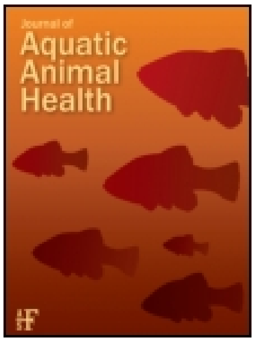

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ARTICLE

Distribution of Infectious Pancreatic Necrosis Virus (IPNV) Based on Surveillance Programs in Freshwater Trout Farms of Mexico

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Abstract
Diagnostic testing was performed between 2000 and 2012 to determine the distribution of infectious pancreatic necrosis virus (IPNV) in the main states of the Mexican Republic with freshwater Rainbow Trout *Oncorhynchus mykiss* (Walbaum) farms. This virus was positively identified from Rainbow Trout farms in seven of the eight states assessed. Due to nonnormal data distribution, a logistic regression model was applied for statistical analysis, the results of which indicated that virus prevalence was variable between states, with moderate but significant differences. Regarding the time periods evaluated, IPNV prevalence was higher during the first years of the study. The susceptible, infected, removed model was used to examine this phenomenon, which indicated that the decreased prevalence during the latter years of the study could be associated with a real elimination of the infection. The information of the cases analyzed also suggests a relationship with the irregularity in the submission of samples to the laboratory and emphasizes other factors that have contributed to the transmission of IPNV throughout the country.

Aquaculture production is the fastest growing source of animal protein worldwide (FAO 2005), and salmonid farming represents a significant proportion of this industry. Atlantic Salmon *Salmo salar* are currently a major focus of research and development, but this species is produced in a restricted number of countries, including Norway, Chile, Scotland, and Canada. In contrast, Rainbow Trout *Onchorhynchus mykiss*, which have been farmed far longer than Atlantic Salmon (Okumus 2002), are produced in many countries, primarily Chile, Norway, France, England, Italy, Iran, Turkey, and the USA (Okumus 2002; Adeli and Baghaei 2013).

Rainbow Trout aquaculture is also used in the Republic of Mexico. This industry, which began in 1888 with imported eggs from the USA (Arredondo-Figueroa 1983), now supports 984 commercial farms and 170 subsistence farms. Current production remains dependent on imported eggs; approximately 17.5 million eggs were imported in 2009 from the USA, Denmark, Chile, England, and Northern Ireland, of which 11.6 million were from the USA alone (National Commission of Aquaculture and Fishing, unpublished data). This practice of ova importation represents a risk for disease transmission.

The aquaculture industry is subject to a variety of negative economic impacts, disease being the most prevalent (Pilay and Kutty 2005). Salmonid production has historically been affected by a variety of bacterial and viral diseases. One of the earliest viruses to affect the salmonid industry was the infectious

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pancreatic necrosis virus (IPNV; M'Gonigle 1941; Wolf 1988). Since its initial detection in farmed Brook Trout *Salvelinus fontinalis* (Mitchill), this virus has been identified in a variety of fish and invertebrate species, its impact being greatest on salmonid species (Wolf 1988; Smail et al. 2006), and its distribution is worldwide. In Mexico, the first clinical outbreak of infectious pancreatic necrosis (IPN), the disease caused by IPNV, occurred in 2000 (Ortega et al. 2002). Despite early diagnosis, animals from this initial outbreak were distributed to grow-out facilities within the country (Ortega et al. 2007; Aguirre-Guzmán et al. 2011). Since this incident, a large number of IPNV-positive populations have been identified in Rainbow Trout facilities throughout Mexico. These findings provide the opportunity to perform an epidemiological study on the occurrence and spread of IPNV within the Rainbow Trout aquaculture sector of Mexico.

Specifically, this manuscript will epidemiologically analyze the results of diagnostic tests for IPNV performed between 2000 and 2012 in the most important Rainbow Trout breeding states in the Republic of Mexico. Data for the study were based on virus isolation from susceptible cells via culture testing and on immunological confirmation of positive tests (Rodriguez Saint-Jean et al. 2003; OIE 2006).

METHODS

Fish sampling.—Between 2000 and 2012, a total of 2,126 Rainbow Trout individuals (body weight 100–250 g) were sampled from farms located in the eight principal Rainbow Trout producing states of Mexico (Table 1). In accordance with an established health surveillance program for monitoring IPNV, samples were provided for analysis by the corresponding health authorities of each state evaluated. Information on the monitoring procedures performed by each authority for cases of IPNV was not recorded for analyses.

