Reaction Patterns of Five Strains of Infectious Pancreatic Necrosis Virus in Enzyme-Linked Immunosorbent Assay

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(Received 14 June 1984/Accepted 3 August 1984)

ABSTRACT. Comparison of reaction patterns among the five strains of infectious pancreatic necrosis virus (IPNV) and an infectious bursal disease virus (IBDV) was done by using enzyme-linked immunosorbent assay (ELISA) with antiserum against purified VR299 strain of IPNV. Five strains of IPNV were divided into three groups by the reaction pattern: i) VR299 strain, ii) Buhl and Powder Mill strains, and iii) d'Honninchtun and Bonnamy strains. IBDV could not be detected by ELISA using the anti-IPNV serum. Molecular weight comparison of virion proteins revealed that size of β proteins of IPNV was unique in three groups of IPNV. Antigenic relationships among IPNV strains examined by immunodiffusion (ID) showed that Buhl and Powder Mill strains were similar to VR299 strain, but d'Honninchtun and Bonnamy strains were distinct from other three strains of IPNV. No cross reaction was observed between IBDV and IPNV-VR299 strain by ID. These results indicate that ELISA can be used to distinguish antigenic differences among IPNV strains and to differentiate IPNV from antigenically unrelated virus such as IBDV which is classified in the same virus family as IPNV.—**KEY WORDS:** ELISA, infectious bursal disease virus, infectious pancreatic necrosis virus, viral polypeptide.

Jpn. J. Vet. Sci. 46(6): 823–830, 1984

Infectious pancreatic necrosis (IPN) is an acute, highly contagious disease mainly affecting young salmonid fish [37]. Isolation of IPN virus (IPNV) has been reported in North America [25, 38], Europe [3, 4, 33] and Japan [29]. IPNV is now characterized with bisegmented double-stranded RNA genomes and a naked, icosahedral particle [6, 8, 23]. Many strains of IPNV have been isolated and cross neutralization test can be employed for serotyping them [13, 21, 22, 26, 28, 29, 34, 37]. Three major serotypes have been identified [22, 28]. Two of these serotypes are Ab and Sp strains [35] which are found in Europe, and another serotype is VR299 strain isolated in North America [37]. A number of viruses which possess similar morphological and biochemical properties to IPNV were isolated from a variety of non-salmonid fishes [1, 2, 29, 31] and marine

invertebrates [17, 32]. Infectious bursal disease virus (IBDV) of chickens and drosophila X virus (DXV) of the fruit fly are also considered to belong to the same virus family as IPNV [12]. The antigenic relationship of these IPNV-like viruses to IPNV are also not well understood.

In the previous paper, we described the double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of IPNV in tissue culture and in rainbow trout infected with the virus [14]. The sensitivity and the specificity were demonstrated by using highly purified IPNV antigen and hyperimmune serum. The present study was conducted to evaluate the antigenic relationships among 5 strains of IPNV by ELISA and by immunodiffusion (ID). The reaction patterns of ELISA were also compared with the differences in the size of viral polypeptides

analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Further, antigenic relationships of IPNV to IBDV were examined by ELISA and ID.

MATERIALS AND METHODS

Cells: Chinook salmon embryo cell line (CHSE-214) and steelhead trout embryo cell line (STE-137) were kindly provided by Dr. T. Nagabayashi (School of Fisheries Science, University of Kitasato, Iwate, Japan). Culture media (Eagle's minimum essential medium; MEM, pH7.2) and the method for propagation of line cells are described in the previous paper [14]. Chick embryo fibroblasts (CEF) were propagated as monolayers in MEM supplemented with 10% fetal calf serum, 200 units/ml of penicillin and 200 μ g/ml of streptomycin at 37°C.

Virus and purification of virus: IPNV strains of VR299, Buhl, Powder Mill, Bonnamy and d'Honninchtun were kindly supplied by Dr. T. Nagabayashi. Propagation of the virus in cell lines was described in the previous paper [14]. Strain of IBDV labeled RF-1 was kindly provided by Dr. M. Higashihara, Research Center for Veterinary Science, Kitasato Institute, Kashiwa, Japan. The IBDV was propagated at 37°C in CEF for 3 days until an extensive cytopathic effect was observed. Infected culture fluids and cells with these viruses were collected and frozen at -20°C or -80°C until use.

