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NOTE

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Virulence of infectious pancreatic necrosis virus (IPNV) isolates from Mexico

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ABSTRACT. Infectious pancreatic necrosis virus (IPNV) causes economic losses in Mexican rainbow trout industry. In this study, virulence and genetic fingerprints of Mexican IPNV isolates was investigated for the first time. Two Mexican IPNV isolates were analyzed in rainbow trout fry and the Sp strain was included as high virulence. One of the Mexican IPNV isolate was obtained from diseased fish and the other from fish without clinical signs. The infection was performed using a standardized immersion. Clinical signs were observed at 4 days post infection in fry group infected with strain Sp, two days earlier than in trout infected with IPNV isolates Mexican. Severe lesions were found in 100% of the individuals of Sp group, but only in 25% of each isolated Mexican group. Results suggest that Mexican IPNV isolates are pathogenic, but less virulent than strain Sp. The amino acid motif residues of both Mexican isolates, corresponded to a subclinical disease. Nevertheless, the accumulated motility observed in the field, suggest that other factors play a role in the virulence of the disease.

KEY WORDS: genetic fingerprints, infectious pancreatic necrosis virus (IPNV), Mexico, rainbow trout, virulence

Infectious pancreatic necrosis virus (IPNV) belongs to the genus *Aquabirnavirus* and *Birnaviridae* family. IPNV isolates have been classified into two serogroups (A and B) and in 10 serotypes (A1 to A9 and B1) [8]. To date, seven genogroups (I to VII) have been proposed after the sequence analysis of the VP2/VP4 genes [13]. Infectious pancreatic necrosis (IPN) is a disease that causes high economic loss due to the morbidity and mortality 90% [11]. Actually, the virulence of IPNV isolates were quite variable [11], VP2 has been identified as molecular marker of IPNV virulence [6, 18, 23, 24] and VP5 with anti-apoptotic activity in cell culture [9]. Furthermore, IPNV serotypes exhibit different virulence also as serotype A2 (Sp strain), which is virulent for trout [10]. By using reverse genetics, confirmed that amino acid residues, Thr at position 217 (Thr217) and Ala221 of VP2 are the major determinants of virulence in IPNV of the Sp serotype [26]. Recently, translated amino acid sequence alignments of virus isolates revealed that isolates associated with clinical and subclinical infections had different genetic fingerprints in the VP2 [12]. In Mexico, epidemiological studies identified IPNV by the principal national zones of production of rainbow trout [17, 21], and in 2009 was reported a prevalence of 11.90% [21]. Genotyping and phylogenetic studies by VP2 gene from Mexican IPNV isolates have been performed [2, 7, 22], however its biological virulence is unknown. The objective of the present study was to determine the virulence of Mexican isolates in rainbow trout fry and related the results to VP2 amino acid fingerprint of the virus.

Two IPNV isolates from Mexican rainbow trout farms were included in the study. The isolate MEX3-CSM-05 was obtained from fish with typical clinical signs of IPN and the MEX2-CSM-07 was isolated from clinically healthy rainbow trout fry. The Sp strain (ATCC® Number: VR-1318™), was included in the study as positive control. Cell line CHSE-214 (Chinook salmon embryo, *Oncorhynchus tshawytscha*) (ATCC® Number: CRL-1681™) was used for viral isolation, and BF-2 (Bluegill fry, *Lepomis macrochirus*) (ATCC® Number: CCL91™) to propagate and quantify viruses. Cells were grown in Minimum Essential Medium Eagle (MEM) (in vitro s. a., D. F., Mexico) supplemented with 10% fetal bovine serum (FBS) (HyClone, Thermo Scientific, Logan, UT, USA), penicillin 100 IU/m*l*, streptomycin 100 μg/m*l* and amphotericin B 0.25 μg/m*l* (in vitro s. a., D. F., Mexico) [15, 16].

