Virulence Comparison of Three Buhl-Subtype Isolates of Infectious Pancreatic Necrosis Virus in Brook Trout Fry

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Abstract.—Infectious pancreatic necrosis virus (IPNV) is an important aquatic pathogen that can cause high mortality in populations of young salmonids. To determine the molecular basis of virulence, the fry of brook trout Salvelinus fontinalis were experimentally infected with three different A1-serotype, Buhl-subtype isolates of IPNV. The three isolates were selected on the basis of results of previously completed virulence assays, which indicated that the isolates had substantially differing virulence levels. To confirm this, mortalities from each treatment were recorded for the duration of the experiment (62 d), along with observation of any clinical disease signs. Mortalities began on day 5 postexposure, peaked on day 7, and then rapidly decreased for all three isolates tested. Diseased fry exhibited whirling, ascites, abdominal hemorrhaging, and prostration on the bottom of the tank. Daily virus titers from live fish were determined for 10 d postexposure (dpe), as well as at 28 and 62 dpe. Viral titers were correlated with fish weight to determine statistical significance. Fish weight was found to correlate negatively to virus titer for the two least-virulent isolates. Initial sequencing results demonstrated sequence homology for the viral capsid protein VP3, whereas slight differences among isolates were observed for the viral capsid protein VP2. The VP2 region was sequenced for each isolate at three times—before being introduced into the fish, during the epizootic, and 2 months after exposure—to determine whether major changes existed in the VP2 region that might account for the differences in virulence. Data indicate that two amino acid differences in the VP2 region exist, at residues 217 and 286, distinguishing the least-virulent isolates and the most-virulent isolate. These amino acid differences might account for the disparity in expressed virulence.
capsid protein VP2. Therefore, the sequencing efforts were focused on the VP2 protein. The purpose of this study was to characterize virulence differences between IPNV isolates, at a molecular level.

**Methods**

*Experimental animals.*—Brook trout *Salvelinus fontinalis* were obtained as swim-up fry from the Oregon Department of Fish and Wildlife, Wizard Falls Fish Hatchery, Camp Sherman, Oregon. The IPNV has not been detected at this facility since 1976. The fish were held at the Oregon State University Salmon Disease Laboratory (SDL) in Corvallis, Oregon, in ultraviolet-irradiated water. Fish were fed Rangen trout starter diet ad libitum and averaged 0.08 g in size, initially.

*Viruses.*—Three IPNV Buhl-subtype isolates were used in the experiment: 035-85, 91-114, and 91-137. Isolate 035-85 (S. LaPatra, Clear Springs Foods, Inc., Idaho) was isolated in 1985 from fingerlings of rainbow trout *Oncorhynchus mykiss* during an epizootic of IPN disease at Box Canyon, Idaho. The latter two isolates, 91-114 and 91-137 (K. Hauk, Idaho Department of Fish and Game) were isolated in 1991 from asymptomatic carrier adult rainbow trout collected at Sawtooth, Idaho, from a blind passage of tissue homogenates. Case 91-114 was isolated from a single-brood Pahsimerei A strain male, whereas 91-137 was isolated from four-brood Sawtooth A strain males. None of the isolates had been passaged in cell culture more than three times.

The IPNV Buhl-subtype isolates were selected because of differences in viral titers in previous experiments (Maret 1997). The isolates were selected to represent a high-virulence isolate (035-85), a medium-virulence isolate (91-114), and a low- or no-virulence isolate (91-137), on the basis of the mortality previously produced in brook trout fry. In the experiment described in Maret 1997, the cumulative mortalities caused by the isolates were 99%, 81%, and 33%, respectively. Cumulative mortality observed in control fish was 30%, primarily due to high residual chlorine levels in the water just before experimentation. These levels were stabilized by the time of the actual experiment.

The 035-85 isolate was obtained from infected fingerlings (frozen at −70°C for 1 year) with an average titer of $10^{7.25}$ tissue culture infectious dose with 50% endpoint per milliliter (TCID50/mL) and passaged twice in embryo cells of chinook salmon *Oncorhynchus tshawytscha* (CHSE-214; Lannan et al. 1984). The 91-114 and 91-137 isolates were obtained as infected CHSE-214 cell cultures (frozen at −70°C for 1 year) and passaged twice to increase the quantity and titrate the viral concentration.

