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Case Report: Viral Infectious Pancreatic Necrosis in Farmed Rainbow Trout from Mexico

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Abstract.—This case report provides pathologic and confirmatory diagnostic documentation of the first reported clinical epizootic of infectious pancreatic necrosis (IPN) in farmed rainbow trout *Oncorhynchus mykiss* from central Mexico. Both the gross and microscopic pathology were consistent with IPN. A virus was isolated in cell culture with the cytopathic effect typical of the IPN virus (IPNV). Positive identification as IPNV was achieved by means of an IPNV-specific indirect fluorescent antibody test and reverse transcription–polymerase chain reaction. Further genotyping identified this isolate as the Buhl strain of IPNV, which is a member of the West Buxton (A1) serotype of aquatic birnavirus serogroup A.

Infectious pancreatic necrosis (IPN) is an acute, contagious disease that in salmonids can cause substantial mortality among first-feeding fry. Affected fish are characterized by abnormal spiraling behavior when agitated, along with the accumulation of ascites, marked acute coagulative necrosis of the exocrine pancreas, and often enteric, vascular, renal, and muscular pathologies. Once infected, survivors can carry the virus for life and transmit the etiologic agent both horizontally and vertically within salmonid populations (Wolf 1988). For this reason, control strategies generally promote prevention through intense diagnostic screening.

Although IPN was observed in salmonids in the first half of the 20th century (M'Gonigle 1941), the IPN virus (IPNV) was not isolated until the late 1950s. It was first isolated from clinically ill brook trout *Salvelinus fontinalis* (Wolf et al. 1960) and subsequently from rainbow trout *Oncorhynchus mykiss* (Parisot et al. 1963). IPNV has since been identified in many different species of salmonids, including fish from South Africa (Bragg 1991), Spain (Rodriguez et al. 1994), Scotland (Smail et al. 1992), Korea (Lee et al. 1996), Japan (Jung et al. 1999), Norway (Jarp 1999), and Australia (Crane et al. 2000), as well as in other finfish (Wolf 1988; Rodriguez et al. 1997) and shellfish (Hill 1982). In the Western Hemisphere, this disease is endemic to regions of the United States, Canada, and South America (McAllister and Reyes 1984), but at the time of the outbreak reported in this article IPN had not been officially confirmed in farmed or wild fish from Central America or Mexico (Contreras et al. 2000; C. Ortega, Universidad Autónoma del Estado de México, unpublished data; J. A. Pérez, Secretaria de Agricultura, Ganaderia, Pesca, y Alimentación).

In Mexico, the culture of rainbow trout began at the end of the 19th century, when eyed eggs were introduced from the United States. Since then, it has become firmly established in the more temperate central regions of the country, as shown by the increase in both the number and productivity of farms (Camacho et al. 2000; Pérez, unpublished data). Like intensive fish farming in other areas, trout culture in Mexico has not been exempt from the typical husbandry-associated diseases. Currently, the Mexican government requires reporting of all exotic infectious diseases of salmonids, including bacterial kidney disease (caused by *Renibacterium salmoninarum*), piscirickettsiosis (caused by *Piscirickettsia salmonis*), and whirling disease (caused by *Myxobolus cere-*

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bralis) (government of Mexico 1993a, 1993b; Contreras et al. 2000).

In January 2000, a rainbow trout farm in the central region of Mexico near Toluca reported clinical signs typical of IPN in rainbow trout fry 5–5.5 cm in total length and weighing approximately 3 g. At the time of the clinical site visit by the regional aquaculture veterinarian, the workers reported that mortalities had been occurring for 5 weeks, resulting in the loss of approximately 5,000 fry. This group of fish was originally imported from the United States as a single lot of 300,000 eyed eggs in November 1999 and had subsequently been reared in springwater at 12°C.

Methods

Necropsy.—Approximately 25 fish were necropsied and examined for gross external and internal lesions. Wet preparations of skin, gills, and intestines were evaluated for the presence of parasites and microbial agents. In addition, representative moribund fish were cultured for routine bacteriology and stored frozen (−20°C) for virology.

