Short Communication

Presence of Infectious Pancreatic Necrosis Virus on Rainbow Trout (*Oncorhynchus mykiss*) by Histopathology, ELISA and RT-PCR

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Abstract

Trout (*Oncorhynchus mykiss*) culture in Mexico experiences sporadic and significant fish mortalities, where disease signs are associated with infectious pancreatic necrosis virus disease (IPNV). The purpose of the present work was to support the potential of ELISA, histopathology, and RT-PCR as routine techniques for IPNV detection. Fourteen trout farms were monitored after a disease outbreak. Positive results were confirmed in different collected organisms from 21% of tested farms by all 3 techniques. Virus detection by ELISA and RT-PCR was successfully performed in only two days after the initial signs of disease were observed in fish, since these methods are faster when compared to histopathology, which only detected signs of disease such as necrosis of the pancreatic acinus and intestine after 5 days. The results suggest that ELISA and RT-PCR offer an early, sensitive, faster and cheaper alternative for the routine detection of IPNV compared to cell culture and histopathology. These techniques can be performed for fish health monitoring and are reliable tools in the control, prevention and timely detection of IPNV.

Keywords: ELISA, infectious pancreatic necrosis, trout, reverse transcription polymerase chain reaction

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บทคัดย่อ

การพบ Infectious Pancreatic Necrosis Virus ในปลาเรนโบว์เทราท์ (*Oncorhynchus mykiss*) โดยวิธีทางจุลพยาธิวิทยา ELISA และ RT-PCR

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อุตสาหกรรมการเพาะเลี้ยงปลาเรนโบว์เทราท์ (*Oncorhynchus mykiss*) ในประเทศเม็กซิโก เคยประสบปัญหาการตายของปลา ซึ่งพบว่าอาการดังกล่าวมีความสัมพันธ์กับโรคติดเชื้อไวรัส infectious pancreatic necrosis virus disease (IPNV) วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาศักยภาพของวิธีการวินิจฉัยโรค IPNV โดยวิธี ELISA จุลพยาธิวิทยา และ RT-PCR. โดยทำการศึกษาในฟาร์มปลาเรนโบว์เทราท์ จำนวน 14 ฟาร์มที่เคยพบการระบาดของโรค พบว่าร้อยละ 21 ของตัวอย่างทั้งหมดที่เก็บจากฟาร์มให้ผลบวกทั้ง 3 วิธี การตรวจหาแอนติเจนของไวรัส โดยวิธี ELISA และ RT-PCR สามารถที่ให้ผลบวกภายในวันที่ 2 ของวันที่ปลาเริ่มป่วย ซึ่งทำการยืนยันผลโดยวิธีทางจุลพยาธิวิทยาที่สามารถตรวจพบรอยโรคในวันที่ 5 จากผลการศึกษาพบว่าวิธี ELISA และ RT-PCR มีความไว รวดเร็ว และเป็นแนวทางเลือกที่ดีในการวินิจฉัยโรค IPNV เมื่อเปรียบเทียบกับการเพาะแยกเชื้อไวรัส และการตรวจรอยโรคทางจุลพยาธิวิทยา ซึ่งสามารถนำวิธีดังกล่าวมาใช้ในการเฝ้าระวัง ควบคุมและป้องกันโรคในฟาร์มปลาได้เป็นอย่างดี

คำสำคัญ: ELISA, infectious pancreatic necrosis, ปลาเรนโบว์เทราท์, reverse transcription polymerase chain reaction

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Introduction

Infectious pancreatic necrosis virus (IPNV) of the Aquabirnavirus genus and Birnaviridae family is a well-known pathogen of rainbow trout (*O. mykiss*). This virus is one of the major etiological agents found in farms and wild salmonid fish, and its genome has a double-stranded RNA packaged in a nonenveloped icosahedral shell, 60 nm in diameter (Cutrín et al., 2000; Zhang and Suzuki, 2004). This is a highly contagious disease initially detected in North America (Roberts and Pearson, 2005) with mortalities ranging from 70-100% in fingerlings and early juvenile fish, usually following culture stress (Evensen and Lorenzen, 1997). The susceptibility to this virus decreases with age, but asymptomatic organisms facilitate horizontal and vertical transmission (Brown and Bruno, 2002). Clinical signs include skin hyperpigmentation, distended abdomen with ascites, empty gut, presence of clear or milky mucus in stomach and anterior intestine, long, thin white fecal casts, spiral swimming, pale spleen, heart, liver, and petechiae observed in the viscera (Crane et al., 2000; Brown and Bruno, 2002).