Both IPNV and IBDV were purified according to the method by Macdonald and Yamamoto [23] with a slight modification [14]. Briefly, virus materials were centrifuged for 90 min at 80,000 $\times$ g (B-60 Ultracentrifuge, Damon/IEC, Needhamhts, Mass.) and the pellet was resuspended in a small volume of TNE buffer (0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH7.2). The viral preparation was referred as crude IPNV. This preparation was overlaid on CsCl stepwise gradient solution (3 ml of 40% (W/W) CsCl, 3 ml of 30% CsCl and 1 ml of 20% CsCl) and

centrifuged at 35,000 $\times$ g for 16 hr at 4°C using #488 rotor. The virus band (density 1.33 g/cm³) was harvested and suspended in a small volume of TNE buffer, and then overlaid onto 8 ml of 20% sucrose solution in TNE buffer. Virus collected by centrifugation at 100,000 $\times$ g for 60 min was dissolved in a small volume of 0.15 M phosphate buffered saline (PBS; pH7.2) and referred as purified IPNV.

Antisera: CHSE-214 cell line was used for the propagation of VR299 strain for preparation of antiserum. The crude IPNV (1 mg protein/ml) was emulsified in an equal volume of Freund's complete adjuvant and was inoculated subcutaneously into the hind foot pads of guinea pigs. A booster injection was given 3 weeks later with the same amount of the antigen and blood was taken one week after the second injection. Anti-VR299 rabbit serum was prepared by the same procedure except that purified IPNV was used and the rabbits were inoculated subcutaneously on the back. Virus neutralization titers of these sera were 1:62,500 (anti-VR299 guinea pig serum) and 1:51,200 (anti-VR299 rabbit serum) as estimated by the method of Behrens and Kärber [18] using microplate (Falcon, Oxnard, Calif.).

Anti-IBDV chicken serum used for ID was supplied by the courtesy of Dr. M. Higashihara. The serum was collected from chickens inoculated with homogenate of bursa of Fabricius infected with RF-1 strain of IBDV. Virus neutralization titer of the serum was 1:40,960.

Sandwich ELISA: The method of ELISA described by Voller *et al.* [36] and Katze and Crowell [19] was employed with a slight modification. The detailed procedure of ELISA was described previously [14]. Polystyrene microtiter plates (Linbro, Hamden, Conn.) were coated with 0.2 ml of 0.1% anti-VR299 guinea pig serum in 0.06 M carbonate buffer, pH9.6. Plates were placed overnight in a humidified chamber at 4°C and

were washed three times with 0.15 M PBS, pH7.2, containing 0.05% Tween 20 (PBS-Tween). The plates were then recoated with 0.2 ml of 1% gelatine in PBS and were incubated for 1 hr at 37°C. After three additional washes, 0.2 ml of serial dilutions of purified IPNV or IBDV in PBS containing 0.1% ovalbumin (Grade V, Sigma, St. Louis, Mo.) (PBS-ovalbumin) was added to the wells. After incubation for 1 hr at 37°C, the plates were washed and added with 0.2 ml of 1% anti-VR299 rabbit serum in PBS-ovalbumin. The plates were incubated at 37°C for 1 hr and washed. Anti-rabbit IgG goat Ig conjugated with alkaline phosphatase diluted 1:1,500 in PBS-Tween was added (0.2 ml) to each well and incubated at 37°C for 1 hr. After washing the plates, 0.2 ml of substrate was added to each well and incubated for 1 hr at 37°C. The substrate solution consisted of 2.5 mM *p*-nitrophenyl phosphate in 0.06 M carbonate buffer containing 1 mM MgCl₂. The yellow color produced by the enzyme-substrate reaction was measured in a spectrophotometer (Titertek Multiskan, Flow Lab., McLean, Virg.) at wavelength of 405 nm. To express the antigenic relationships among five strains of IPNV, reactivity of each strain was expressed as a positive-to-negative (P/N) ratio. The ratio was calculated by the following formula:

$$P/N = \frac{\text{OD of test sample (10 } \mu\text{g/ml)}}{\text{OD of uninfected STE-137 cell culture fluid (10 } \mu\text{g/ml)}}$$

SDS-PAGE of viral proteins: Virion proteins were analyzed in 10% polyacrylamide slab gels, using the discontinuous SDS-gel system of Laemmli [20]. Purified IPNV was denatured with 2.5% SDS and 10% 2-mercaptoethanol for 1 to 2 min at 100°C. Fifteen to 20 μ g of sample was loaded into the wells and electrophoresis was performed for 5 hr at 15 mA. After the run, the gels were stained in 0.2% Coomassie brilliant blue in 50% methanol, destained in acetic acid-methanol-

water (10:10:80). To estimate the molecular weight of viral proteins, standard kit purchased from Pharmacia (Uppsala, Sweden) was used. This is a mixture of phosphorylase b (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), soybean trypsin inhibitor (20.1K), and α -lactoglobulin (14.4K).

