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Normal rainbow trout serum (RTS)-resistant variants of the infectious pancreatic necrosis virus (IPNV)-Jasper differ with respect to inhibition by RTS, serotype and cDNA sequence*

K. C. Park[1,3,], S.-J. Lee[2], P. W. Reno[1]**

[1]Department of Microbiology and Coastal Oregon Marine Experimental Station, Hatfield Marine Science Center, Oregon State University, 2030 SE Marine Science Drive, Newport, Oregon 97365, USA

[2]Human Metabolism Section, Lab of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, National Institutes of Health, 111 Alexander Drive, MD C3-01, PO Box 12233, Research Triangle Park, North Carolina 27709, USA

[3]*Present address:* Institute for Marine Biosciences, National Research Council Canada, 1411 Oxford Street, Halifax, Nova Scotia B3H 3Z1, Canada

ABSTRACT: In order to determine if the infectious pancreatic necrosis virus isolate IPNV-Jasper (Ja-ATCC) is homogeneous or heterogeneous with respect to inhibition by normal rainbow trout serum (RTS), 50 clones were tested for sensitivity to RTS. The initial isolate was very sensitive to RTS, losing from 10^4 to 10^8 50% tissue culture infection dose ($TCID_{50}$) ml^{-1} with a 1:100 dilution of RTS. The sensitivity of the clones ranged from highly sensitive to completely resistant (0 to 10^8 $TCID_{50}$ ml^{-1} reduction). Eight percent of clones (4/50) were very sensitive to RTS (Ja-S) and 84% of clones (42/50) showed a mid-range of sensitivity to RTS. The final 8% of clones (4/50) were resistant to RTS (Ja-R). Enzyme immunodot assay revealed that Ja-S clones showed a monoclonal reaction identical to the parents, Ja-ATCC; however, Ja-R clones differed by several epitopes from the parental strain. Analysis of Ja-S and Ja-R revealed that there were significant differences in their nucleic acid sequences for the capsid protein VP2. These 2 strains shared 80.7 and 86.5% identity in nucleic acid and in amino acid sequences, respectively. Ja-S had 99.7 and 91.0% identity in nucleic acid sequences, and 99.5 and 95.9% in amino acid sequences with Ja-ATCC and Jasper-Dobos (Ja-D), respectively, while Ja-R showed 80.6 and 79.8% identity in nucleic acid sequences and 86.5 and 87.0% in amino acid sequences with Ja-ATCC and Ja-D, respectively. In conclusion, the Ja-ATCC population was heterogeneous in terms of RTS sensitivity, serotype and cDNA sequences from the VP2 coding region.

KEY WORDS: IPNV-Jasper variants · RTS inhibition · Serotype · cDNA sequence

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) belongs to the family *Birnaviridae* (Dobos et al. 1979) and is an agent of an acute, contagious fish disease causing high mortality not only in juvenile salmonids but also in non-salmonid fishes (Reno 1999). IPNV has 2 segments of double-stranded RNA: segments A (2.5×10^6 Da) and B (2.3×10^6 Da). The longer one encodes a 106 kDa polyprotein that is cotranslationally cleaved by a viral protease (NS or VP4, 29 kDa) into 2 proteins, pVP2 (62 kDa) and VP3 (31 kDa) (MacDonald & Dobos 1981, Mertens & Dobos 1982, Duncan et al. 1987). VP3 was thought to be an internal protein of the virus (Dobos & Rowe 1977), but at least a portion of VP3 is exposed on the surface since it reacts with a number of monoclonal

*The nucleotide sequences reported in this paper have been deposited in the GenBank data base (Accession No: AF399925 for Ja-S and AF399926 for Ja-R).

**Email: kyoung.park@nrc.ca

antibodies (Caswell-Reno et al. 1989). pVP2 is further processed during viral maturation into VP2, which is a major external protein and responsible for the reaction of type-specific neutralizing monoclonal antibodies (Dobos et al. 1977, Nicholson 1993). A universal, group-specific epitope has been reported to be located near the amino terminus of VP2, whereas the polypeptide responsible for a serotype-specific epitope has been mapped in the middle of VP2 (Dobos 1995). It has been reported that an anti-IPNV inhibitor, called '6S inhibitor or RTS inhibitor', is present in rainbow trout serum (RTS) unexposed to IPNV (Vestergard-Jørgensen 1973, Dorson & de Kinkelin 1974). The inhibitor has a sedimentation coefficient of approximately 6S by ultracentrifugation and is not thought to be an antibody as inhibition was not dependent on the specific serotypes of IPNV (Macdonald & Gower 1981, Ögüt 1995). Park (2000) suggested that it is not interferon due to its strongest inhibition activity at a molecular weight of approximately 150 kDa. It has also been demonstrated that IPNV can be directly affected by the RTS inhibitor before viral attachment and that pretreatment of tissue culture cells with RTS does not provide protection against subsequent viral infections (Kelly & Nielson 1985, Park 2000). This suggests that inhibition does not result from the stimulation of tissue culture cells to release cytokines. Not all virus isolates tested were inhibited by RTS and the inhibition was not dependent on the specific serotypes of IPNV (Macdonald & Gower 1981, Ögüt 1995). Cell culture-adapted virus strains were more susceptible to RTS than were wild type virus (Dorson & de Kinkelin 1974, Hill & Dixon 1977, Dorson et al. 1978). It has also been reported by some researchers that RTS sensitivity was correlated with virulence of IPNV and that virulence was changed by cell passage (Hill & Dixon 1977, Hill 1982, Ögüt 1995). The RTS sensitivity of IPNV is also dependent on the cell line in which the virus replicates. It has been reported that IPNV sensitivity to RTS was increased rapidly with multiple viral passages in a cyprinid cell line (EPC) compared to RTG-2 or bluegill fry (BF) cell lines (Hill & Dixon 1977) but decreased with passage in the Chinook salmon embryo (CHSE)-214 cell line (Park 2000).