**Experimental exposure of fish to IPNV.**—Approximately 400 brook trout fry were placed into each tank containing 10 L of water at 10°C. Three tanks were used for each treatment (or isolate) and control. Virus was diluted in 20 mL of HEPES-buffered (pH 7.2) minimum essential medium (HMEM) to give a final tank concentration of $10^5$ TCID50/mL (McAllister and Owens 1986). A sample of water from each tank was titered to assure that the correct viral dosage was given. Control fish were immersed in 10 L of water to which 20 mL of HMEM had been added. The fish were held for 5 h in static-flow conditions and supplied with a high volume of air to maintain oxygen levels. After the immersion period, the water in the tanks was replaced with 25 L of virus-free water, the air was removed, and the tank water was allowed to circulate as usual. All tanks at the SDL were connected to a common flow-through system; an approximate flow rate was 2.2 L of 10°C well water/min.

To conserve space, fish from replicate tanks were combined into a single 100-L tank (flow rate = 8.8 L/min) for each isolate and control at 15 d postexposure (dpe), after the primary sample-gathering period was completed and it had been determined that variability in mortality among replicate tanks was not significantly different (analysis of variance: $F_{3,43} = 0.01, P = 0.9904$).

**Sampling schedule.**—After exposure to IPNV, five live fish were collected daily from each replicate tank for each isolate during days 1–10 postexposure. Each sample of five fish was pooled, weighed, and homogenized 1:5 in phosphate-buffered saline (pH 7.3) for determining viral titer. For days 1–4 postexposure, before signs of IPN disease developed, fish were randomly sampled. For days 5–10 postexposure, fish displaying clinical signs of IPN disease were sampled for all three isolates. These data were censored for survival analysis.

At 28 dpe, five live fish were collected for each of the three isolates, and at 62 dpe, nine live fish were collected for each of the three isolates for determining virus titer. For the 62-dpe sampling, three of the fish examined were of small size (0.23–0.55 g), three were of medium size (1.00–1.48 g), and three were of large size (2.02–2.54 g). These fish were titered individually for virus.
Dead fish were collected daily from each tank and recorded over the entire 62-d course of the experiment. Mortalities among replicate tanks (first 15 d postexposure) and isolates (entire 62 d) were statistically analyzed to determine whether there was a significant difference among replicate tanks. The analysis was performed by use of a generalized linear- modeling program (Glimstat, Perth, Australia) after logistic transformation of the mortality data.

**Cell culture and virus titration.**—Virus was propagated in CHSE-214 cells grown in HMEM containing 10% fetal bovine serum (HMEM-10), on 96-well plates, as previously described by Caswell-Reno et al. (1986). Samples for virus titer were processed on the same day they were collected, within 2 h, to eliminate loss of virus titer due to freeze-thaw cycles.

The titer of virus in both cell culture and fish tissue was determined by use of the mathematical model of Spearman-Karber as the TCID50 per gram (TCID50/g). After determination of the virus titer, any sample remaining was frozen at −70°C and later used for sequencing. Virus titers among replicate tanks and isolates were statistically analyzed by use of analysis of covariance (ANCOVA) to determine whether there was a significant difference assessed by the Statview program (Abacus Concepts).

For the first 10 d postexposure, average viral titers were also compared with the corresponding average fish weight (groups of five fish each) for replicate tanks of each isolate to determine whether there was a statistically significant correlation. For samples collected at 62 dpe, individual viral titers were compared with the corresponding average fish weight (groups of five fish each) for replicate tanks and isolates were statistically analyzed to determine whether there was a statistically significant correlation.

**Reverse transcription.**—The IPNV RNA was extracted from either infected tissue culture cells or fish homogenates as described above and used as a template for the reverse transcriptase reaction. The IPNV RNA was diluted 1:10 in RNase- and DNase-free water, heated at 95°C for 5 min, centrifuged briefly, and then placed on ice for 2 min. The following reagents were added together: 1 L of viral RNA (1:10 dilution), 1 L of 25 mM MgCl₂, 1 L (200 units) of reverse transcriptase (RT), 1 L of 5× RT buffer, 1 L (33 units) of RNasin, 1 L (55 pmol) of 3’ primer, 1 L (55 pmol) of 5’ primer, 1 L (ATP, CTP, GTP, and TTP each at 1 mM) of deoxynucleotides, and 12 L of water. The solution was placed at 37°C for 1 h. After 1 h, the mixture was heated at 95°C for 3 min, centrifuged briefly at 12,000 × gravity, and iced for 2 min; then an additional 200 units of RT were added. The solution was placed at 37°C for an additional hour to increase the amount of cDNA produced.

