with feral lake trout, Salvelinus namaycush (Walbaum). J Fish Dis 1994;17(5):461-471.