Histopathology.—Whole moribund fingerlings were euthanized, cut open along the ventral body wall, and fixed in 10% neutral buffered formalin. Fixed tissues were subsequently processed for routine histology, stained with hematoxylin and eosin, and examined by light microscopy at both the Centro de Investigación y Estudios Avanzados en Salud Animal in Toluca and the Aquatic Diagnostic Services Unit of the Atlantic Veterinary College, Prince Edward Island.

Virus isolation.—Frozen tissues were shipped on ice from Mexico to the regional diagnostic virology unit at the Atlantic Veterinary College. Briefly, a 10% suspension of tissues in minimum essential medium with Earles' salts was prepared and centrifuged at 500 × gravity for 20 min. The supernatant was then collected, diluted 1:10 and 1:100, and inoculated onto a 1-d-old monolayer of chinook salmon embryo (CHSE-214) and rainbow trout gonad (RTG) cell lines. The negative control consisted of phosphate-buffered saline inoculated onto the monolayer. The monolayer was examined daily for cytopathic effect (CPE) under a tissue culture microscope.

The virus was confirmed using an indirect fluorescent antibody test (IFAT) and reverse transcription–polymerase chain reaction (RT–PCR). The IFAT was performed with rabbit anti-IPN polyclonal antisera provided by Phillip McAllister of the National Fish Health Laboratory, Kearneysville, West Virginia, and the fluorescein-isothiocyanate-labeled polyclonal antibodies against rabbit IgG (Sigma). The RT–PCR was done using a pair of primers developed by Zhou and Cepica (1992), which were on the 5′ flanking region of a large open reading frame of segment A of the virus.

Virus genotyping.—For genotyping, supernatants from CPE-positive tissue cultures were frozen to −80°C and shipped on ice to the Department of Biochemistry, Microbiology, and Molecular Biology at the University of Maine, Orono. The viral genomic RNA was extracted using the methods described by Blake et al. (1995). Oligonucleotide primers for PCR and cDNA sequencing were designed from the published cDNA sequence of genome segment A of the Jasper–Dobos (Duncan and Dobos 1986) strain of aquatic birnavirus and used to amplify a 1,338-base-pair cDNA fragment within the VP2 coding region of the large open reading frame of genome segment A, as described previously (Blake et al. 2001).

For cDNA sequencing, the amplified fragment was cut out of the gel and transferred to a microcentrifuge tube, melted by being heated to 65°C for 5 min, and treated with 5 U of Agarase (Sigma). The samples were then incubated at 37°C overnight. The purified PCR products were cycle-sequenced using *Taq*-polymerase-mediated incorporation of dye-labeled dideoxy terminators in an ABI model 373A DNA sequencer at the University of Maine DNA Sequencing Facility, as described previously (Blake et al. 2001)

The sequences were edited using the Sequence Navigator Program (Applied Biosystems, Inc.). Deduced amino acid sequences were derived with the DNASTAR EditSeq computer program (Lasergene, Inc.). Multiple alignments of the deduced amino acid sequences of the new virus isolates, together with the known sequences of 28 other aquatic birnavirus isolates, were performed using the DNASTAR MegAlign program, with the suggested parameter of 10 for both the unweighted gap penalty and the gap length penalty. A phylogenetic tree was constructed with the Clustal algorithm of the MegAlign program. The serotype and strain of the new aquatic birnavirus isolates in this study were then determined from this tree.

(The nucleotide and deduced amino acid sequence reported in this paper for the aquatic birnavirus isolate from Mexico has been deposited in GenBank [accession number AF537269]).

