Epitope analysis of IPN virus isolates from feral and hatchery salmonid fishes by neutralizing monoclonal antibodies

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A panel of ten neutralizing monoclonal antibodies (MAbs) were raised against IPN virus associated with feral lake trout (LT-IPNV) from Cornwall Lake, Alberta, western Canada. The antibodies were used for resolving antigenic relationship between IPN virus isolated from hatchery reared trout in Jasper (Ja-IPNV) in Alberta and from feral Arctic char (AC-IPNV) from Northwest Terrorities in western Canada. Three MAbs neutralized only LT-IPNV indicating its distinct epitopes, and clearly differentiated it from AC-IPNV. Seven MAbs recognized common epitopes present on LT-IPNV and AC-IPNV in neutralization assay and none of the antibodies neutralized Ja-IPNV isolate. LT-IPNV was found to be a distinct isolate, closely related to AC-IPNV than to Ja-IPNV from Alberta.

INFECTIOUS pancreatic necrosis virus (IPNV) causes serious disease and mortality in hatchery reared salmonid fishes[1]. Viruses similar or identical to IPNV have been isolated worldwide from various salmonid and non-salmonid fishes, marine molluscs and even from rotifers[2–8]. Based on the relative degree of cross-neutralization by rabbit antisera, three main serotypes of IPNV namely VR 299 (North America), Sp and Ab (Europe) were designated[9–11]. Further examination of several isolates from fish and shellfish by cross-neutralization with rabbit antisera indicated six additional serotypes[12]. Significant antigenic difference among isolates of the North American serotype VR 299 has also been demonstrated by polyclonal and monoclonal antibodies[13, 14]. Four serotypes of IPNV have been proposed from Canada based on 50% plaque reduction by rabbit antisera[15], which have been confirmed by monoclonal antibody studies[13]. Recently, Lecomte *et al.*[16] have shown antigenic difference among IPNV isolates of Canada using monoclonal antibodies reacting with VP_3 protein.

In western Canada three IPNV isolates have been recorded namely the Jasper (Ja-IPNV) from a trout hatchery in Alberta[17], Arctic char (AC-IPNV) from feral char of the Northwest Territories[18] and lake trout (LT-IPNV) from feral lake trout of Cornwall Lake, Alberta[19]. The location of LT-IPNV isolation is geographically closer to that of Ja-IPNV, than to AC-IPNV location (Figure 1). The LT and AC-IPNV were isolated from adult feral chars from two different remote regions in western Canada which have no records of introduction of hatchery reared salmonids and hence it is likely that the viruses are evolved indigenously[20]. In cross-neutralization studies using rainbow trout antisera