In our previous experiments, we found much variation in the sensitivity of IPNV to RTS inhibitory activity even though we used the same RTS source, the same stock of virus and the same host cell condition (confluent) (Park 2000). In experiments using a member of the A_1 serotype (IPNV-Buhl), we found that sensitivity was altered after 5 passages in RTG-2 cells (Park 2000). Sequencing of the sensitive and resistant variants revealed genetic differences in the VP2 region of sensitive and resistant variants, but not in VP3. Therefore, we questioned whether the virus population was heterogeneous in terms of RTS sensitivity. At present, no information is available about whether virus clones have different RTS sensitivity and different genetic information. Therefore, in this work, we first cloned an RTS-sensitive strain of IPNV originally isolated from trout in a facility in Jasper-Alberta, Canada (Hill & Way 1995) and tested the progeny for RTS sensitivity. Second, the epitope pattern on VP2 and VP3 of clones with 2 different sensitivities was determined. Third, the cDNA sequences of the VP2 region of 2 strains of IPNV-Jasper, highly RTS-sensitive (Ja-S) or RTS-resistent (Ja-R), were compared.

MATERIALS AND METHODS

Virus. The IPNV-Jasper isolate (ATCC VR-1325), Ja-ATCC, used in this study belongs to serotype A_9 (Hill & Way 1995). This virus was originally isolated from diseased brook trout *Salvelinus fontinalis* at the Maligne River Hatchery, Alberta, Canada (Yamamoto 1974). It was kindly provided by Dr. B. Nicholson, University of Maine, Orono, ME, USA, who originally received it as a donation from Dr. Barry Hill (Department of Fisheries and Food [DAFF], Weymouth, UK). The virus has been passaged for 20 yr in the laboratory. Brook trout were obtained from Wizard Falls Hatchery, Camp Sherman, OR. IPNV has not been detected at this hatchery since 1976. The fish were held at the laboratory for Fish Disease Research at Hatfield Marine Science Center, Newport, OR in dechlorinated city water. Prior to use in these experiments, the virus was passaged 2 times through brook trout fry by immersion in water containing 10^4 $TCID_{50}$ ml^{-1} for 5 h at 14°C, isolated in CHSE-214 cells (Lannan et al. 1984) and frozen until use.

Isolation of clones sensitive or resistant to RTS. IPNV-Jasper (10^7 $TCID_{50}$ ml^{-1}) was serially diluted to 10^1 $TCID_{50}$ ml^{-1} and 0.1 ml of the final dilution was inoculated into 96 wells of each of 15 replicate 96-well plates. Fifty virus clones were isolated from 96-well plates showing CPE in fewer than 5 wells. The virus contained in these wells had a high probability of arising from a single virus. The isolated clones were tested for sensitivity to RTS *in vitro* as described below. Aliquots of isolated clones were kept in liquid nitrogen for later enzyme immunodot assay and sequencing.

***In vitro* virus sensitivity to RTS.** The RTG-2 cells (Wolf & Quimby 1962) were propagated in 24-well plates as described by Caswell-Reno et al. (1989). In this experiment, 3 types of media were used: Eagle's Minimum Essential Medium (MEM) without serum (MEM-0); MEM + 10% fetal bovine serum (MEM-10); MEM + 10% fetal bovine serum + 1%RTS (MEM-RTS). Two wells were prepared for each clone; one well was for MEM-RTS and the other one was for MEM-10. Virus was diluted with MEM-10 or MEM-RTS to give a final virus concentration

of 10^4 $TCID_{50}$ ml^{-1}. A total of 100 µl of diluted virus were added onto a confluent monolayer of cells in each well and incubated at room temperature for 2 h. After the incubation time, the inoculated monolayers were washed 3 times with MEM-0 and then 1 ml of either MEM-10 or MEM-RTS was added. Uninoculated controls were treated with either medium, MEM-10 or MEM-RTS, as appropriate. Cells were incubated at 18°C for 7 d in an incubator to which 5% CO_2 was supplied. The level of cytopathic effect (CPE) was monitored daily and scaled from 0 (no CPE) to 4 (complete CPE) on an ordinal scale. On the 7th day after exposure, cell culture supernatant from each well was harvested, pooled and held in liquid nitrogen until they were titrated.

Virus titration. CHSE-214 cells (Lannan et al. 1984) were grown with MEM-10 in 96-well plates. The endpoint dilution method as described in Caswell-Reno et al. (1986) was used for virus titration. Virus samples were serially diluted 10-fold with MEM-0 and then 100 µl of each of diluted virus suspension were added to each of 4 wells of a 96-well plate (Lannan et al. 1984). After incubation at 18°C for 7 d, wells showing CPE were counted to determine 50% tissue culture infectious dose ($TCID_{50}$ ml^{-1}) (Spearman 1908).

RT-PCR. Viral RNA was extracted from 2 types of IPNV-Jasper clones, Ja-S or Ja-R, using TRIzol reagent according to the manufacturer's instructions (Life Technologies) as described earlier (Lee et al. 1998). Primers (F31 and R1212), shown in Table 1, were designed based on published sequences of the cDNAs of genomic segment A of the Jasper-Dobas (Ja-D) clone (Duncan & Dobos 1986). Primers for PCR and sequencing were constructed at the Oregon State University (OSU) Center for Gene Research. Extracted RNA was diluted in RNase, DNase-free water to a concentration of 100 ng $µl^{-1}$ RNA and heated at 95°C for 5 min, and placed on ice for 2 min. The reaction components for the first-strand synthesis included viral RNA (200 ng), R1212 primer (200 pmol), a reaction buffer (final reaction contains 50 mM Tris-HCl, pH 8.3, 30 mM KCl, 8 mM $MgCl_2$, 1 mM dithiothretital [DTT]), each dNTP at 2 mM, RNasin (40 units) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (400 units) in 50 µl reaction. The RT reaction was performed at 37°C for 1 h. The cDNA was stored at −70°C. Ten µl of cDNA (150 ng $µl^{-1}$) was heated at 95°C for 5 min, then cooled on ice and briefly centrifuged at 10 000 × *g*. The PCR contained primers at 10 pmol each (R1212 and F31), each dNTP at 2.5 mM, cDNA (300 ng), 15 mM $MgCl_2$ and PLATINUM *Taq* DNA polymerase at 2.5 U (Life Technologies). Amplification was performed in a Programmable Thermal Controller (PCT-100, MJ Research). PCR cycling parameters included initial denaturation of 4 min at 94°C (1 cycle), 35 cycles each of; a denaturation/annealing/extention (94°C, 1 min / 60°C, 1 min / 72°C, 2 min) and a final extention at 72°C for 10 min.