**Polymerase chain reaction.**—The cDNA produced from the reverse transcription reaction was amplified by PCR run on a Thermolyne Temptronic (Barnstead/Thermolyne) for 35 cycles of the following dissociation-annealing program: 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. The PCR product was analyzed on a 1% agarose gel made with buffer containing 40 mM tris, 20 mM acetic acid, and 2 mM EDTA.

**Purification of PCR products.**—The PCR DNA products were purified by use of the QIAquick PCR purification kit (Qiagen, Inc., Chatsworth, California) according to the manufacturer’s instructions.

**Sequencing.**—Purified PCR products were sequenced by the OSU Center for Gene Research by use of the dideoxynucleotide chain termination method (Sanger et al. 1977) on an automated sequencer. Each virus isolate was sequenced from virus isolated under the following conditions: preinfection (passaged fewer than three times in CHSE-214 cells), peak of epizootic (directly from day 6–7 infected fish), and postepizootic (day 62 infected fish). Each sample was sequenced three times, from both the 5’ and 3’ ends, to ensure sequence accuracy. The sequences were aligned and analyzed by use of the Genetics Data Environment (GDE) editor, UNIX version (S. Smith, Harvard Genome Laboratory and University of Il-
Nucleotide discrepancies among the three replicates obtained at each sampling point were resolved by determining which sequence represented the majority consensus. After a consensus was determined, the nucleotide sequence was translated into amino acids by use of the GDE editor.

**Protein secondary structure.**—Prediction of protein secondary structure, after conversion of the sequencing results to amino acids, was made by use of the ProtPlot Program (Ross and Golub 1989).

**Results**

**Mortalities**

Acute mortalities characteristic of an IPN epizootic occurred with all three isolates. No elevated mortalities were noted in negative control fish. No signs of disease or deaths were observed in the first 4 dpe, with the exception of one control fish and one fish infected with isolate 91-137, both on 2 dpe. Daily mortalities for the three isolates are charted in Figure 1. Moribund fish displaying clinical signs of IPN disease were observed for all three isolates on 5 dpe, and mortalities were observed for isolates 035-85 and 91-137. The majority of the moribund fish exhibited typical external and behavioral signs of IPN disease: prostration on the bottom of the tank, rapid gilling, ascites, and petechial hemorrhaging in the abdominal area. Whirling of fish was observed infrequently. Mortalities for all three isolates in all nine treatment tanks were observed on 6 dpe. There were no control fish mortalities on this day. Mortalities peaked in the treatment tanks on day 7 and then immediately decreased and continued to decline but did not cease entirely. The experiment was terminated at 62 dpe; no mortalities occurred in any of the tanks past 56 dpe.

Cumulative mortalities for isolates 035-85, 91-114, and 91-137 at the conclusion of the experiment were 93, 67, and 78%, respectively. Cumulative mortality for control fish was 3%. Figure 2 depicts cumulative mortalities over the course...
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FIGURE 2.—Percent cumulative mortality of brook trout challenged with the IPNV Buhl-subtype isolates 91-137, 91-114, and 035-85. Triplicate tanks of approximately 400 fish each were infected with a challenge of $10^5$ TCID50/mL. The average percent cumulative mortality for each virus isolate is shown on the ordinate and plotted against days postexposure. The range did not vary appreciably among tanks and thus is not shown.

of the experiment. There was a statistically significant difference in cumulative mortalities among isolates. Specifically, there was a significant difference between cumulative mortalities for isolates 035-85 and 91-114 and isolates 035-85 and 91-137, but there was not a significant difference in cumulative mortalities between isolates 91-114 and 91-137 (log-rank test = 321.5, 2 df; $P < 0.0001$).