On average, 30 live fish were collected from each farm, placed in plastic bags with water and oxygen, and transferred to the Laboratory for Aquatic Animal Health at the Universidad Autónoma del Estado de México. In all cases, the initial sample groups received were composed of moribund and healthy fish. Fish were killed with an overdose of ethyl-*p*-aminobenzoate (BZ-20; Veterquimica, Chile). Samples from the states of Chihuahua and Durango were preserved in minimal essential medium (MEM) supplemented with 2% fetal bovine serum (FBS) and transported to the Laboratory for Aquatic Animal Health in refrigerated conditions.

Diagnosis of IPNV.—Viral isolates were obtained from kidney samples according to the methodology of the OIE (2006), with minor modifications. Importantly, the applied method is highly sensitive and able to detect subclinical infections of IPNV. Kidney samples of three to five fish were pooled to obtain approximately 1 g of tissue. This was mixed with 9 mL of MEM supplemented with 2% FBS in 15-mL Eppendorf tubes to a final 1:10 dilution. The tube contents were macerated in a mortar with sterile sand and centrifuged at 4,000 × *g* (4,500 rpm) for 15 min at 4°C. The supernatant was filtered through a 0.2-μm pore-size filter membrane and stored at 4°C ± 2°C until further use. Afterwards, 100 μL of each sample was inoculated in duplicate in 1:10 and 1:100 dilutions on a 24-well plate containing Chinook Salmon *Oncorhynchus tshawytscha* embryo cells (CHSE-214) at 90% confluence. As a positive control, two wells were inoculated with 100 μL of the reference virus (Ortega et al. 2002), while two wells were inoculated with only 100 μL of MEM with 2% FBS as a negative control. After absorption for 1 h, 1 mL of MEM was added to each well, and the plates were incubated at 15°C with monitoring every 24 h for the appearance of cytopathic effects. In the samples showing cytopathic effects, the presence of IPNV was confirmed through indirect fluorescent antibody testing (OIE 2006; Munro et al. 2010) using the commercial IPNV-Fluoro Test Kit (Grupo Bios, Chile).

The proportion of positive cases in relation to the total number of tested farms was determined. Binary data were used for each year and state to represent the number of positive and negative cases obtained (with 0 negative and 0 positive for years without observations in a state). As a simple

TABLE 1. Relationship between the number of Rainbow Trout samples submitted for IPNV analysis by each of the evaluated states in Mexico and the resulting number of validated IPNV infections.

	2000		2001		2002		2003		2004		2005		2006		2007		2008		2009		2010		2011		2012		
State	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	Total
México	6	20	0	42	10	29	1	5	55	54	80	102	19	152	28	192	15	231	1	92	13	99	9	115	0	0	1,370
Hidalgo	0	2	16	8	26	2	0	1	1	0	1	2	4	45	3	13	2	37	3	18	0	17	3	30	3	37	274
Morelos	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	4	0	0	0	0	0	0	5
Michoacán	0	0	0	3	1	1	0	2	12	15	36	50	7	73	4	25	0	0	0	0	0	21	0	14	0	1	265
Puebla	0	2	0	1	3	3	0	1	0	1	0	0	19	32	0	0	0	0	0	0	0	0	0	0	0	0	62
Chihuahua	0	1	2	8	1	2	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	22
Durango	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	3
Veracruz	0	0	0	0	0	0	0	0	0	0	0	0	12	7	7	37	0	0	0	0	6	16	0	14	10	16	125
Total	6	25	18	62	42	37	1	9	68	70	117	155	61	309	43	276	17	268	4	114	19	153	12	173	13	54	2,126

test to determine whether there were statistically significant temporal and spatial variations in this binary data (infected or noninfected), a logistic regression model was used as follows:

$$\mathrm{Logit}[p/(1-p)] = \mu + x_1 y + x_2 S,$$

where y is the year (a continuous variable) and S is a state factor for infection variations in proportion to IPNV-positive relative to Michoacán state.

Moreover, an epidemiological susceptible, infected, removed (SIR) model was used to simulate an IPNV epidemic. The S variable represents susceptible, uninfected farms, whereas the I represents infected farms. The inclusion of removed sites, R, is required to simulate the proportional decrease in positive cases following the peak of an outbreak (Murray 2006a). These farms probably resist infection due to immunity or improved biosecurity.

The model equations are

$$dS/dt = -bSI$$
$$dI/dt = bSI - rI$$
$$dR/dt = rI$$

The model was fitted using the least-squares difference between the observed and predicted proportion of infected farms, weighted by the number of observations in a given year. Both the logistic regression and SIR models were programmed in the R platform (http://www.r-project.org/).

RESULTS

The IPNV was isolated in seven of the main states where Rainbow Trout is produced in Mexico (Figure 1). Findings for the state of Morelos, which had only five farms, were negative. Rainbow Trout samples were received at irregular intervals over the period analyzed. Hidalgo was the only state that submitted samples for all of the 13 years studied (Table 1). The highest number of submitted samples (1,370) came from farms located in the state of Mexico, thus representing 64.4% of all samples. The state of Mexico was followed in sample quantity by Hidalgo (274) and Michoacán (265). However, as previously stated, samples were not submitted for every year analyzed. Of all the examined states, Durango submitted the few samples (3).