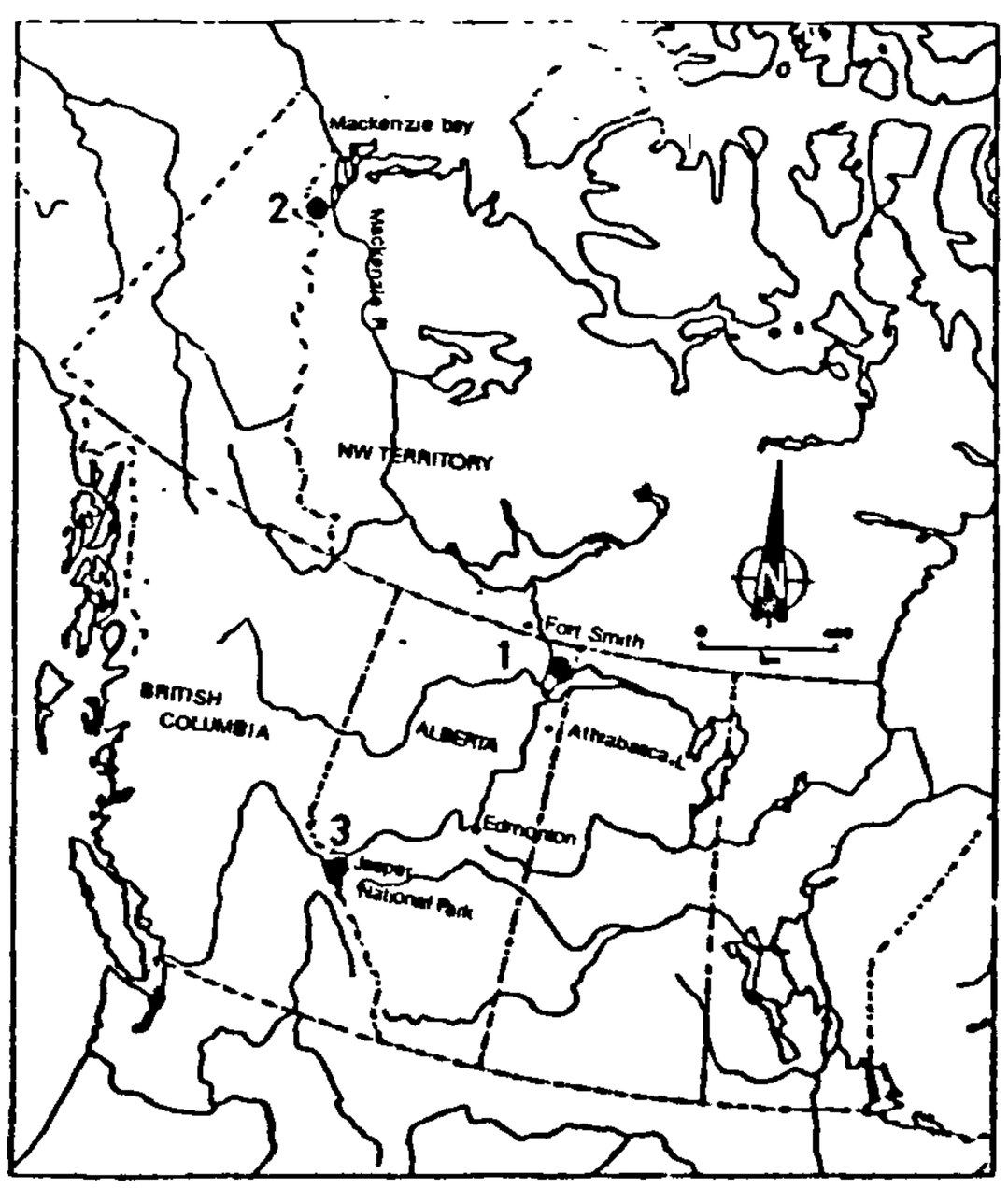

Figure 1. Map of western Canada showing the geographical locations of IPNV isolations. 1, LT-IPNV from Cornwall Lake in northern Alberta, 2, AC-IPNV from western Mackenzie River Delta in Northwest Territory; 3, Ja-IPNV from Jasper National Park in Alberta.

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LT and AC-IPNV were found to be identical[21], but our studies with rabbit antisera demonstrated difference between them (results communicated). Further, there was difference between LT and AC-IPNV in molecular weight of VP_2 bearing neutralizing epitopes and in degree of pathogenicity to salmonids[22]. Hence, there was a general interest about the antigenic relationship between the IPNV isolates from hatchery and wild salmonids in western Canada. In this study antigenic relationships between LT, AC and JA-IPNV isolates are analysed using neutralizing monoclonal antibodies (MAbs) raised against LT-IPNV.

Materials and methods

Virus stocks and their purification

LT, AC and Ja-IPNV isolates were available in our Edmonton laboratory. The isolates were plaque purified thrice and propagated in CHSE-214 cells according to Lannon *et al.*[23]. For immunization LT-IPNV was purified in $CsCl_2$, whereas sucrose purified virus[24] was used in ELISA.

Production of MAbs to LT-IPNV

Hybridomas were produced as detailed in Yamamoto and Shankar[22]. Eight-week-old female Balb/C mice were injected (IP) with 0.06 ml of CsCl purified LT-IPNV ($10^{10\,5}$ $TCID_{50}$/ml) in 0.01 M PBS buffer. A second dose of 0.15 ml of purified virus was injected (IP) on the 21st d, followed by a third dose of 0.15 ml (IV) on the 28th d. Three days later spleen from mice showing higher titers of antibody against 10^8 $TCID_{50}$/ml of antigen in ELISA was used for fusion with SP_2 myeloma cells. Fused cells were maintained at 37°C with 5% CO_2 and 80% relative humidity in a Steri-cult incubator. Supernatants of hybridomas were assayed for LT-IPNV antibodies by ELISA according to Caswell-Reno *et al.*[24]. Positive hybridomas were minicloned thrice, and antibodies from stable clones were isotyped using mouse monoclonal sub isotyping kit (Hyclone laboratories, Logan, Utah, USA).

Initial microneutralization assay[25] was performed with supernatant from all the positive clones, to determine neutralization of the virus. Briefly, supernatant from ELISA positive hybridoma from 24 well plates was mixed with an equal volume of $10^{2.2}$ TCID 50/ml of LT-IPNV, and 0.1 ml inoculated to wells of CHSE-214 cells in 96 well plates. Cell monolayer was observed for CPE for 14 d to determine neutralization of the virus.