Quantitation of Virus in Exposed Fish

The virus titer of water in each tank at the initiation of the experiment was approximately $10^5$ TCID50/mL ($10^{4.95} - 10^{5.1}$ TCID50/mL). The harmonic mean virus titers for each isolate in fish for the first 10 d postexposure are shown in Figure 3. Virus titer increased overall for all three isolates during the first 10 d postexposure, with a range from $10^5$ to $10^{11}$ TCID50/g. Statistically, there was no significant difference in virus titer among tanks or isolates during the epizootic period (ANCOVA with days as covariant, $F_{2,80} = 0.284, P = 0.754$).

Viral titers determined at 28 dpe were $10^{3.95}$, $10^{3.95}$, and $10^{5.2}$ TCID50/g for isolates 91-137, 91-114, and 035-85, respectively. This indicated a marked drop in virus titer from the levels seen during the epizootic. Average viral titers at 62 dpe were $10^{3.84}$, $10^{4.76}$, and $10^{5.7}$ TCID50/g for isolates 91-137, 91-114, and 035-85, respectively, which are similar to the 28-dpe virus titer levels. Virus was never detected in control fish.

Weight of Fish versus Titer

For the first 10 d postexposure, there was a positive correlation between weight of the fish and virus titer (Figure 4) for all three isolates ($R^2 = 0.336, 0.148$, and $0.079$ for 91-137, 91-114, and 035-85, respectively). As the weight of the fish increased, so did the virus titer, per gram of tissue. The size of the fish collected ranged from 0.08 to

![Graph showing cumulative mortality over days postexposure for different isolates.](image)
0.23 g over the 10 d, among all three isolates. There was no significant difference between the size of sampled infected fish and the size of control fish during this time period (Scheffé’s test, \( P > 0.67 \)).

Similar analysis of samples collected at 62 dpe indicated a significant negative correlation between weight of the fish and virus titer (Figure 5) for the isolates 91-137 and 91-114 (91-137: \( R^2 = 0.826, F_{1,7} = 33.28, P = 0.0007 \); 91-114: \( R^2 = 0.557, F_{1,7} = 8.79, P = 0.021 \)). The smallest fish collected for these isolates carried the highest concentration of virus, per gram of tissue. Isolate 035-85, the most virulent isolate, did not demonstrate a significant correlation between weight of the fish and virus titer (\( R^2 = 0.007, F_{1,7} = 0.048, P = 0.832 \)). Virus titers at 62 dpe for this isolate were statistically similar, regardless of the size of the fish. The size of the fish collected for the isolates ranged from 0.23 to 2.54 g; 0.38 g was the average size of the smallest fish, 1.14 g was the average size of the medium fish, and 2.27 g was the average size of the largest fish collected. The average fish weight of the remaining survivors for each isolate at 62 dpe was the following: for 91-137, 1.18 g (\( N = 166 \)); for 91-114, 1.08 g (\( N = 230 \)); and for 035-85, 1.16 g (\( N = 52 \)). The average weight of the remaining control fish at 62 dpe was 2.42 g (\( N = 738 \)).

**Viral RNA and Protein Sequencing**

The deduced amino acid sequence of the VP3 protein was constructed at pre- and peak-epizootic, with 100% homology exhibited at both sampling times among all three isolates (Figure 6). The deduced amino acid sequence of the VP2 protein was constructed for the three virus isolates at pre-, peak-, and postepizootic (Figures 7–9). The isolates, which react identically in immunodot blots with a panel of 11 MAbs (Maret 1997), showed considerable homology of the VP2 amino acid sequence, around 99%. There are two amino acid differences in the VP2 region, at positions 217 and 286, between the least virulent isolates (91-114 and 91-137) and the most virulent isolate (035-85). The differences are demonstrated at all three of the sequence sampling points (pre-, peak-, and postepizootic). At amino acid 217, the 91-114 and 91-137 isolates have an alanine residue, whereas the 035-85 isolate has a threonine residue. At ami-
no acid 286, the 91-114 and 91-137 isolates have an arginine residue, whereas the 035-85 isolate has a lysine residue. Although the amino acids belong to different classes, they do not appear to result in a change in the protein secondary structure (Figure 10a).