Rainbow trout farming in Mexico is a growing and new industry whose production increased from 1612 to 11,792 metric tons in one decade (1998-2008); with most of the rainbow trout farms and hatcheries located in Chihuahua, Durango, Mexico, Michoacán, Morelos, and Puebla (CONAPESCA 2008). However, other states such as Hidalgo, Jalisco, Nuevo León, and San Luis Potosi have small scale extensive farms with potential to increase their production because of their association to eco-tourism and proximity to big cities. The first report of IPNV in Mexico occurred in 2002, from O. mykiss samples obtained from a farm located in Central Mexico (Ortega et al., 2002); however, IPNV distribution in farm and wild stock in Mexico has been little understood and poorly evaluated (Barrera-Mejia et al., 2002).

The standard protocol for the routine detection of IPNV is usually based on virus isolation (Milne et al., 2006), complemented by histopathology, immunofluorescence, serology, and molecular biology (Barrera-Mejia et al., 2002; Bowden et al., 2002; Jenčič et al., 2002). Viral isolation is considered as the gold standard, but it is laborious, expensive and time consuming (Kerr and Cunningham, 2006; Milne et al.,

2006). The purpose of the present study was to evaluate the use of RT-PCR and ELISA for the routine diagnosis of IPNV in aquatic health labs.

Materials and Methods

Fourteen rainbow trout farms in the Mexican state of Hidalgo (Fig. 1) were monitored for the presence of IPNV virus. Four hundred and ninety six fish (*O. mykiss*) showing clinical signs such as erratic swimming, loss of appetite, skin darkening, distended abdomen, exophthalmia, and long thin white fecal casts were collected. Fingerlings and early juveniles were collected without dissection and immediately submerged into 10% neutral buffered formalin (Evensen and Lorenzen 1997). Older juveniles were dissected in situ and their gills, brain, heart, spleen, kidney, liver, pancreas, and intestine samples were collected (Barlic-Maganja et al., 2002; Kerr and Cunningham, 2006). The ELISA samples were washed with sterile PBS and stored at -80ºC until used. Samples for RT-PCR were washed with PBS and placed in RNAlater® (Ambion, USA) for storage (Phelan et al., 2005).

The fixed samples (whole organisms or tissues) were processed for routine histology; embedded in paraffin according to standard procedures, sectioned at 4-6 µm, stained with H&E. The samples were examined by light microscopy (Carl Zeiss/Axiostar) coupled to a digital camera (Canon, Powershot G6 PC1089) to look for IPNV-associated morphologic changes such as necrotic lesions and ulcers in the pancreatic acini, esophagus, stomach, intestine, and renal hematopoietic elements (Bowden et al., 2002; Roberts and Pearson, 2005).

Fish samples from the same pond were pooled in groups of three or four organs, and homogenized with a pellet mixer (VWR, USA) at 200 rpm for 1 min with sterile PBS, and independently analyzed. All samples were tested with an ELISA kit for IPNV (Test-line®, Czech Republic) according to the manufacturer's instructions (Bowden et al., 2002; Jenčič et al., 2002). The samples were analyzed with a Bio-Rad (USA) microplate reader at 415 nm, and were positive when absorbance was greater than 0.5. Positive controls used were supplied by Laboratorio de Biotecnología 1, Facultad de Ciencias Químicas, Universidad Autonoma de Chihuahua (LB1-FCQ-UACH), and the test-line kit.

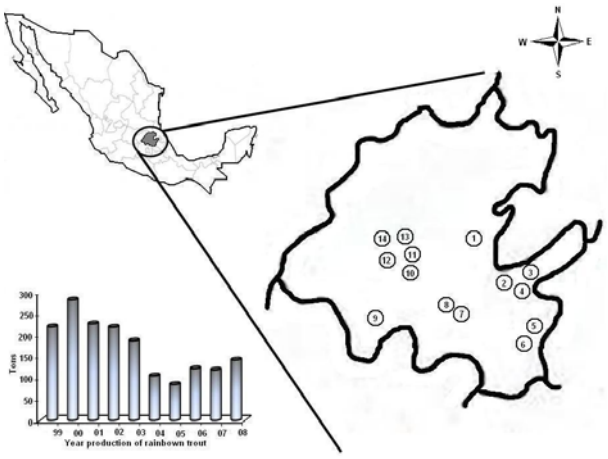

Figure 1 Trout (*Oncorhynchus mykiss*) farm locations in Hidalgo state, Mexico, and yearly aquaculture yield.