Minicloned hybridomas were grown in RPMI medium with 5% fetal bovine serum in the absence of antibiotics. Cell culture supernatants were harvested and their antibody titer determined by ELISA. Appropriate dilution of culture supernatants giving an ELISA absorbance of approximately 1.5 against 10^8 $TCID_{50}$/ml of antigen was used in neutralization assay. Ascites of hybridomas were produced in 18-month-old male Balb/C mice primed with pristane.

Determination of epitope specificity of MAbs by reciprocal blocking ELISA

Epitope specificity was determined by the method of Friguet *et al.*[26] Antigen saturation point of ascites was determined at an antigen concentration of $10^{7\,5}$ $TCID_{50}$/ml. A 1:500 dilution of ascites of a hybridoma producing non-specific antibody to LT-IPNV (LTO) was used as negative control. ELISA absorbance of MAb_1 and MAb_2 together and separately at antigen saturation point were determined. Reciprocal ELISA was carried out for all the MAb pairs and additivity index (AI) calculated as follows.

$$AI = 100 \times ([(2 \times A \text{ of } MAb_1 \text{ and } MAb_2 \text{ together})/(A \text{ of } MAb_1 \text{ alone}) + (A \text{ of } MAb_2 \text{ alone})] - 1)$$

Two-way analysis of variance (ANOVA) test was applied to determine AI for homologous MAb pairs at which statistical additivity is zero. MAb pairs giving higher reciprocal AI values were considered as reacting with two different epitopes on the antigen.

Epitope analysis of IPNV isolates by neutralization assay

The concentration of virus isolates was adjusted to $10^{2\,2}$ $TCID_{50}$/0.1 ml in MEM-HEPES, and 0.4 ml aliquots mixed with an equal volume of serial two-fold dilutions of LT-IPNV neutralization positive MAbs as determined before by microneutralization assay. Hybridoma culture supernatants giving an ELISA absorbance of 1.5 against 10^8 $TCID_{50}$/ml of LT-IPNV were used for serial dilution. The mixture was thoroughly mixed and incubated for 60 min at room temperature. One tenth of 1 ml from the above mixture was inoculated on to CHSE-214 cell monolayer in 96 well microtiter plates. The inoculated cell monolayer was observed for cytopathic effect for 14 d and neutralizing antibody titer protecting 50% of inoculated cell culture (ND_{50}) calculated[27].

Results

Epitope specificity of MAbs

A total of 300 ELISA positive hybridomas were obtained from eight fusions, from which 20 stable monoclones producing neutralizing antibodies of IgG isotypes were selected for determining epitope specificity by reciprocal blocking ELISA.

Table 1. Additivity index (AI) of 10 different LT-IPNV monoclonal antibodies

MAb_2 / MAb_1	LT3	LT4	LT5	LT6	LT7	LT8	LT10	LT11	LT12	LT13	LT0
LT3	7 95	45 87	58 97	33.39	60 90	121 85	33 20	79 68	140 76	165 31	79 00
LT4	74 85	9 88	30 68	79 67	42 59	55 56	46 76	31 70	42 61	115 70	93 00
LT5	63 39	88 31	NA	33 42	67 29	48 80	78 20	76 67	45 06	119 50	78 00
LT6	66 60	30.40	31.50	11.90	39 15	37 19	36 90	31 90	56 96	88 46	98 00
LT7	56 39	43 68	70.96	80 60	9 88	45 86	65 41	66.02	90 00	156 49	90 00
LT8	97 47	50 38	84 54	58 44	149 00	10 50	112 00	86.76	36 03	152.00	102 00
LT10	30.94	41 30	63 61	44 42	35 00	33 92	7 11	31 21	41.00	108.00	85 00
LT11	76.18	32 14	33 20	70 40	30 71	97 05	32 17	8 42	86 92	137.50	104 00
LT12	49 20	59 20	30 68	70 65	44 56	63 20	83 17	95 60	NA	61 86	98 00
LT13	68 20	43 98	71 20	66 47	74 89	56 62	73 00	46 39	81 36	11 00	113 00
LT0	109 00	87 00	87 00	93 00	85 00	94 00	107 00	107 00	105.00	78 00	NA

AI values were derived from reciprocal blocking ELISA where AI = 100 × ([(2 × A of MAb_1 and MAb_2 together)/(A of MAb_1) + (A of MAb_2)] – 1) MAb_1 – MAb applied first, MAb_2 – MAb applied second, NA – No additivity.