There are also two amino acid substitutions at amino acids 194 and 203 that occur for all three isolates, between the pre- and peak-epizootic stages. The arginine residue that was present initially in the preepizootic stage changes to a lysine residue by the peak-epizootic stage. This change is still seen when the samples were sequenced for the postepizootic data and does not appear to affect the secondary structure. There were no sequence changes observed between peak- and postepizootic samples for any of the isolates.

Additionally, there is one amino acid difference for the 91-114 isolate that is only observed in the preepizootic sequencing, at amino acid 256, and that does appear to result in a change in the protein secondary structure (Figure 10b). The 91-114 isolate has a serine residue initially, whereas both of the other isolates have a phenylalanine residue. However, by the time the isolates are sequenced at 7 dpe during the epizootic stage, the serine residue appears to have been replaced by a phenylalanine residue, resulting in a sequence identical to that of the other two isolates.

Discussion

Although there were differences in virulence levels among the three isolates, it is unknown at this point why isolate 91-137 caused very low mortality (33%) in brook trout fry in previous experiments (Maret 1997) and caused 78% mortality in brook trout fry in this experiment. The fry were from the same hatchery and the same stock of fish and had a 1-year difference in hatch. There have been a few comparable studies reported in the literature. Working from a hatchery population of brook trout fry, Sonstegard and McDermott (1971)
isolated an apparently avirulent IPNV that induced heavy mortalities when used to artificially infect another population of brook trout fry. Similarly, Hill (1982) reported isolating an apparently avirulent strain of IPNV from asymptomatic rainbow trout in England. This IPNV strain produced heavy mortalities in other rainbow trout fry, as well as in the rainbow trout fry from which it was originally isolated, after experimental challenge. This suggests that it is possible for a virulent strain of IPNV to infect susceptible fry without causing clinical disease. Both of the previously cited cases originated in hatchery fish, and the original level of virus exposure is unknown. Certainly there are other factors, such as environmental conditions, host physiology, and genetics, that influence the final outcome between a virus and its host. There was, however, a statistically significant difference in the mortalities caused by the isolates 91-137 and 91-114 versus isolate 035-85, which has consistently caused greater than 90% mortality in experimentally infected brook trout fry less than 6 months of age.

Despite the difference in virulence indicated by the mortality data, there was no appreciable difference in the amount of virus produced in infected fish for the first 10 d postexposure. This implies that the virus quantity in fish does not account for differences in virulence between isolates. This concurs with results published by Silim et al. (1982), which reported similar virus titers (<1 log\textsubscript{10} difference) in several species of trout infected by virus isolates with differing virulence levels. Therefore, it appears that the ability of the virus to replicate in the host is not related to its ability to kill fish.

It is interesting to note that fish weight was negatively correlated with virus titer for the two less virulent isolates, after the epizootic stage of the infection. It does not seem likely that the fish are smaller because of the increased amount of virus they are carrying, because the fish infected with

**Figure 5**—Fish weight versus virus titer of brook trout fry infected with the IPNV Buhl-subtype isolates 91-137, 91-114, and 035-85. Fish were sampled at day 62 postexposure. The viral titer expressed as (log\textsubscript{10} TCID\textsubscript{50})/g for each individual fish is shown on the ordinate and plotted against the weight (wt.) of the fish in grams. Linear trend lines are added for each virus isolate.
the most virulent isolate 035-85 all had higher virus titers and demonstrated a similar size distribution of fish. It is more likely that these virus isolates have a propensity for more effective replication in smaller fish, possibly because the tissues of these fish are at a stage of more rapid development than are the tissues of the larger fish. Alternatively, larger fish may have a more developed nonspecific (innate) or specific immune response and can neutralize virus more effectively. This might explain the fact that IPNV rarely causes epizootics in susceptible fish greater than 6 months of age; size is a more important factor than age. Okamoto and Sano (1992) concluded that fish body weight was the principal factor influencing mortality of rainbow trout fry experimentally infected with an IPNV Buhl isolate. They observed that the larger fry in groups of infected fish of the same age displayed greater survival rates, over a 2-month sampling period. A similar observation was reported by Bierling et al. (1994) after experimental infection of small (0.1 g) and large (1.0 g) fry of Atlantic halibut *Hippoglossus hippoglossus* with the N1 isolate of IPNV. At 12°C, the small fry had considerably higher mortality than did the other size-group and controls. Additionally, the larger fry seemed to clear the IPNV infection after 3 weeks, whereas the small fry remained IPNV positive during the entire experimental period. However, it is clear that the IPNV isolates used in the current study all had an effect on fish growth, because the average weight of control fish at the end of the experiment was twice the average weight of infected fish.