Results and Discussion

Clinical observation and necropsy findings.—Initial sampling of moribund fish occurred on January 26, 2000. Affected fish displayed erratic

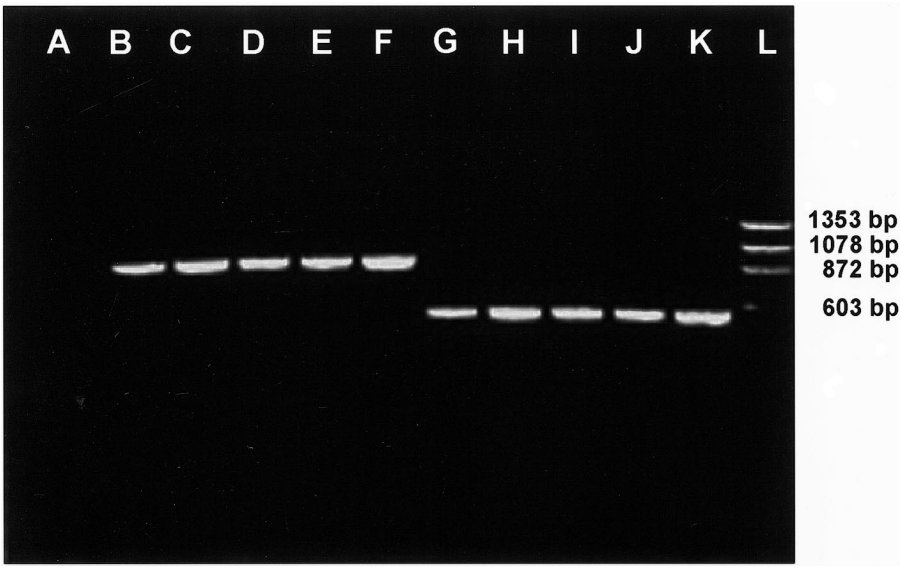

FIGURE 1.—Agarose gel electrophoresis of reverse transcription–polymerase chain reaction products of the viral RNA of a Mexican aquatic birnavirus using two different primer sets, one for the products in lanes B–F and another for the products in lanes G–K. Lanes are as follows: A, negative control; B–D, Mexican IPNV isolate; E–F, aquatic birnavirus positive control; G–I, Mexican IPNV isolate; J–K, aquatic birnavirus positive control; and L, DNA base-pair (bp) marker standards.

swimming behavior characterized by rotations in their longitudinal axis (i.e., spiraling). External examination of the more severely affected fish showed them to be in good condition but dark in color, with marked abdominal distension, pale gills, severe fin erosion, and clear mucus strands (pseudofeces) protruding from the anus as well as floating in the water. Internally, the peritoneal cav-

ACATCCAGAGCTCAACCCTTCCCGCTGGTCTGTATGCACTCAACGGGACCCTGAACGCTG
CCACCTTCGAAGGAAGTCTGTCTGAAGTAGAGAGCCTAACCTACAATAGCTTGATGTCCCT
AACAACAAACCCACAGGACAAGGTCAACAACCAACTAGTGACCAAAGGAATCACCGTCCT
GAATTTACCAACTGGGTTTGACAAACCATACGTCCGCCTAGAGGACGAGACGCCGCAGG
GCCCCCAGTCCATGAACGGAGCAAGGATGAGGTGCACAGCTGCAATCGCACCAA
GGAGGTATGAAATCGACCTCCCATCCGAACGCCTGCCGACCGTGGCCGCGACTG
CGACCCCAACAACAATCTACGAGGGGAATGCCGACATCGTGAACTCCACAACAGT
TACCGGGGACATAACATTCCAGCTCGAGGCCGAACCCGCCAATGAGACAGTGTTC
GACTTCATTCTACAGTTCCTGGGGCTGGACAACGACGTCCCCGTGGTCACCGTGA
CAAGCTCCACGCTAGTCACAGTGGACAACCACAGGAAGGCGTCAGCCAAGTTCAC
CCAGTCAATCCCAACAGAAATGATTACTAAACCAATTACACGGGTCAAGCTGGCCT
ACCAGCTCAACCAGCAGACCGCCATTGCAAACGCAGCAACGCTCGGAGCCAATG
GGCCGGCATCAGTCTCATTCTCCTCCGGGAACGGCAATGTGCCGGGAGTCCTAC
GACCCATAACCCTAGTGGCGTACGAGAAGATGACCCCCCAGTCAATCCTGACCGT
GGCTGGCGTATCCAACTATGAGCTGATCCCAAACCCAGACCTACTGAAGAACATG
GTCACCAAGTATGGAAAGTACGACCCTGAAGGCCTTAACTATGCCAAGATGATCCT
ATCCCACAGAGAGGAGCTGGACATCAGAACCGTCTGGAGGACCGAGGAATACAAA
GAAAGGACAAGAGCATTCAAAGAGATCACTGACTTTACAAGTGACCTACCAACCTC
AAAGGCATGGGGATGGAGGGACCTAGTCAGAGGCATCAGAAAAGTGGCAGCCCC
CGTGCTGTCAACGCTCTTCCCAATGGCGGCTCCCCTTATAGGAGCGGCCGACCAA
TTCATTGGGGACCTCACCAGGACCAACTCAGCCGGGGGACGCTACCTGTCACACG
CAGCCGGAGGCCGCTACCATGATGTCATGGACTCATGGGCCAGCGGGTCCGAGG
CAGGAAGCTACTCCAAGCACCTCAAGACCCGGCTTGAGTCCAACAACTATGAGGA
AGTGGAGCTTCCAAAGCCAACAAAGGGAGTCATCTTTCCTGTGGTGA