ID: IPNV and IBDV antigens used for ID were prepared as follows. After removal of virus particles from culture fluids by ultracentrifugation, the supernatants which contained degraded virus particles were salted out by 50% saturated ammonium sulfate. The sediment collected by centrifugation at 12,000 $\times$ g for 30 min at 4°C was suspended in a small volume of 0.15 M PBS (pH7.2) and was dialyzed extensively against PBS. Ouchterlony plates were prepared with 1% Noble agar (Difco, Detroit, Mich.) in PBS. The plates containing antigens and antiserum were incubated at room temperature for 2 days and observed for precipitin lines.

RESULTS

Reactions of IPNV and IBDV in sandwich ELISA: The antigenic relationships in five strains of IPNV were examined by ELISA. Threefold dilutions of 5 strains of purified IPNV were added to microplate wells coated with anti-VR299 guinea pig serum. After incubation, the plate was reacted with anti-VR299 rabbit serum. Figure 1 represents the response curves for five strains of IPNV and Table 1 shows P/N ratios of these strains. These results show that homologous viral antigen (VR299 strain) gives the highest reaction in this test and other four strains of IPNV are reacted with anti-VR299 rabbit serum with the titer in the order of Buhl, Powder Mill, d'Honninethun and Bonnamy strains.

A test was performed to determine whether IBDV could be identified by ELISA using antiserum against IPNV-VR299 strain. The results showed that IBDV could not be identified in the present system because value of

Fig. 1. Response curves of five strains of IPNV in ELISA. Each symbol represents VR299(○), Buhl(□), Powder Mill(■), d'Honninchtun(△) and Bonnamy(●) strains.

Fig. 2. Comparison of viral polypeptides of five strains of IPNV in SDS-polyacrylamide gel electrophoresis. (A) VR299; (B) Bonnamy; (C) Buhl; (D) Powder Mill; and (E) d'Honninchtun strains. Molecular weights of marker proteins are indicated on the left side. α , β , γ_1 , and γ_2 indicate 4 viral proteins of IPNV.

absorbancy at 405 nm was 0.07 when 10 μ g of purified IBDV antigen was applied, whereas the value for purified IPNV-VR299 strain

Table 1. Identification of IPNV by the sandwich ELISA

IPNV strain	OD at 405 nm ^{a)}	P/N ratio ^{b)}
VR299	1.82	26.0
Buhl	1.01	14.4
Powder Mill	0.89	12.7
d'Honninchtun	0.42	6.0
Bonnamy	0.32	4.6
None ^{c)}	0.07	1.0

a) Optical density of purified antigen (10 μ g/ml).

b) $P/N = \frac{\text{OD of test sample}}{\text{OD of uninfected STE-137 cell culture fluid}}$

c) Uninfected STE-137 cell culture fluid (10 μ g protein/ml).

was 1.20 (data is not shown). This indicates that IBDV is antigenically distinct from IPNV.

SDS-PAGE of IPNV polypeptide: Four distinct proteins are known in IPNV virions [5, 9, 10, 12]. The result of the present study showed that β protein was the most prominent polypeptide in all the five strains of IPNV (Fig. 2). Further, there were at least 3 polypeptides of β size; namely 1) molecular weight (MW) 49,000 in Bonnamy and d'Honninchtun strains, 2) MW 50,000 in VR299 strain and 3) MW 52,000 in Buhl and Powder Mill strains. The experiments were repeated 3 times and the same results were obtained.

ID: Antigenic relationships between five strains of IPNV were examined by using anti-VR299 rabbit serum (Fig. 3). The results indicate that antigenic characters of VR299 strain were similar to that of Buhl and Powder Mill strains but differed from Bonnamy and d'Honninchtun strains. Precipitin lines formed against Buhl strain were almost identical to that of Powder Mill strain, and the lines formed against Bonnamy and d'Honninchtun strains were similar to each other. These results were consistent with the differences in the reaction patterns of ELISA and the differences of the molecular weight of β polypeptides of the 5 strains of IPNV.