Purification of PCR products and cDNA cloning. Twenty µl of each of the RT-PCR products were analyzed by electrophoresis through a 1% (w/v) agarose gel containing ethidium bromide (0.5 µg ml^{-1}) in TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA). The gel was electrophoresed at 75 V for 1.5 h and visualized with a UV light. The DNA band of expected size (1.2 kb) was excised and purified using the QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's protocol. Purified PCR products were cloned using TOPO TA Cloning kit (Invitrogen) according to the manufacturer's instructions. A QIAprep Spin Miniprep Kit (QIAGEN) was used to purify plasmid DNA from transformed *Escherichia coli* cells.

Sequencing and phylogenetic analysis. Sequencing of the DNA was carried out at OSU Center for Gene Research using an automatic DNA sequencer (ABI PRISM Model 377). Each base in the sequence was determined at least 3 times in both directions using synthetic oligonucleotide primers (Table 1) designed from determined nucleotide sequences. Chromatograms (ABI PRISMS) of sequenced data were analyzed and assembled by the use of the MacVector software and nucleotide discrepancies among sequence replicates were determined by majority consensus. The nucleotide sequence was translated into amino acids using MacVector software. The nucleotide sequences of 2 strain types of IPNV-Jasper clones in this paper have been deposited in GenBank with the following accession numbers: Ja-S, AF399925; Ja-R, AF399926. The similarity and phylogenetic relationships among these strains and the other 14 IPNV strains available in GenBank were analyzed by the Clustal method with DNASTAR MEGALIGN program

Table 1. Primers for RT-PCR and cDNA sequencing. Map positions of the primers are based on the sequence of segment A of Jasper (Duncan & Dobos 1986): position 31 in this paper corresponds to position 151 of segment A of Jasper

Primer name	Orientation	Position[a]	Sequence (5' to 3')
F31[2]	Sense	31–53	TTGAGATCCATTATGCTTCCCGA
F37	Sense	37–60	TCCATTATGCTTCCCGAGAATGGA
R148	Antisense	148–125	TTCCTGAGTCTGAGACCTCTAAGT
F417	Sense	417–437	CAGCTTGATGTCCCTGACAAC
R669	Antisense	669–649	TGTTGGGGTCCCGGTTGCCAT
F735	Sense	735–754	GCTAGAAGCCAAACCCGCCA
R1208	Antisense	1208–1182	AGGATCATCTTGGCATAGTTTAGGCCC
R1212[2]	Antisense	1212–1190	GGACAGGATCATCTTGGCATAGT

[a]Map positions of the primers used for RT-PCR

(Lasergene). The strains utilized in this analysis have the accession numbers: Ja-D, NC001915; Ja-ATCC, AF342735; N1, D00701; He, AF342730; ASV, AY026490; C1, AF342732; EEV, AY026486; VR-299, AF343572; WB, AF342727; C2, AF342733; Sp, AF342728; Ab, AF342729; C3, AF342734; Te, AF342731.

Protein structure analysis. Protein composition and antigenic index (James-Wolf method) of amino acid sequences were analyzed using the DNASTAR Protean program (Lasergene). Hydropathic plots for 2 strains were conducted by the Kyte-Doolittle method using the DNASTAR Protean program (Lasergene) and more detailed information was obtained using the SeqView program.

Enzyme immunodot assay. To determine whether Ja-S and Ja-R clones of IPNV-Jasper (Ja-ATCC) have different epitopes, an enzyme immunodot assay was performed for the parent virus population (Ja-ATCC), cloned Ja-S and cloned Ja-R following the procedure of Caswell-Reno et al. (1989). As positive controls, 6 serotype isolates including Canada 1 were used. A total of 100 µl of each virus (10^7 $TCID_{50}$ ml^{-1}) was added for the reaction with monoclonal antibodies. Supernatant collected from uninfected CHSE-214 cell culture was used as a negative-control antigen.

RESULTS

In vitro virus sensitivity to RTS

In order to determine whether IPNV-Jasper (Ja-ATCC) clones are homogeneous or heterogeneous in terms of RTS sensitivity, 50 clones were isolated from a virus population which had been passaged twice through brook trout, then once in CHSE-214 cells and tested for RTS sensitivity. The RTS sensitivity of the clones ranged from highly sensitive to resistant. The parent isolate (Ja-ATCC) was extremely sensitive to RTS, losing 10^8 $TCID_{50}$ ml^{-1} after treatment. Eight percent of clones were also very sensitive (Ja-S) showing no CPE during 7 d incubation in the presence of RTS and were inhibited as high as $10^{-4.8}$ $TCID_{50}$ ml^{-1} reduction. Eighty-four percent of clones showed a mid-range of RTS sensitivity. CPE levels of these clones (as measured on an ordinal scale of 0 to 3) in the presence of RTS were 1 to 2, while it was 3 in the absence of RTS. The mean inhibition level was $10^{-2.3}$ $TCID_{50}$ ml^{-1}. Eight percent of clones were resistant to inactivation by RTS (Ja-R). They were not significantly inhibited by RTS (mean $10^{-0.8}$ $TCID_{50}$ ml^{-1} reduction). The level or intensity of CPE-development with these clones was the same in the presence or absence of RTS (3 on our scale). CPE had developed by the second day post-infection in these clones.