FIGURE 2.—Nucleotide sequence of the Mexican IPNV isolate (GenBank accession number AF537269).

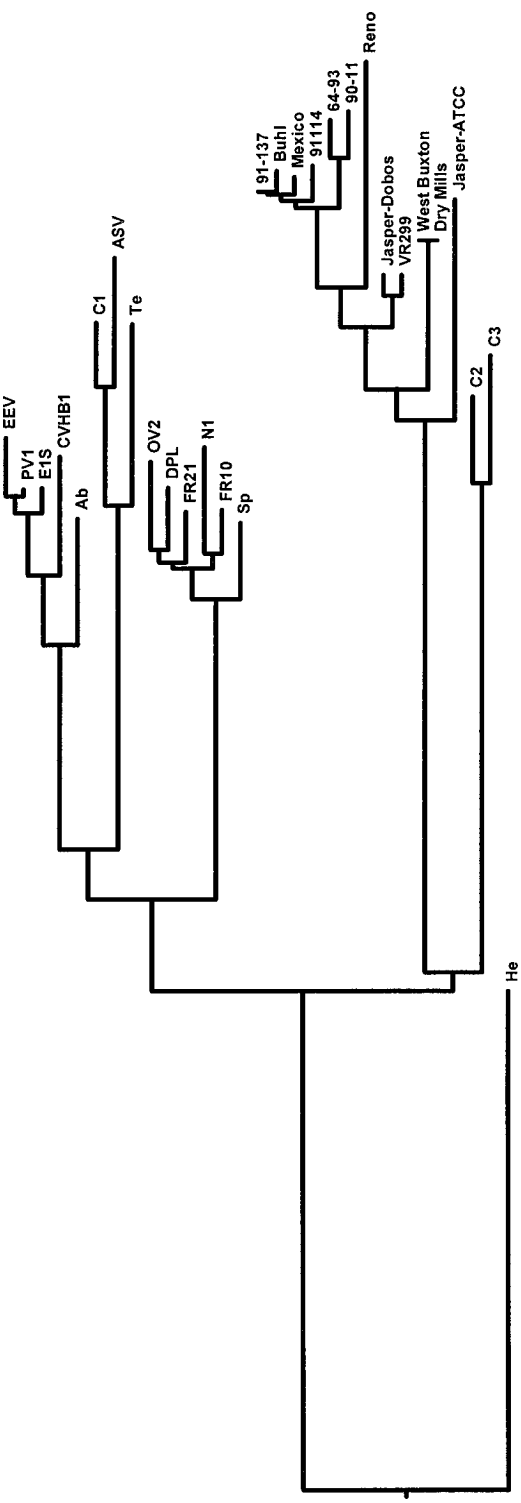

FIGURE 3.—Phylogenetic tree based on deduced amino acid sequences showing the relationship of the Mexican IPNV isolate to other aquatic birnaviruses (Blake et al. 2001). The tree was constructed using the Clustal algorithm of the DNASTAR Megalign program; the length of each pair of branches represents the distance between sequence pairs.