Rainbow trout samples (whole organisms or tissues) were sent for RT-PCR analysis to the LB1-FCQ-UACH. All samples were pooled in groups of three or four organs, homogenized with a pellet mixer at 200 rpm for 1 min with sterile RNAlater® at 4ºC. All samples were processed with a RNA isolation kit (Quantum Prep AquaPure Genomic) according to the manufacturer's instructions (Holmes et al., 2003). Reverse transcription reactions were performed using the protocol of SuperScriptTM II ARNse reverse transcriptase (Invitrogen Inc., USA)(Cutrín et al., 2000). For IPNV detection, PNF primers [ATCTGCGGTGTAGACATCAAA (Forward) and TGCAGTTCCTCGTCCATCCC (Reverse)](Taksdal et al., 2001; Barrera-Mejia et al., 2002) were used for the PCR reaction where the visualization of a 224 bp band was considered as a positive result (Fig. 2). PCR was carried out in a total volume of 25 µl of FSB buffer (10 mM dNTP's, 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8.3) containing 15 pmol of each primer, and 2.5 U of Taq polymerase (Promega, Inc.). The PCR reactions were carried out in a thermocycler (Corbett Research) under the following conditions: 96ºC for 5 min followed by 94ºC for 60 sec (denaturalization), 55ºC for 30 sec (annealing) and 72ºC for 60 sec (extension). After 35 cycles, the reactions were cooled down to 4ºC, and PCR products were analyzed on 2% agarose gel in 1x TBE buffer (89 mM Tris HCl, 89 mM boric acid, 2 mM EDTA, pH 8) at 80 V for 60 min. The gels were stained with ethidium bromide, and analyzed with imaging system software from Kodak (Model Gel logic-200).

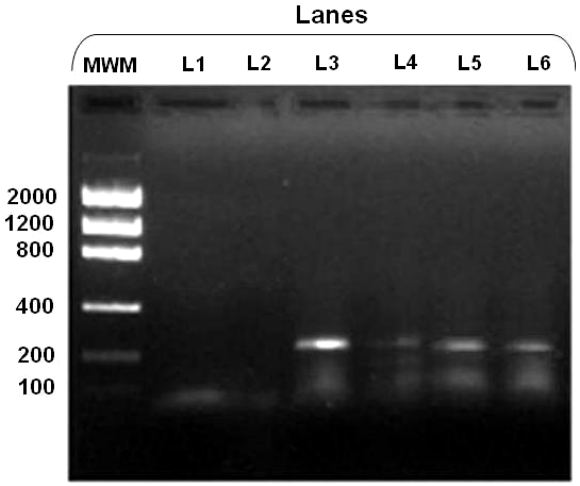

Figure 2 IPNV amplification from trout samples in 2% agarose gel electrophoresis in 1x TBE at 80 volts by 60 min. MWM: molecular weight marker; Lane 1 and 2: negative control (trout tissue sample and distillate sterile water, respectively); Lane 3: positive control from LB1-FCQ-UACH; Lane 4-6: trout samples.

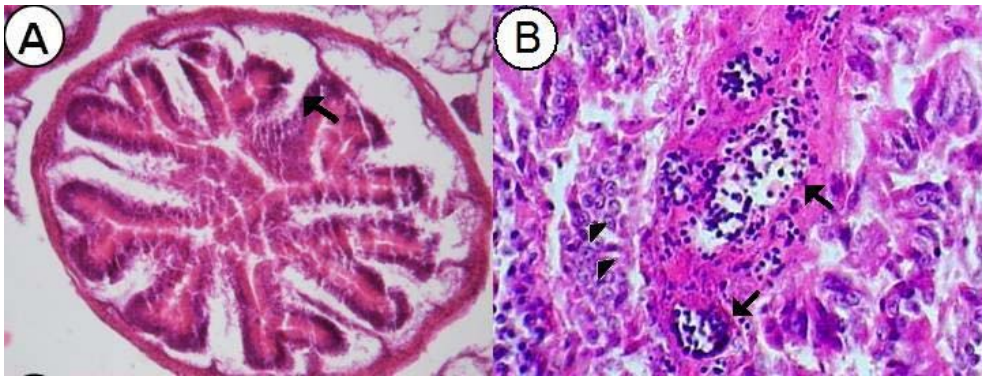

Figure 3 Histological sections from *Oncorhynchus mykiss*. (A) Acute necrosis in pancreas (arrow) (H&E, 4x). (B) Generalized necrosis in intestine (arrow) and cells with nuclear pyknosis and karyorrhexis (arrow head)(H&E, 40x).