The amino acid difference between virus isolates 91-114 and 91-137 and isolate 035-85 at residue 217 on protein VP2 is the most likely cause of the considerable variation in virulence among the IPNV isolates used for this particular experiment. Although it does not result in a change in the predicted secondary structure of the VP2 protein, the amino acids do belong to different classes. The alanine residue found in 91-114 and 91-137 is a hydrophobic amino acid, whereas the threonine residue found in 035-85 is a polar amino acid.
FIGURE 7.—Deduced amino acid sequence of the viral capsid protein VP2 for the IPNV isolate 91-137. The viral sequences were determined preinfection (PRE), when each virus isolate had been passaged fewer than three times in cell culture; at the peak of the epizootic (PEAK), directly from day 6–7-infected fish; and postepizootic (POST), directly from day 62-infected fish. Amino acids that varied among the three sampling times are shown, whereas identical amino acids are indicated by an asterisk.

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main of the viral-binding region. It has been suggested by Frost et al. (1995) that minor changes in the amino acid sequence in this area could result in conformational variation. Pryde et al. (1993) found only two amino acid variations after sequencing a Scottish Sp virus strain and comparing the results with the N1 virus strain of Norway, both of which belong to the same serotype. It is possible that the change in amino acids at residue 217 hinders efficient binding of IPNV to receptors on specific cell types, resulting in a lowered virulence for isolates 91-114 and 91-137. The VP2
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Figure 8.—Deduced amino acid sequence of the viral capsid protein VP2 for the IPNV isolate 91-114. The viral sequences were determined preinfection (PRE), when each virus isolate had been passaged fewer than three times in cell culture; at the peak of the epizootic (PEAK), directly from day 6–7-infected fish; and postepizootic (POST), directly from day 62-infected fish. Amino acids that varied among the three sampling times are shown, whereas identical amino acids are indicated by an asterisk.

protein has been implicated previously as an important factor for virus attachment to the cell surface (Darragh and MacDonald 1982). It is less likely that the amino acid difference at 286 is a factor, because the difference is merely a substitution of one charged amino acid residue for another (arginine to lysine). However, residues 275–286 have been designated a hypervariable region by Heppell et al. (1995).

It is equally unlikely that the amino acid changes at 194 and 203 that occur for all isolates between the preepizootic sample and the peak- and postepiz-
**Figure 9.** Deduced amino acid sequence of the viral capsid protein VP2 for the IPNV isolate 035-85. The viral sequences were determined preinfection (PRE), when each virus isolate had been passaged fewer than three times in cell culture; at the peak of the epizootic (PEAK), directly from day 6–7-infected fish; and postepizootic (POST), directly from day 62-infected fish. Amino acids that varied among the three sampling times are shown, whereas identical amino acids are indicated by an asterisk.

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<td>LVAYE KTMPQSI LTVAGS NYELI PNDLKLKN M V T</td>
<td></td>
<td>385</td>
</tr>
<tr>
<td>393</td>
<td>KYGKYDPE</td>
<td></td>
<td>393</td>
</tr>
</tbody>
</table>

Izoo tic samples are relevant, because the changes also involve a substitution of one charged amino acid residue for another (arginine to lysine). The difference between an arginine residue and a lysine residue is one nucleotide, and it is quite possible that a mutation of this sort would occur without affecting the performance of the virus. Because both of the changes were from arginine to lysine residues, there is a possibility that the change from replicating in cell culture to fish somehow invoked the change.

The serine residue at position 256 originally se-
The data obtained from this experiment indicate...
the possible importance of individual amino acid residues located in the VP2 viral capsid protein hypervariable region, which might partially account for the widely varying virulence differences displayed among IPNV isolates. The fact that viral replication appears to be influenced partially by the size of the host, depending on the isolate used, indicates another possible connection with viral virulence. This research illustrates the overall complexity of IPNV and the ensuing host–viral interactions.

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References