The proportion of infected farms clearly showed temporal and spatial variations between 2000 and 2012. The proportion of IPNV infection was higher in the initial years of the study period, the proportion being highest in 2002 followed by 2004 and 2005. The lowest proportion occurred in 2009, when only four positive cases were found (Figure 2).

The general tendency of infection proportion over the study period can be described as an initial increase followed by a decrease, with a slight increase at the end of the period (Figure 2). This pattern reflects a typical epidemic wave when the data are adjusted to a SIR model. The best fit for the observations was obtained with a transmission coefficient of b = 1.28 site^{-1} y^{-1} and a recovery rate of r = 0.33 y^{-1}. Note that 2003 had little weight in fitting the model, given the small number of samples received for that year.

FIGURE 1. Map of the Republic of Mexico showing states analyzed: (1) Mexico; (2) Hidalgo; (3) Morelos; (4) Michoacán; (5) Puebla; (6) Chihuahua; (7) Durango; and (8) Veracruz.

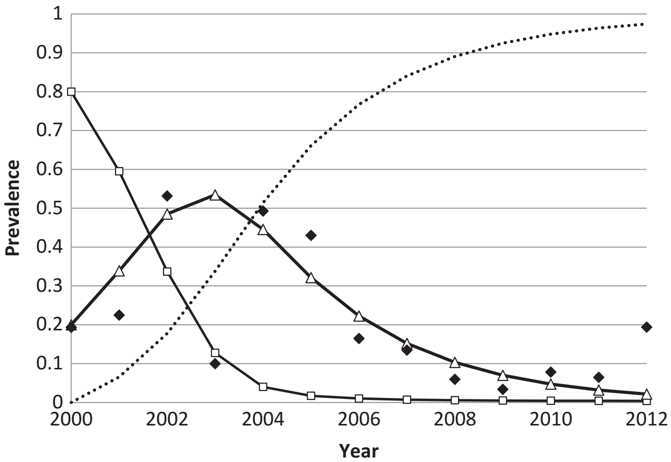

FIGURE 2. Results of SIR model fitted to the observed infection prevalence (black diamonds) using the least-squares difference weighted by the number of observations. Squares = susceptible, triangles = infected, dashed line = removed.

TABLE 2. Values of the logistic regression model, parameters of the odds ratios, and 90% confidence range for the evaluated states with respect to the baseline (Michoacán) and the number of observations per state (*N*).

Parameter	Odds ratio (range)	p	*N*
Year	0.76 (0.74–0.79)	2×10^{-16}	
State			
Chihuahua	0.22 (0.07–0.66)	0.02	22
Durango	6.51 (0.80–52.96)	0.14	3
Hidalgo	1.21 (0.84–1.73)	0.37	265
Mexico	0.81 (0.58–1.13)	0.20	1,370
Morelos	< 0.01 (0–∞)	0.98	5
Puebla	1.60 (0.96–2.66)	0.13	62
Veracruz	2.24 (1.43–3.50)	0.03	119

In addition to varying over time, prevalence also significantly differed between states, as determined through the simple logistic regression model. This model had an intercept of μ = 540, a binomial distribution of –0.25 per year, and an Akaike's information criterion of 1,950. This was improved to a binomial distribution of –0.27 per year and an Akaike's information criterion of 1,930 by including state information. This parameter indicated a highly significant odds ratio of 0.76, where values between 0.74 and 0.79 fall within a 90% confidence range (Table 2). In other words, reported IPNV prevalence declined by nearly a quarter year for year.

The variation between states was also significant (Table 2), the proportion of positive IPNV samples from Chihuahua being significantly lower and those from Veracruz being significantly higher than the mid-ranked Michoacán. Data for the state of Morelos were consistent with an absence of IPNV (an odds ratio of 1 to infinity), resulting in an extreme range on the calculated odds ratio. Because data were not available for each sampled farm, the model was not corrected to farm-level effects.

During the analyzed period, only four of the cases studied were clinical diseases affecting the first feeding offspring.

DISCUSSION

The presence of IPNV was confirmed for seven of the eight main states in Mexico that produce Rainbow Trout. The proportion of positive infections within each state varied over time, forming a typical epidemic wave of infection. Moreover, smaller, but still significant, differences in the proportion of infected farms were detected between states. However, this result is based on the proportion of infected individuals from sampled farms and does not encompass samples from all Rainbow Trout farms within each evaluated state. Unlike prior studies documenting infection behavior in a particular area (Murray et al. 2003; Bruno 2004; Munro et al. 2010), the current study may present biases due to irregular sample submissions from each state, a lack of samples from all Rainbow Trout farms within each state, and a lack of follow-up in cases of positive infection. However, the proportion of positively infected farms can be determined from the evaluated sample group.