Nucleotide and deduced amino acid sequences

The conserved initiation codon (ATG) of the large open reading frame (ORF) as published by Duncan & Dobos (1986) is defined as position 1 in our sequence analysis. Our sequences span the region from position 31 to position 1212. There was an 80.7% nucleotide identity between Ja-S and Ja-R. Upon comparison of these data with information on IPNV-Jasper strain (Ja-D) (Duncan & Dobos 1986) and IPNV-Jasper (ATCC VR-1325) (Ja-ATCC) (Blake et al. 2001), we obtained a 99.7% nucleotide identity for Ja-S and 80.6% identity for Ja-R when compared to Ja-ATCC. When compared to Ja-D, both Ja-S and Ja-R had slightly lower nucleotide identities (91.0 and 79.9%, respectively). With respect to percent nucleotide divergence, Ja-S showed 0.3, 9.0 and 19.3% divergence from Ja-ATCC, Ja-D and Ja-R, respectively. Our sequence for Ja-R showed a 19.4% divergence from Ja-ATCC and a 20.2% divergence from Ja-D.

The results of our comparison of the deduced amino acid sequences of Ja-S and Ja-R to each other, as well as to published sequences for the parent strain (Ja-ATCC) and other strains are given in Fig. 1 & Table 2. There was an 86.5% amino acid identity between Ja-S and Ja-R. Our RTS-sensitive group of clones (Ja-S) had a higher amino acid identity to Ja-ATCC (99.55%) than to Ja-D (95.9%), West Buxton (ATCC VR-877) (95.9%) or VR-299 (ATCC VR-299) (95.9%). With respect to percent amino acid divergence, Ja-S showed a divergence of 0.5% with Ja-ATCC, 4.2% with Ja-D and 14.9% with Ja-R (Table 2). It is not surprising that our phylogenetic analysis places Ja-S within a clade formed by Ja-D, VR-229, WB and Ja-ATCC (Fig. 2).

Our resistant group (Ja-R) showed relatively low amino acid identities with Ja-ATCC (86.5%) and Ja-D (87.0%), and higher amino acid identities with ASV (95.9%) and C1 (96.7%). With respect to percent amino acid divergence, Ja-R showed a divergence of 14.9% with Ja-ATCC and 14.3% with Ja-D. The results of our phylogenetic analysis places Ja-R within a clade formed by ASV and C1.

Protein structure analysis

The predicted molecular weights of the proteins encoded by these sequences were 41 836 Da for Ja-S and 41 723 Da for Ja-R. Both proteins had the same predicted isoelectric point of 5.0. Further analysis of these proteins revealed that Ja-S had 32.5 and 32.2% of polar residues and hydrophobic residues, respectively, while Ja-R had 32.5 and 31.7%, respectively. Comparison of the hydrophilic plots of the 2 strains