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Viruses were inoculated onto a confluent monolayer of BF-2 cells with MEM containing 2% FBS, 100 IU/m*l* penicillin, 100 μg/m*l* streptomycin and 0.25 μg/m*l* amphotericin B (in vitro s. a., D. F., Mexico). IPNV isolates were quantified by the 50% end point tissue culture infectious dose ($TCID_{50}$/m*l*) method [19]. One hundred rainbow trout fry (average weight 3.51 g and size 6.42 cm) was provided from a certified disease-free hatchery (Secretaría de Agricultura, Ganadería Desarrollo Rural, Pesca y Alimentación, State of Mexico, Mexico). Fish were divided in four groups of 25 organisms, each one, maintained in separate 100 *l* closed re-circulating aquaria at the laboratory, supplied with dechlorinated water, with mechanic, biological and chemistry filtration (Elite Hush 55, Hagen, Montreal, Quebec, Canada). Water temperature was maintained at 15°C ± 1 during the experiment. Artificial light was provided (12 hr with light and 12 hr dark). Fish were fed at 5% body weight per day [3] with pelleted commercial feed (El Pedregal, Silver Cup, Toluca, Mexico). All fry was acclimated for four weeks and seven fish of each group were screened for the presence of IPNV by standard virology methods [16]. Presence of bacteria and parasites were identified by current methods [14]. Neither viral, bacteria or parasites were found. The fish were placed in fasted the day before infection. On day 0, three fish from each group were euthanatized with an overdose of tricaine methanesulfonate (300 mg/*l*) [1] (SIGMA, St. Louis, MO, USA). Tissue samples from spleen, liver, pancreas and kidney were collected and processed for viral isolation [16], and for identification by RT-PCR and nested PCR (nPCR). Culture medium from infected BF-2 cells with IPNV isolates with an approximate viral titer 5×10^5 $TCID_{50}$/m*l* was added and perfectly diluted to a new fish tank of 2 *l* supplied with dechlorinated water with additional oxygenation. Control immersion fish (group 4) were mock immersed in water containing a 1 m*l* MEM (equal inoculum volume as administered to the IPNV immersion groups). Fish were exposed for 3 hr and 30 min at 15°C under static flow but with aeration. After infection and before being returned to their tanks (100 *l*), the fish were rinsed with water. IPN clinical signs: anorexia, darkening of the skin, exophthalmia, abdominal swelling and unusual swimming motion of infected fish were counted daily (score: 0= no sign observation, 1= sign observation), during 10 days after infection. Degree of severity of clinical signs was determined as follows: slight (+, fish with anorexia, darkening, unilateral exophthalmia and mild abdominal swelling), moderate (++, fish with anorexia, darkening, bilateral exophthalmia and marked abdominal swelling), severe (+++, fish with anorexia, darkening, bilateral exophthalmia, marked abdominal swelling and unusual swimming motion). Fish were monitored three times daily for mortality. Dead fish were removed daily and average cumulative mortality was calculated. On days 2, 4, 6, 8 and 10, three fish (moribund and with clinical signs) from each group were euthanatized (overdose of tricaine methanesulfonate, 300 mg/*l*) [1] (SIGMA). Samples spleen, liver, pancreas and kidney were obtained and were processed for viral isolation [16]. The care and use of fish in research were approved by the Institutional Subcommittee on Experimental Animal Care, Universidad Nacional Autónoma de México (UNAM), Mexico. On days 0, 2, 4, 6, 8 and 10, two water samples (15 m*l*) were taken (upper and lower) at the four experimental tanks for the identification of IPNV. The water samples were filtered using 0.20 μm membrane. Virus isolation was performed in BF-2 cell culture and confirmed by RT-PCR and nPCR. From cell culture supernatant was extracted total RNA using TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA), according to manufacturer's protocol. Two sets of DNA oligonucleotide primers were designed to amplify the VP2 gene of the Sp strain, and of the Mexican isolates MEX3-CSM-05, MEX2-CSM-07 (GenBank accession numbers: AF342728.1, MH708133.1, MH708132.1, respectively) [22]. The oligonucleotide primers used were: INFE1+ 5′-ATGAGCACATCCAAGGCAA-3′ (position 137–160) and INFE1- 5′-TCTGATGCCTCTGACTAGGTCC-3′ (position 796–818), with an amplified PCR product of 682 bp and INFE2+ 5′-ATTCGACCAGTGGCTAGAGACG-3′ (position 482–503) and INFE2- 5′-GCGGTCTGCTGGTTGAGCTGG-3′ (position 688–710), with an amplified PCR product of 229 bp. First strand cDNA of the total RNA was synthesized using a First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) with random primers according to the manufacturer's instructions. Subsequently, the regions of interest were amplified by PCR and nPCR using a Thermo Scientific DreamTaq DNA Polymerase kit (Thermo, Foster City, CA, USA). Amplification was performed in 25 μl volumes containing 2.5 μl of Dream Taq Buffer (10X), 0.5 μl of dNTP's mix (2 mM each), 0.5 μl of forward and reverse primers (5 μM), 2.5 μl of cDNA, 0.13 μl of DNA Polymerase (5 U/μl) and 18.37 μl of PCR grade water. The cycling conditions were as follows: an initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C (to INFE1+ and INFE1- primers) and 65°C (to INFE2+ and INFE2- primers) for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 15 min. The PCR products were electrophoresed in 1% agarose gels and stained using ethidium bromide. Gels were digitized using Quantity One 1-D Analysis System software (Bio-Rad, Hercules, CA, USA). The score by the total of fish with clinical signs for each group for all experiments was calculated. From the data of clinical signs, a χ^2 statistic for proportions was used. To compare multiple proportions statistical Tukey type was used with an angular transformation. For statistical tests were considered significant at the probability of $P<0.05$ [28].