The prevalence of IPNV can vary (Bruno 2004), and the virus may even be ubiquitous (Murray et al. 2003). However, implementing sanitary measures can reduce the incidence and spread of this and other infectious agents (Murray et al. 2003; Peeler et al. 2007; Munro et al. 2010). Indeed, the spread of IPNV and its ongoing prevalence in Mexican Rainbow Trout farms can be associated with an absence of adequate sanitary measures to control infection. Following the first recorded outbreak (Ortega et al. 2002), IPNV was later reported at other farms and in different states of Mexico (Ortega et al. 2007; Aguirre-Guzmán et al. 2011). While there are no contact networks between the states by waterways in Mexico, the traditional custom of commercializing serving-size live trout (pan size 250–300 g) results in a live-fish transportation network from rearing farms to consumption areas where farms also exist (Ortega et al. 2007), a practice that can result in the spread of diseases (Green et al. 2009). Additionally, several analyzed samples originated from hatcheries that incubate domestic or imported eggs, which could lead to the dissemination of a viral agent in the progeny or through the water used in the breeding process (Ortega et al. 2007; Peeler et al. 2007; Munro et al. 2010).

An *SI* (susceptible–infected) model was previously used to evaluate IPNV infections in Atlantic Salmon (Murray 2006a), and the present study expanded upon this model by including an *R* (removed) state to simulate decreased infection after an epidemic peak. This SIR model showed an epidemic tendency, the proportion of sampled sites infected being higher in the initial years of the study period. This result could be associated with higher infection susceptibility in the population followed by a subsequent reduction or elimination of infection in the later years of the sampling period. Given that Hidalgo was the only state to provide follow-up on its samples, it is not possible to determine whether the *R* state corresponds to negative, noninfected farms or if these farms are presenting a nondetectable level of infection that would subsequently aid in preventing infection outbreaks. Another possibility is that farms eliminated infection by implementing cleaning and biosafety measures but later became susceptible again.

Based on the SIR model, the decrease in the proportion of positive samples could be associated with a real elimination of infection during the evaluated period. However, there were considerable irregularities in the submission of samples. In 2002, for example, 28 farms from the state of Hidalgo were sampled, and 26 were positive (Ortega et al. 2007). In contrast, the state of Mexico has 451 farms, but only 39 were sampled, of which 10 were positive. In 2003, the state of Mexico submitted samples from 1 positive farm out of 6, but in 2004, this same state submitted samples from 99 farms, of which 55 were positive. These results were also influenced by the fact that some states did not send samples every year. In contrast to the present investigation, other studies have been able to clearly indicate the behavior of infection as a result of systematic surveillance programs (Murray et al. 2003).

The results of the present study can only indicate the dissemination of IPNV among the main Mexican states where Rainbow Trout are farmed. Due to the absence of a program for epidemiological monitoring and a defined health policy, the infection dynamics and possible production, economic, and ecological impacts of IPNV are unknown. Moreover, the effect of this virus has not been assessed in states with limited production or in wild fish. Taking into account the resistance of IPNV and its high propagation rate (Wolf 1988; Rodriguez Saint-Jean et al. 2003), this virus may be present in both the environment and wild organisms (Murray 2006b; Peeler et al. 2007).

During the time period analyzed, only four cases corresponded to clinical disease with lesions typical of IPN (Roberts and Pearson 2005; Smail et al. 2006) on offspring in the first feeding stage. This finding can be related to molecular studies (Barrera-Mejía et al. 2011) that have found that Mexican IPNV isolates are closely related to the North American VR-299 strain. Initially reported in Mexico by Ortega et al. (2002), this strain is considered as having low virulence (Rodriguez Saint-Jean et al. 2003). However, the potential risk of introducing other IPNV strains into Mexico's Rainbow Trout farming industry is ever-present since approximately 80% of the Rainbow Trout eggs required for continued production are imported. Although the USA provides most of the imported eggs, countries in Europe, Africa, and South America also provide eggs (National Commission of Aquaculture and Fishing, unpublished data). This widens the possibility of importing other IPNV strains that may significantly affect Rainbow Trout aquaculture in Mexico.

This study was performed based on procedures recommended by the OIE for the diagnosis of viral diseases in fish (OIE 2006). Despite the irregularity of sample submissions, this diagnostic process is an invaluable tool for the Mexican fish farming industry as it can be applied to confirm whether other viral diseases currently affecting salmonids, such as viral hemorrhagic septicemia (VHS) and infectious hematopoietic necrosis (IHN), among others (OIE 2006), are present.

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