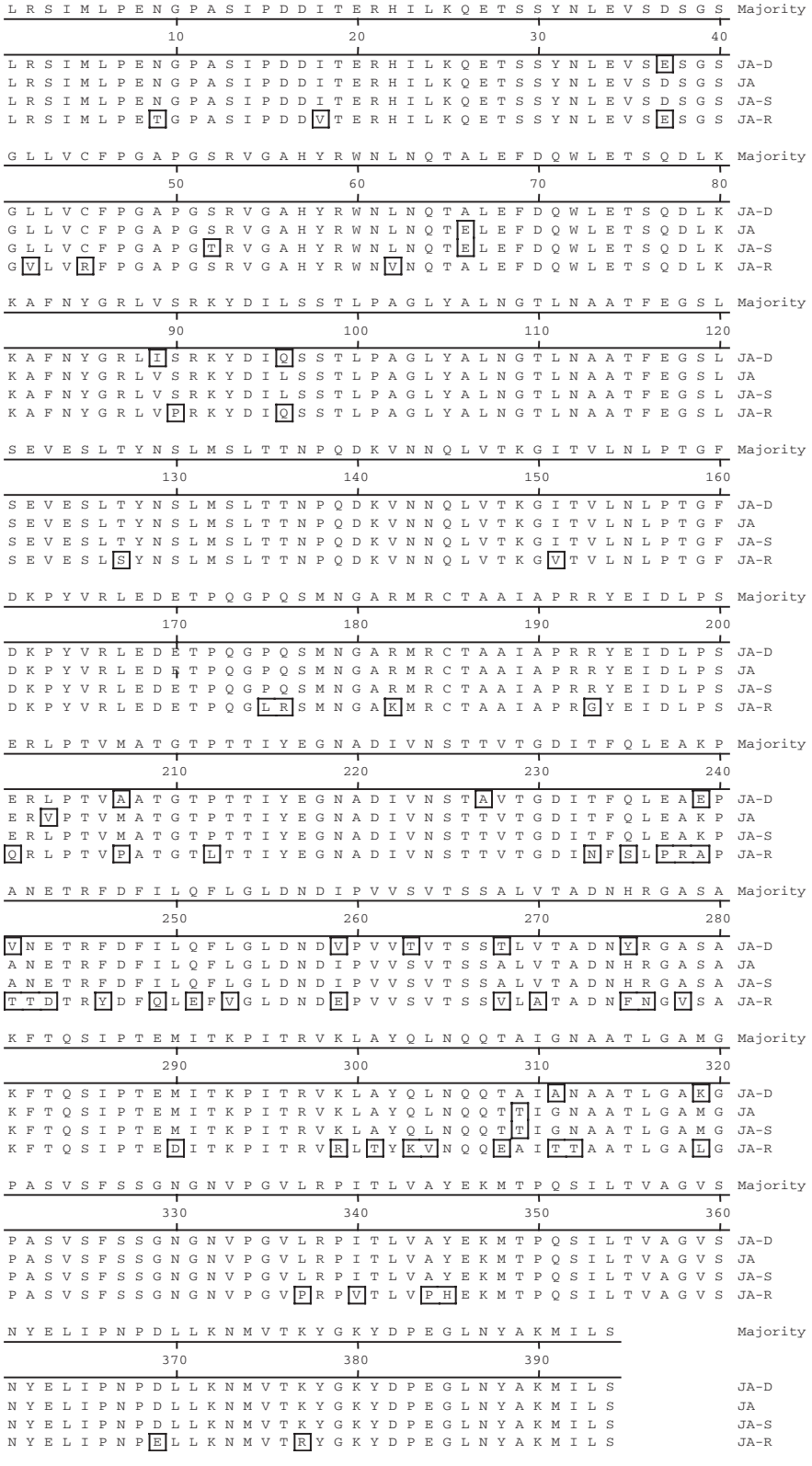

Fig. 1. Multiple alignment of deduced amino acid sequences of the 1182 bp cDNA fragment representing most of the VP2 protein of IPNV. Boxes indicate differences with the consensus. Each isolate is indicated as follows: JA-D = Jasper isolate (NC001915; Duncan & Dobos 1986); JA = Jasper-ATCC (AF342735; Blake et al. 2001); JA-S (AF399925) = Jasper isolate which is sensitive to RTS; JA-R (AF399926) = Jasper isolate which is resistant to RTS

revealed marked differences in hydrophilic region found in Ja-S and Ja-R: 64–69 (R), 74–75 (S), 258–259 (S), 276–278 (R), 340–343 (R) (Fig. 3). At these positions, only strain Ja-S had at least 3 consecutive hydrophilic residues, while Ja-R did not show any hydrophilic residues. The difference between hydrophilic regions on the protein of the 2 strains was closely related to the estimated antigenic difference between the 2 clones (Fig. 3). Significant antigenic differences were found at these positions: 61–70, 126–130 and 231–340.

Enzyme immunodot assay

Differences in the monoclonal antibody reaction profile were determined for parent Ja-ATCC, Ja-S and Ja-R (Table 3). The parent Ja-ATCC and Ja-S showed identical epitope patterns with a panel of 11 anti-IPNV MAbs having positive reactions with the monoclonal antibodies AS-1, W-1, W-2, W-3, W-4, W-5, E-1 and E5. The monoclonal antibody reaction profile was significantly different for Ja-R with positive reactions seen with the monoclonal antibodies AS-1, E-1, E-5 and E-6.

DISCUSSION

The use of modern molecular techniques has demonstrated that populations of serologically identical RNA viruses are extremely heterogeneous (Steinhauer & Holland 1987, Steinhauer et al. 1989). This extreme heterogeneity of RNA virus has also been reported among clones from single isolates of foot-and-mouth disease viruses (Domingo et al. 1980, Schiappacassi et al. 1995). This is thought to be due to an inefficient nucleic acid repair system that retains transcription errors at a high rate compared to those in DNA virus. Despite heterogeneity within virus populations, termed quasispecies, high mutation rates

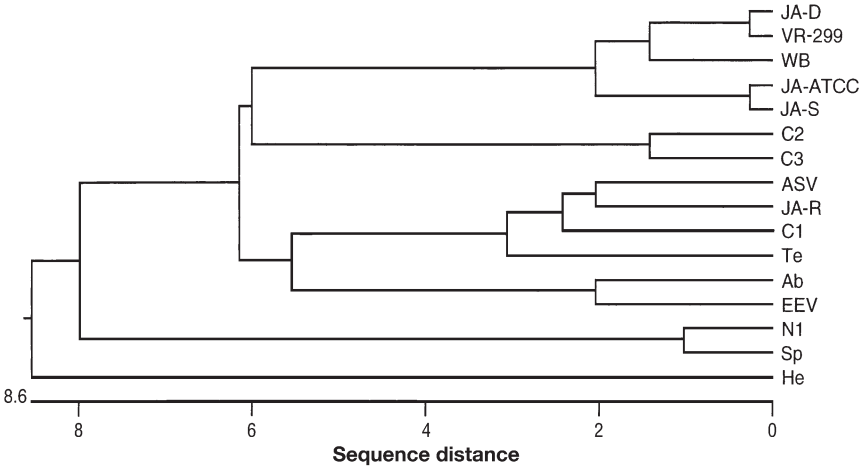

Fig. 2. Phylogenetic tree of selected aquatic birnaviruses based on deduced amino acid sequences of the 1179 bp cDNA fragment encoding most of VP2. GenBank accession numbers of 16 IPNV used for phylogenetic analysis as follows: Ja-S (AF399925); Ja-R (AF399926); Ja-D (NC001915); Ja-ATCC (AF342735); N1 (D00701); He (AF342730); ASV (AY026490); C1 (AF342732); EEV (AY026486); VR-299 (AF343572); WB (AF342727); C2 (AF342733); Sp (AF342728); Ab (AF342729); C3 (AF342734); Te (AF342731)

Table 2. Percent similarity and divergence of the amino acid sequences of the 1179 bp cDNA fragment within the VP2 coding region of aquatic birnaviruses. Upper triangle indicates percent similarity; lower triangle indicates percent divergence. Ja = Jasper-ATCC, Ja-D = Jasper-Dobos, Ja-R = Jasper isolate which is resistant to RTS, Ja-S = Jasper isolate which is sensitive to RTS