ity contained serosanguineous ascites and showed petechial hemorrhages of the mesenteric fat (in 2 of 25 fish), empty digestive tracts, and pallor of the spleen, liver, and kidney. Wet mounts of the intestinal contents and skin mucus revealed no parasites or microbes, and macroscopic examination of the gills showed mild branchial hyperplasia but no parasites, bacteria, or fungi.

Bacteriology.—*Pseudomonas fluorescens* was isolated in culture from the intestines of a few fish.

Histopathology.—The primary morphological changes included low-frequency granulocyte infiltrate with single-cell necrosis of exocrine pancreatic tissue and the adjacent mesenteric fat, mild congestion of the mesenteric vasculature, skeletal muscle degeneration (myopathy), lifting of the serosal epithelium from the surfaces of visceral organs, empty digestive tracts, and the sloughing or necrosis of intestinal mucosa. The morphological diagnoses included (1) necrotizing pancreatitis with ascitic trauma to the serosa of visceral organs (classified as acute to subacute [in terms of lesion age], multifocal [in terms of distribution], and mild [in terms of severity]); (2) degenerative enteropathy (acute to subacute, diffuse, moderate); and (3) degenerative myopathy of the body wall skeletal muscle (acute to subacute, multifocal, mild).

Virology.—All samples showed CPE by day 2 postinoculation, which became fully developed by day 4. This CPE was characterized by the contraction and detachment of individual cells followed by disruption of the entire monolayer, which is typical for IPNV-infected tissue cultures. Confirmatory testing by the IFAT and RT–PCR methods showed the specific IPN intracytoplasmic fluorescence in all CPE-positive samples, along with the 97-base-pair PCR product consistent with IPNV (Figure 1).

Genotyping.—Based on a sequence comparison with phylogenetic data from 40 known IPNV genomic sequences representing all nine serotypes of serogroup A, the CPE-positive Mexican isolate showed 99.5% similarity in amino acid sequence to the Buhl strain of IPNV (Figure 2), which is a member of the West Buxton (A1) serotype of aquatic birnavirus serogroup A (Figure 3).

The initial local report of this epizootic was provided to the Mexican authorities by Cesar Ortega in January 2000 and subsequently reported by Contreras et al. (2000), but at that time diagnostic confirmation of IPNV had not been obtained. The subsequent diagnostic work described above confirmed IPNV as the causative agent of this epizootic. It is our belief that this constitutes the first confirmed case of clinical IPN in Mexico. According to Pérez (1998) and Ortega (unpublished data), IPN had not been reported as an endemic disease problem in Mexican aquaculture prior to this outbreak. With the confirmation of IPNV as the causative agent in this case, Mexican veterinary authorities and fish farmers have refocused their attention on the need for thorough infectious disease surveillance programs (Enriquez and Ortega 2000)

We do not know the source of the IPNV, and additional epidemiological testing of trout populations in this region of Mexico will be needed to determine its origin. As noted by Wolf (1988), IPNV can be transmitted both vertically (through reproductive fluids) and horizontally (through virus shedding and coprophagia). Recent publications describing its potential transmission via virus-laden hatchery effluents (McAllister and Bebak 1997) and the feces of wild piscivorous birds (McAllister and Owens 1992) demonstrate the complexities of this epidemiological issue and suggest that a simple explanation as to the etiology of this outbreak will not be forthcoming.

Acknowledgments

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