Table 2. Neutralization of IPNV isolates by LT-IPNV MAbs

	MAbs									
Virus	LT3	LT4	LT5	LT6	LT7	LT8	LT10	LT11	LT12	LT13
LT	++	+++	+++	+++	+++	+	++	++	+++	+++
AC	+	+++	–	+++	–	+	++	+++	++	–
Ja	–	–	–	–	–	–	–	–	–	–

Equal quantities of $10^{2\cdot2}$ $TCID_{50}$/0 1 ml of IPNV isolates and serial two-fold dilutions of hybridoma culture supernatants were mixed and 0 1 ml inoculated to CHSE-214 cell monolayers in 96 well microtiter plates Inoculated monolayers were observed for CPE for 14 d and ND_{50} calculated according to the method of Karber[27]. Log $ND_{50} > 3$, +++; log $ND_{50} > 2$, ++; log $ND_{50} > 1$, +; No detectable neutralization, –.

Reciprocal AI values were calculated for all the 20 pairs of MAbs. There was no additivity up to AI 25 with the distribution of AI from 0 to 150. At 95% confidence interval it was found that when AI was 25 each observed value was subject to variation of 0 ± 23.97. The LT-IPNV non-specific ascetic fluid used as a control neither gave a background nor interfered with the binding of the specific antibodies. Finally, 10 MAbs (Table 1) which had reciprocal AI values > 25% were chosen as having specific reactions with different epitopes on the virus.

Neutralization of IPNV isolates by LT-IPNV MAbs

Of the ten neutralizing MAbs (Table 2) LT5, LT7 and LT13 neutralized only LT-IPNV and not AC, or Ja-IPNV. However, seven MAbs-LT3, LT4, LT6, LT8, LT10, LT11 and LT12 neutralized both LT and AC-IPNV. LT3 and LT12 weakly neutralized AC compared to that of LT-IPNV. Ja-IPNV isolate was not neutralized by any of the LT-IPNV neutralizing MAbs. Thus these neutralizing MAbs could clearly distinguish LT, AC and Ja-IPNV isolates.

Discussion

The external protein VP_2 of IPNV carries major neutralizing epitopes and type specific antigenic determinants[28, 29] and hence neutralization test is much more specific and sensitive to clearly differentiate the IPNV isolates. Therefore, to resolve antigenic relationship between the LT, AC and Ja-IPNV isolates neutralizing monoclonal antibodies were raised for epitope analysis. Ten neutralizing MAbs to LT-IPNV were selected by virtue of their high AI values in reciprocal ELISA which indicated their specificity to different epitopes[24]. These MAbs recognized VP_2 protein of LT-IPNV in Western blot[22]. AI value of some MAb pairs was less than 0 while for some pairs it was more than 100 indicating inhibition or augmentation respectively of binding of MAbs. This may be due to steric hindrance where binding of one antibody influences the subsequent binding of another[30] or attachment of one antibody causing allosteric alteration of the adjacent antigenic determinant[30]. AI values less than 100 were noticed due to interference in binding between MAbs which indicate overlapping epitopes or close proximity between them. The AI values of LT0 with other MAbs were higher indicating the validity of the values obtained in the experiment.

Among the ten neutralizing MAbs three MAbs LT5, LT13 and LT7 specifically neutralized LT-IPNV. LT5 and LT13 have been successfully used in an immunodot assay for epidemiological analysis of IPN outbreak in Alberta[22] Seven neutralizing MAbs neutralized both LT and AC indicating presence of large numbers of

common neutralizing epitopes on these isolates. LT and AC-IPNV were found to possess similar type of VP_2 protein in SDS-PAGE distinct from that of Ja-IPNV[22]. However, LT and AC differed clearly by the difference in molecular weight of VP_2 protein. Further, a difference in site of persistence in host and also in degree of pathogenicity to salmonids was observed between them (communicated). The reaction pattern of these two isolates with neutralizing MAbs indicates that the isolates are not identical but closely related to each other. This observation is contrary to the findings of Kelly and Nielson[21] that LT and AC are identical.

LT-IPNV neutralizing MAbs did not neutralize Ja-IPNV. This indicates that the hatchery isolate Ja-IPNV is distinct from LT-IPNV isolate from feral char. The locations of isolation of LT-IPNV and Ja-IPNV are close to each other. Though the distance between AC and LT-IPNV locations is more than that between LT and Ja-IPNV, the former two are closely related antigenically. Further both AC and LT-IPNV isolates are isolated from the same char genus *Salvelinus*. It appears that they have undergone minor changes in epitopes to suit their host.