Virus	Ab	ASV	EEV	He	Ja	Ja-D	Ja-R	Ja-S	N1	Sp	Te	VR-299	WB	C1	C2	C3
Ab	**	88.8	95.9	83.7	88.8	90.3	87.0	88.8	89.8	90.3	89.3	90.3	90.3	89.3	87.5	88.5
ASV	12.2	**	89.3	82.1	86.5	87.8	95.9	86.5	88.8	89.3	95.2	87.8	87.0	97.7	87.0	87.5
EEV	4.2	11.6	**	84.9	87.0	88.0	87.8	87.0	91.1	91.1	90.6	88.0	88.0	90.1	87.5	88.3
He	18.5	20.5	16.8	**	82.9	83.7	81.9	82.9	87.2	87.8	84.4	83.7	82.9	83.2	83.7	82.9
Ja	12.2	14.9	14.3	19.5	**	95.9	86.5	99.5	87.2	88.3	87.8	95.9	95.9	87.2	88.3	87.8
Ja-D	10.4	13.4	13.1	18.5	4.2	**	87.0	95.9	88.3	89.3	88.0	99.5	97.2	88.5	88.3	88.3
Ja-R	14.3	4.2	13.4	20.8	14.9	14.3	**	86.5	87.2	87.8	93.6	87.2	86.2	96.7	85.7	86.2
Ja-S	12.2	14.9	14.3	19.5	0.5	4.2	14.9	**	87.2	88.3	87.8	95.9	95.9	87.2	88.3	87.8
N1	11.0	12.2	9.5	14.0	14.0	12.8	14.0	14.0	**	98.0	89.8	88.3	87.8	89.5	89.5	88.8
Sp	10.4	11.6	9.5	13.4	12.8	11.6	13.4	12.8	2.1	**	89.8	89.3	88.8	90.1	89.3	88.5
Te	11.6	5.0	10.1	17.5	13.4	13.1	6.7	13.4	11.0	11.0	**	88.0	87.8	95.4	88.0	88.0
VR-299	10.4	13.4	13.1	18.5	4.2	0.5	14.0	4.2	12.8	11.6	13.1	**	97.2	88.5	88.3	88.3
WB	10.4	14.3	13.1	19.5	4.2	2.9	15.3	4.2	13.4	12.2	13.4	2.9	**	87.8	88.0	87.5
C1	11.6	2.3	10.7	19.1	14.0	12.5	3.4	14.0	11.3	10.7	4.7	12.5	13.4	**	88.5	89.0
C2	13.7	14.3	13.7	18.5	12.8	12.8	15.9	12.8	11.3	11.6	13.1	12.8	13.1	12.5	**	97.2
C3	12.5	13.7	12.8	19.5	13.4	12.8	15.3	13.4	12.2	12.5	13.1	12.8	13.7	11.9	2.9	**

coupled with low repair rates do not always induce rapid evolution. Wild-type virus can predominate through an extensive passage history even though one variant can be dominant at any one time by immunological selection (Schiappacassi et al. 1995).

It has been reported that IPNV is highly heterogeneous at the genome level (Heppell et al. 1992, 1993, Zhang & Suzuki 2003). In this study, we demonstrated the heterogeneity in clones of IPNV-Jasper. The parental virus, ATCC VR-1325 ('Jasper', prototype of the A_9 serotype of Hill & Way 1995) was chosen due to its sensitivity to inactivation by normal RTS. Clones of this strain varied considerably in their sensitivity to RTS, with 8% exhibiting total resistance to inactivation by RTS. The deduced amino acid sequences of the VP2 region of the sensitive clones (Ja-S) had high sequence

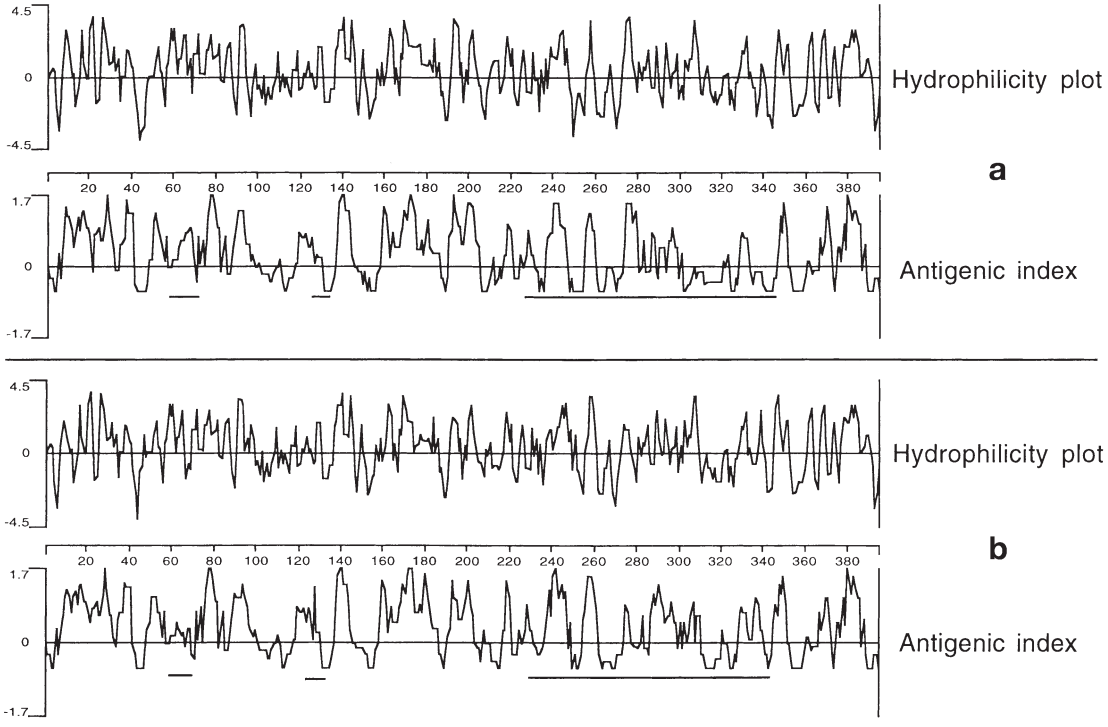

Fig. 3. Hydrophilicity plots and antigenic index of amino acid sequences of VP2 from 2 IPNV-Jasper strains. (a) Jasper RTS-sensitive strain; (b) RTS-resistant strain. Hydrophilicity plots were determined by the Kyte-Doolittle method. Antigenic indexes were determined by the James-Wolf method. Horizontal ruler represents the amino acid position. Lines under the antigenic index show the major antigenic differences between the RTS-sensitive and the RTS-resistant strain