Results and Discussion

The detection protocols for different pathogens (virus, bacteria, fungi, etc.) on aquatic organisms help to increase the knowledge about their presence, biogeographical distribution, effects, and control on aquaculture and fisheries production settings (Ghittino et al., 2003). In Mexico, rainbow trout production areas have shown a sudden and significant increase in mortality in fingerlings and early-juveniles during 2003-2006 (CONAPESCA 2008), which had a direct effect on fish farms. The international diagnosis protocol for IPNV is based on virus isolation by cell culture; which is a laborious, expensive and time-consuming activity. Histology, immunofluorescence, immunohistochemistry (Ellis et al. 2010), ELISA (Bowden et al., 2002; Jenčič et al., 2002), and RT-PCR (Taksdal et al., 2001; Barrera-Mejia et al., 2002) are other methods that have been suggested as complementary for IPNV detection, because of their sensitivity, speed and economy.

In this study, the organisms collected (496) from rainbow trout farms (14) consisted of fingerlings (13%), early-juveniles (26%), juveniles (52%) and adults (9%). Significant histopathological lesions were observed in the different fish samples examined; however, only samples from farms 1, 4 and 8 showed lesions associated to IPNV, such as pancreatic necrosis, necrotizing enteritis; mucosal epithelial necrosis and catharrhal enteritis (Fig 3). Renal tissues displayed moderate degenerative tubules with necrotic areas (Brown and Bruno, 2002; Roberts and Pearson, 2005). However, the usefulness of histopathology in IPNV diagnosis is only effective when: i) a specific sign is associated to a specific pathogen; ii) this specific sign is expressed at a high enough level; and iii) the aquatic pathologist experience. The histopathological changes observed in the collected samples were not exclusive of IPNV diseases and the use of other diagnostic protocols were necessary for a confirmed diagnosis of the specific pathogen affecting the organism.

Table 1 shows the results of the ELISA and RT-PCR analyses for all trout samples. It can be seen that samples from three farms (1, 4, and 8; = 21% of tested farms) were positive to IPNV (100%, pool of samples) by ELISA (1.12, 1.17, and 1.13 of S/P ratio mean, respectively) and RT-PCR, in a much shorter time frame compared to histopathology and exhibited a higher sensitivity to IPNV detection.

Prevention of pathogen exposure is the most effective way of controlling disease, with farms including routine diagnostic examinations for their aquatic health programs. These techniques may represent an advantage for the routine and timely diagnosis of IPNV in trout culture and wild stock studies, since early pathogen identification is crucial in the prevention of spread of infection, and in decision making for treatment and disease control (Barrera-Mejia et al., 2002), which represent possible effective containment and elimination of an emergency disease outbreak and epidemic.

Table 1 ELISA and RT-PCR results from trout farm of Hidalgo state, Mexico.

Trout farm	Sample number	ELISA*				RT-PCR		
		K+	T+	W-	Sample	T+	W-	Sample
1	40	R	R	ND	R	+	-	+
2	8	R	R	ND	ND	+	-	-
3	5	R	R	ND	ND	+	-	-
4	14	R	R	ND	R	+	-	+
5	96	R	R	ND	ND	+	-	-
6	8	R	R	ND	ND	+	-	-
7	10	R	R	ND	ND	+	-	-
8	155	R	R	ND	R	+	-	+
9	14	R	R	ND	ND	+	-	-
10	16	R	R	ND	ND	+	-	-
11	16	R	R	ND	ND	+	-	-
12	16	R	R	ND	ND	+	-	-
13	60	R	R	ND	ND	+	-	-
14	38	R	R	ND	ND	+	-	-

*Reactive (R) where the mean absorbance in wells with positive antigen is < 0.5. ND: not determined.
Control positive: T+ (sample from LB1-FCQ-UACH), K+ (control positive from test-line kit)
Control negative: W- (distilled water).

Acknowledgements

This study was funded by grants from the Mexican Council of Science and Technology (CONACyT), project SAGARPA-CONACYT-2003-002-227). We thank Laboratorio de Biotecnología 1, Fac. de Ciencias Químicas, Universidad Autonoma de

Chihuahua (Dr. G. Erosa de la Vega) for supplying the positive control and the laboratory facilities for this work.

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