In summary, we report that a panel of ten monoclonal antibodies recognizing different epitopes on LT-IPNV virus was produced. In neutralization tests these antibodies clearly distinguished the Ja-IPNV isolate from the hatchery and LT and AC-IPNV isolates from feral salmonid fishes. The feral IPNV isolates were found to be closely related to each other, distinct from the hatchery IPNV isolate and may have been evolved indigenously in the remote regions of western Canada.

1 Wolf, K., Snieszko, S. K., Dunbar, C. E and Pyle, E., *Proc. Soc Exp. Biol. Med.*, 1960, **104**, 105–112
2. Chang, N., Macdonald, R. D and Yamamoto, T., *J. Microbiol.*, 1978, **24**, 19–24.
3 Comps, M., Menu, B., Brenil, G. and Bonami, J. R, *Aquaculture*, 1991, **93**, 1–5.
4 Hedrick, R P., Okamoto, N., Sano, T and Fryer, J. L., *Eur J. Gen Virol.*, 1983, **64**, 1421–1429.
5 Hill, B. J, *Wildlife Diseases*, Plenum Press, New York and London, 1976a, p. 445.
6. McAllister, P. E, U S. Fish and Wildlife Service Fish Disease Leaflet, 1983, p. 65.
7. Mortensen, S. H, Hjeltness, B., Rodseth, O., Krogsurd, J K. and Christie, K. E., *Bull. Eur. Assoc. Fish Pathol.*, 1990, **10**, 42–48
8 Mortensen, S H, Bachere, E, Legal, G. and Miahle, E., *Dis. Aqua Organisms*, 1992, **12**, 221–226.
9 Hill, B J., Queens University Press, Kingston, Canada, 1976b, p 25
10. Macdonald, R. D. and Gower, D. A, *Virology*, 1981, **114**, 187–193
11. Okamoto, N., Sano, T., Hedrick, R. P. and Fryer, J. L., *J Fish Dis*, 1983, **6**, 19–26.
12. Hill, B J and Way, K., in Abstracts of First International Conference of the European Association of Fish Pathologists, 1983, p. 10.
13. Caswell-Reno, P., Lipipun, V., Reno, P W and Nicholson, B L., *J. Clin Microbiol*, 1989, **27**, 1924–1931.
14. Nicholson, B L. and Pochebit, S, in Int. Symp. on Fish Biologics: Serodiagnostics and Vaccines (eds. Anderson, D. P. and Hennersen, W), S Karger, Basel, 1981, p. 35.
15. Hill, B. J and Way, K., in Abstracts of American Fisheries Society International Fish Health Conference, 1988, p 151.
16. Lecomte, J, Arella, M. and Berthiaume, L, *J Fish Dis.*, 1992, **15**, 431–438.
17. Yamamoto, T., *J. Fish Res. Bd Canada*, 1974, **31**, 397–406.
18. Souter, B. W., Dwilow, A. G., Knight, K. and Yamamoto, T., *J. Wildlife Dis*, 1984, **20**, 338–343.
19 Larson, B., *Am Fish Soc Fish Health Sec. Newsl.*, 1985, 13.
20. Sonkr, B. W, Dwilow, A G., Knight, K and Yamamoto, T., Canadian Technical Report of Fisheries and Aquatic Services, 1986, pp. 1441–1445.
21. Kelly, R. K. and Nielsen, O, *J. Aqua Animal Health*, 1990, **2**, 56–61.
22. Yamamoto, T. and Shankar, K. M., in *Diseases in Asian Aquaculture I* (eds. Sharif, M, Subasinghe, R. P. and Arthur, J. R.), Fish Health Section, Asian Fisheries Society, Manila, Philippines, 1992, p. 253.
23 Lannon, C. N., Winton, J. R. and Fryer, J. L., *In Vitro*, 1984, **20**, 671–678.
24 Caswell-Reno, P., Reno, P. W. and Nicholson, B L, *J. Gen. Virol.*, 1986, **67**, 2193–2203
25. Winton, J. R, Arakawa, C. K., Lannan, C. N. and Fryer, J. L., *Dis. Aqua Organisms*, 1988, **4**, 199–203
26. Friguet, B., Ohaniance, L. D., Pages, J., Bussard, A and Goldberg, M, *J. Immunol. Meth.*, 1983, **60**, 351–361.
27. Karber, G., *Pathol. Pharmacol.*, 1931, **162**, 480–486.
28. Dobos, P. and Roberts, T. E., *Can. J Microbiol.*, 1982, **29**, 377–383.
29. Wolski, S. C., Robertson, B S. and Hetrick, F. M, *Vet. Immunol. Immunopathol.*, 1986, **12**, 373–381.
30. Lubek, M D. and Gerhard, W., *Virology*, 1981, **113**, 64–73.

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