Table 3. Monoclonal reaction patterns of IPNV in an immunodot assay. Jasper-parent = parent IPNV-Jasper; Jasper-RTS-S = progeny Jasper-RTS sensitive clones; Jasper-RTS-R = progeny Jasper-RTS resistant clones

IPNV-isolate	Monoclonal antibodies (epitopes)											RTS sensitivity
	AS-1	W-1	W-2	W-3	W-4	W-5	E-1	E-2	E-3	E-5	E-6	
Jasper-ATCC	+	+	+	+	+	+	+	–	–	+	–	Sensitive
Jasper-parent	+	+	+	+	+	+	+	–	–	+	–	Sensitive
Jasper-RTS-S	+	+	+	+	+	+	+	–	–	+	–	Sensitive
Jasper-RTS-R	+	–	–	–	–	–	+	–	–	+	+	Resistant
VR-299	+	–	–	+	+	+	+	–	–	+	+	Resistant
West Buxton	+	+	+	+	+	+	+	–	–	+	+	Resistant
Ab	+	+	+	–	–	–	+	+	–	+	+	Resistant
Canada 1	+	–	–	–	–	–	+	+	–	+	+	Sensitive
Canada 2, 3	+	–	–	–	–	–	–	–	–	+	+	Resistant
Buhl[a]	–	–	+	–	+	+	+	–	–	+	+	Resistant
Epitope on:	VP-2	VP-2	VP-2	VP-2	VP-2	VP-3	VP-3	VP-3	VP-2	VP-3	VP-3	

[a]Data of Buhl isolate were taken from Ögüt (1995)

identity with our ATCC VR-1325. Analysis of the same region for the resistant clones (Ja-R) showed highest identity with the ASV and Canada 1 (A_6 serotype) isolates. Our phylogenetic study of the VP2 region and the results of our monoclonal reaction tests suggest that Ja-R is not closely related to either the parent strain or Ja-S strains.

It has been found that the epitope patterns of IPNV proteins, VP2 and VP3, have been stable during multiple viral passages *in vivo* and *in vitro* over long periods of time. For example, viruses (ATCC VR-877) isolated between 1970 and 1999 from chronically infected brook trout at a single facility were identical in their epitope pattern with 11 monoclonal antibodies (Reno 1999). Likewise, virus isolated from rainbow trout from the mid-1960s until 1999 at a rearing facility in the Idaho had an epitope pattern identical to ATCC-VR1430 (Buhl) (Reno 1999). However, these antigenic tests have not been conducted for clones of virus populations. In this study, we report significant heterogeneity among clones with respect to epitope configuration and RTS sensitivity. From a total of 50 clones, 92% were wild-type (Ja-S) with respect to RTS sensitivity. For the 11 epitopes tested, these clones had all 8 of the epitopes that test positive in the ATCC VR-1325. Eight percent of the isolated clones (Ja-R) were resistant to RTS and these clones had an epitope configuration most similar to the A_7, Canada 1 serotype. The Ja-R strain differed from the Canada 1 serotype because it lacked the E-2 epitope. However, the presence of the E-2 epitope has been reported to be variable in the Canada 1 serotype (P. W. Reno unpubl. data). This suggests that the Ja-R strain is antigenically similar to the Canada 1 serotype. This is further supported by growth studies in different cell lines. The Ja-R strain was able to replicate in CHSE-214 but not in EPC cells. This pattern of growth is similar to that reported for Canada 1 by Ögüt (1995). The monoclonal reaction pattern of strain Ja-R is identical to that of IPNV-NEL that belongs to the Canada 1 serotype (P. W. Reno unpubl. data).

Based on the results of our RTS selection and subsequent sequence analysis, and monoclonal antibody tests of our Ja-S and Ja-R clones, it appears that the initial isolate (Jasper-parent) used in this study was heterogeneous.

The presence of the Ja-R strain within the VR-1325 might be accounted for by the exogenous contamination with a Canada 1 serotype isolate. The likelihood of exogenous contamination is small, since our lab has not used the IPNV-NEL isolate for the last 5 yr, including the time during which this experiment was carried out and the viruses were not thawed simultaneously even at that titre. The presence of heterologous IPNV in the Albertan hatchery at the time of the original isolations cannot be ruled out. Two laboratories obtained isolates of IPNV that they designated as 'Jasper' from the Maligne River hatchery at different times. These isolates are now referred to as Ja-D and Ja-ATCC. Berthiaume et al. (1992) reported that Ja-D and Ja-ATCC showed differences in their monoclonal reaction patterns and sequences of their VP2 regions. In facilities with long-term, chronic IPN infection, it is recognized that multiple serotypes of IPNV can be present at a single facility. For example, 4 subtypes of IPNV (A_9, OR A_1, Buhl A_1, A_3) were isolated between 1986 and 1996 from a single facility in northern Idaho (Reno 1995). In 1990 alone, 3 serotypes were isolated from this facility. Two different subtypes were isolated from rainbow trout (VR-299) in 1975 and brook trout (Buhl) in 1974 at the Wizard Falls Hatchery in Oregon (P. W. Reno pers. comm.). Yamamoto (1975a,b) also reported that both rainbow and brook trout were found to harbor IPNV at Maligne River Hatchery, Alberta and the possibility exists that different sero/phenotypes were present. If this were indeed the case, the replication rates of the strains would have to have been very similar to prevent 1 strain from out competing the others. It is also possible that the Ja-R strain has always been present in this isolate at a level below the detection limit for the monoclonal antibodies.

There is some precedent for a genetic shift under the influence of selective pressure. Schiappacassi et al. (1995) reported polyclonal antibody neutralization-resistant (Nr) variants from a population of foot-and-mouth disease virus C_3 Resende strain. Resende original strain (C_0) was passaged 25 times under immunological pressure (F_1, F_2, F_3, F_4 and F_5) or under non-immune pressure as a control (C_1, C_2, C_3). Each of the selected Nr populations (F_1 to F_5) showed a specific antigenic reactivity. Three Nr variants (F_2, F_3 and F_4) acquired 1 reactivity with monoclonal antibody (MAb) which was not positive from the original strain. The F_1 Nr population lost 1 MAb reaction, while all other Nr variants as well as the control showed a positive reaction. Nucleotide sequence analysis from Nr variants and the original strain also showed modifications in the most variable regions (residues 40–60) of VP1.