Characteristic clinical signs of IPN were observed in infected fish. The three IPNV isolates were pathogenic. The strain Sp and Mexican isolated MEX3-CSM-05 showed the highest clinical signs scores, with 66 and 53%, respectively. Group infected with the Mexican isolated MEX2-CSM-07 showed clinical signs with a score 46%. Statistically significant differences were observed in all three IPNV groups of fish infected versus the negative control fish group ($P<0.05$). Clinical signs were observed from 4 dpi in organism infected with the strain Sp and a reached peak of these was observed the 8 dpi. In fish infected with Mexican virus, clinical signs began to the 6 dpi. In Table 1, clinical signs and degrees of severity of the groups are shown. Group infected with the strain Sp showed a cumulative mortality of 20%. In groups MEX3-CSM-05 and MEX2-CSM-07, cumulative fish mortality was 6.67%. Mortality in the Sp group was observed on days 6, 8 and 10 and Mexican groups until day 10. In Table 2, cumulative mortality of fish by groups is shown. No statistically significant differences were observed in the percentages of clinical signs and mortality in the three infected groups ($P<0.05$). At necropsy, gross findings in infected fish were pale liver (Fig. 1). This finding was observed in four fish group Sp (two on day 6 and two on day 8) and one fish in both Mexican isolates at day 8. BF-2 cell line showed cytopathic effect after inoculation of samples of liver and kidneys of the infected fish. The same samples were positive using nPCR. Identification of IPNV of these organs was in the Sp group at day 4, while for Mexican IPNV groups were at day 6. The identification of IPNV VP2

Table 1. Number of fish with clinical signs by days post infection (dpi), total percentage by group and degree of severity

Group	Number of fish with clinical signs (Degree of severity)					
	2 dpi	4 dpi	6 dpi	8 dpi	10 dpi	Total%
MEX3-CSM-05	0	0	3 (+)	3 (+)	2 (+)	53 (8/15)
MEX2-CSM-07	0	0	2 (++)	2 (++)	3 (++)	46 (7/15)
Sp	0	1 (+++)	3 (+++)	3 (+++)	3 (+++)	66 (10/15)
Control negative	0	0	0	0	0	0 (0/15)

Degree of severity: slight (+), moderate (++), severe (+++).

Fig. 1. Fish at 8 day post infection (dpi): Fish up with pale liver (Sp group) and fish below with normal liver (Control negative group).

Table 2. Daily mortality of fish by days post infection (dpi) and percentage by group

Group	Fish mortality					
	2 dpi	4 dpi	6 dpi	8 dpi	10 dpi	%
MEX3-CSM-05	0	0	0	0	1	6.67 (1/15)
MEX2-CSM-07	0	0	0	0	1	6.67 (1/15)
Sp	0	0	1	1	1	20 (3/15)
Control negative	0	0	0	0	0	0 (0/15)

Table 3. Identification of infectious pancreatic necrosis virus (IPNV) VP2 gene by and nested PCR (nPCR) from organs of the fish included in the study

Group	2 dpi				4 dpi				6 dpi				8 dpi				10 dpi			
	S	L	P	K	S	L	P	K	S	L	P	K	S	L	P	K	S	L	P	K
MEX3-CSM-05	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+
MEX2-CSM-07	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+
Sp	-	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
Control negative	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

S, Spleen; L, Liver; P, Pancreas; K, Kidney. Positive (+), Negative (-).

gene from kidney and liver was evident by nPCR, also in all groups to the 8 and 10 dpi (Table 3). IPNV was not detected in any fish from the negative control group. From the water samples, IPNV was detected in both water intakes (up and down) in the three infected groups: on day 4 post-infection in infected groups, but only in the Sp group was also detected by day 6.