Wang (1992) reported monoclonal antibody neutralization-resistant variants from a population of IPNV-West Buxton strain (WB). Two WB variant strains were very different from the parent based on genomic data and serotype; neutralization-resistant strains had diverged from WB and more closely resembled members of the Sp (A_2) serotypes. Neutralization-resistant strains shared less than 80% sequence homology with WB strains.

We found VR-1325 and Ja-S had the same monoclonal reaction pattern (Table 3). If the parental IPNV-

Jasper contained a subpopulation of 8% of Ja-R variants, E-6 monoclonal antibody reaction might be expected to be positive since Ja-R has the epitope, whereas Ja-ATCC does not. So the question arose whether all Ja-R had the same epitope pattern, including E-6. We tested all 4 Ja-R clones and found that all showed the same epitope pattern (data not shown). Originally the concentration of Ja-parent tested for serotyping was 10^7 $TCID_{50}$ ml^{-1}, thus if the Ja-R strain comprised 8% of the population, the concentration of Ja-R would have been approximately 10^6 $TCID_{50}$ ml^{-1}. We found that a reaction with E-6 was not positive at a concentration of 10^6 $TCID_{50}$ ml^{-1}. Our laboratory experience has shown that Ja-ATCC does not generally grow to a high titer (approximately 10^7 $TCID_{50}$ ml^{-1}), compared to other serotypes (approx. 10^{10} $TCID_{50}$ ml^{-1}) (Ögüt 1995, P. W. Reno unpubl. data). For the serotyping of Ja-R, the clones were passaged once more in RTG-2 cells in the presence of RTS and the virus titer was significantly elevated (10^9 to 10^{10} $TCID_{50}$ ml^{-1}) and produced a strong monoclonal reaction at E-6. This elevated virus titer of the Ja-R clones may have been inhibited when Ja-S was present in the mixture originally. It is known that parental wild-type RNA phage consistently outgrew variant clones under normal *in vitro* conditions (Domingo et al. 1978). Maret (1997) also found that one type of IPNV (Buhl) outgrew another type of IPNV (WB) in a superinfection experiment in brook trout. However, the stability between wild-type and variant virus could be changed by some condition such as interference by defecting interfering (DI) particles, different host or cell types, or immune selection (Steinhauer & Holland 1987) and thereby enhance the replication of a normally slower replicating virus. It is known that host immune selection can be a strong factor in driving virus evolution (Clements et al. 1980, Palese & Young 1982, Webstrer et al. 1982). Even though we do not know the whole history of the Jasper-isolate (Ja-ATCC) that was used in this experiment, we passaged the virus 2 times through brook trout just before this experiment, which could select more RTS-resistant strains from the virus population. Even though a 1:100 dilution of brook trout serum (as opposed to RTS) did not significantly inhibit the virus *in vitro* (Park 2000), this could still be one of the possible reasons that a RTS-resistant population could emerge and comprise a relatively high proportion of the clones (8%) *in vitro*.

We found Ja-R had an epitope composition most closely related to the Canada 1 serotype and had highest amino acid homology with genogroup 4, as reported by Blake et al. (2001), which is also the Canada 1 clade (Tables 2 & 3). Based on 11 monoclonal reaction patterns, we could not differentiate between the RTS-sensitive and RTS-resistant strain. All of the RTS-resistant strains tested showed a positive reaction for E-6 MAb and a negative reaction for W5 MAb. The Canada 1, sensitive isolate, also showed a similar reaction for 2 MAbs. Buhl, a resistant isolate, however had a positive reaction for W5 MAb. It is more likely that other epitopes which do not react with these 11 MAbs are more closely linked to RTS sensitivity.

The antigenic differences between ATCC VR-1325 and Ja-R might not be directly tied to amino acid sequence, but might also be associated with glycosylation of the VP2 or VP3. Glycosylation as well as proper folding of the protein are important factors in antigenicity (Caust et al. 1987). Even though contradictory results have been noted (Perez et al. 1996, B. L. Nicholson pers. comm.), the possibility of glycosylation in IPNV has been suggested (Estay et al. 1990, Håvarstein et al. 1990, Hjalmarsson et al. 1999, Espinoza et al. 2000). To date, 2 possible glycosylation sites, N-glycosylation (Estay et al. 1990) and O-glycosylation (Hjalmarsson et al. 1999), have been suggested. In this study, possible glycosylation sites for N-glycosylation and O-glycosylation were deduced from amino acid sequences of 2 IPNV-Jasper strains. The 2 strains had the same number of possible N-glycosylation sites, Asn(N)-X-Ser(S)/Thr(T) (Fig. 1). Three sites were present on the same regions of 2 strains (63–65,108–110, 224–225); however, the possible sites at 63–65 had different hydrophilic characteristics: Ja-S was hydrophilic at this region and Ja-R was not. The last possible site was present at different regions: Ja-S amino acids 242–244 was a hydrophilic region, while Ja-R amino acids 233–235 were not hydrophilic. The 2 strains had similar numbers of possible O-glycosylation sites involving the amino acids, Ser or Thr. The RTS–sensitive strain had 69 possible sites, while Ja-R had 72 sites among 394 amino acid residues. Ja-S had 14 possible O-glycosylation sites on hydrophilic residues, while Ja-R had 10 sites. This indicates that the Ja-S strain has more possible glycosylation sites for both N-glycosylation and O-glycosylation on hydrophilic regions than Ja-R does. However, we need more data to determine the glycosylation of IPNV.

In conclusion, IPNV-Jasper variants (Ja-R) isolated from IPNV-Jasper (Ja-ATCC) which are sensitive to RTS were resistant to RTS and different with respect to the serotype, and cDNA sequence of VP2 coding region.

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