This is the first study of virulence of Mexican isolates of IPNV in rainbow trout fry in an experimental laboratory analysis. The two Mexican IPNV isolates produced a clinical infection with characteristic clinical signs like anorexia, darkening of the skin, exophthalmia, abdominal swelling or an unusual swimming motion but these signs were more evident in the MEX3-CSM-05 fish group. The two Mexican IPNV isolates used in this study showed a low mortality (6%), very similar to that reported by Skjesol *et al.* [25] from Norwegian field outbreaks of IPN that caused 5% mortality. The mortality observed in the MEX3-CSM-05 group was lower than that reported in the field outbreak. The accumulated mortality in the field outbreak for the MEX3-CSM-05 isolate was 25%. Positive group inoculated with Sp strain showed the clinical sings at day 4, while both Mexican isolates until day 6. An increase in clinical signs was observed at day 8 in all three infected groups. The mortality in the Sp fish group was observed (on days 6, 8 and 10), before that in the fish inoculated with Mexican strains (until day 10). The results of the clinical signs and mortality were similar to those reported by Bowden *et al.* [5], they observed fish with clinical signs and mortality started on day 7 in the group injected intraperitoneal (*Atlantic salmon*) with strain Sp. The percent of mortality was less for the Mexican IPNV infected groups, compared with the strain Sp group, considered as a high virulence strain, based on a cumulative mortality. Roberts and Pearson [20], reported acute mortalities, when observed a severe liver lesions at the fingerling state and mortality level is often related to stocking density [4]. At necropsy, the only finding was pale liver: at four fish for the group Sp (two on day 6 and two on day 8) and in two fish in each Mexican group (on day 8). The lesions observed in the fish groups infected with Mexican isolates were slight to moderate, in comparison with the severe lesions observed in the group Sp fish, which were infected with a virulent strain. Of the samples from liver and kidney of infected fish, cytopathic effect was observed and IPNV was identified by nPCR. The identification of IPNV from the organs was first in the group Sp (on day 4) and then to the Mexican isolates (on day 6). Lesions develop very rapidly in fish challenged with virulent isolates [23]. Lesions in pancreas and liver can be mild to days 5 and 7 post-infection, while 9 days after, the

Table 4. Genetic fingerprints of Mexican isolates compared to the reports by Mutoloki *et al.* [12]

	Amino acid position								
	64	137	217	221	247	252	281	282	319
Overt disease, Mutoloki *et al.* [12]	I	T	T	A	T	V	T	N	A
Subclinical infections, Mutoloki *et al.* [12]	V	A	P	T	A	N	S	D	E
MEX3-CSM-05 (GenBank, AYQ58854.1)	V	T	A	T	E	N	T	V	A
MEX2-CSM-07 (GenBank, AYQ58853.1)	V	T	A	T	E	N	T	V	A

most fish may have severe lesions and widespread viral antigen when infected with field isolates highly virulent [23]. Taksdal *et al.* [27] reported a massive liver damage in fry at the IPN outbreaks field and has also they reported in studies of IPNV challenge fry in Norway. The virus was detected in the water at day 4 after infection in the three groups infected with IPNV, and only was detected until day 6 in the group infected with strain Sp. According to Bebak *et al.* [3], it is possible to detect the virus in the water after 2 dpi.

Translated amino acid sequence alignments of both Mexican viruses included in this study, revealed that the isolates have same genetic fingerprints in the capsid protein (VP2). Fingerprint of both Mexican isolates was V64, T137, A217, T221, E247, N252, T281, V282 and A319 (Table 4). In a previous study conducted by Mutoloki *et al.* [12], subclinical diseases were associated with a V64, A137, P217, T221, A247, N252, S281, D282 and E319 fingerprint on the VP2, while isolates associated with overt disease had the fingerprint I64, T137, T217, A221, T247, V252, T281, N282 and A319. Compared with fingerprint found by Mutoloki *et al.* [12], the amino acid motif residues at the positions 64 (V), 221 (T), and 252 (N), correspond to a subclinical disease. However, in the case of MEX3-CSM-05, it was not what was observed in the field outbreak. And only the positions 137 (T), 281 (T), and 319 (A), correspond to an overt disease [12].

This study provides information about the virulence and genetic fingerprints of Mexican IPNV isolates. The IPNV Mexican isolates can be pathogenic and produced the characteristic IPN clinical sings reported in literature. The clinical signs were more evident in fish infected with MEX3-CSM-05 than MEX2-CSM-07; however, no statistically significant differences were observed in the percentages of clinical signs and mortality in the three infected groups ($P<0.05$). These results suggest that MEX3-CSM-05 and MEX2-CSM-07 are pathogenic, but less virulent than strain Sp. The amino acid motif residues of both Mexican isolates, corresponded to a subclinical disease. Nevertheless, the accumulated motility observed in the field (25%) for the case of MEX3-CSM-05, suggest that other factors play a role in the virulence of the disease. Therefore, further studies should be conducted.

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