Figure 4 shows ID patterns of IPNV-VR

Fig. 3. Antigenic relationships of five strains of IPNV in immunodiffusion. (Ab) Anti-IPNV-VR299 strain rabbit serum; (1) VR299 strain; (2) Bonnamy strain; (3) Buhl strain; (4) Powder Mill strain; (5) d'Honnincthun strain; and (F) fetal calf serum.

Fig. 4. Cross-immunodiffusion of IPNV and IBDV. (A) Anti-IPNV-VR299 strain rabbit serum; (B) anti-IBDV-RF-1 strain chicken serum; (1) IPNV-VR299 antigen; and (2) IBDV-RF-1 antigen.

299 strain and IBDV to antiserum against homologous and heterologous virus. Precipitin lines of the two viruses were formed only against homologous but not heterologous antiserum, indicating that there was no anti-

genic relationship between IPNV-VR299 strain and IBDV.

DISCUSSION

Recently, Nicholson and Caswell [27] and Dixon and Hill [7] examined the antigenic relationships of several isolates of IPNV and IPN-like viruses by ELISA using virus-infected tissue culture fluid as virus antigen and found that antiserum against one isolate of IPNV could be used to serotype IPNV strains. Katze and Crowell [19] described that the highly purified virions in ELISA were essential for type-specific identification of a group of Coxsackieviruses. Further, Herrmann *et al.* [16] had difficulty using partially purified virus for identification of enteroviruses in the test. Therefore, we used purified virion and its hyperimmune antiserum to compare the reactivity of 5 strains of IPNV. The results obtained in the present study indicated that the five IPNV strains were cross reacted to

some degree and the results were essentially similar to those reported by Nicholson and Caswell [27] with one exception that the anti-VR299 serum showed the highest reaction against homologous viral antigen by our test. Further, we found that the molecular weight of β polypeptides of five strains of IPNV could be separated into three groups. The differences in molecular weight of β protein between these strains were consistent with the differences in the reaction patterns obtained by ELISA as described above. Macdonald and Gower [22] reported that IPNV strains were divided into 3 groups by the kinetics of their neutralization by antibody and found that molecular weight of viral polypeptide designated as VP 2 (may be equal to β polypeptide by Dobos [9] and Dobos *et al.* [11]) also differed from each other when analyzed by SDS-PAGE. Since viral strains used in their study are different from those used in the present study, direct comparison of the serological relationships of selected strains can not be done. However, it seems that there is a close relationship between serological groups of IPNV and the size of viral proteins from the present and previous studies [15, 22, 24]. The molecular weight of viral polypeptides determined in the present study using 10% acrylamide gel seemed to be smaller than that reported by Dobos *et al.* who used 7.5% acrylamide gel [11]. This may be due to the difference in concentrations of acrylamide gel used, because we estimated that the molecular weight of β protein of VR299 strain of IPNV was 54,000 to 56,000 when 7.5% acrylamide gel was used (unpublished). These results are consistent with the result reported by Dobos *et al.* [11].

When analyzed by ID in the present study, strain-specific antigen and partial common antigen were observed among 5 strains, and the reaction patterns seemed to correspond with the results of ELISA and SDS-PAGE. These results indicate that the response curves of five strains of IPNV obtained by ELISA

may reflect on the biophysical and biochemical differences of viral proteins showed by the two other tests. Therefore, ELISA could be a useful method for serotyping of IPNV.

Many viruses similar or identical to IPNV have been isolated from various animal species [1, 2, 17, 30, 31, 32]. Recently, several investigators reported the serological relationships among these isolates. Hedrick *et al.* [15] and Okamoto *et al.* [28] supposed that European eel virus was closely related to Ab strain of IPNV. Macdonald and Gower [22] and Underwood *et al.* [32] reported that oyster virus (OV) and/or Tellina virus (TV) isolated from bivalve molluscs were included in the group of IPNV. However, Dobos *et al.* [12] reported that IPNV was different from the other four viruses (TV, OV, IBDV and DXV) in their biophysical and biochemical natures. In the present study, we examined the antigenic relationship of IPNV and IBDV, which is one of the important pathogens of chickens, by ELISA and ID. We observed that IPNV was not antigenically related to IBDV by cross neutralization test as well as ELISA and ID (manuscript submitted to Journal).

Since the serological relationships among different strains of IPNV and the serological relatedness of the virus to IPNV-like viruses were difficult to assess by a single serological method [21, 26], more accurate serotyping of IPNV should be determined by using the following criteria: (i) neutralization test [22, 28], (ii) comparison of reaction patterns by ELISA [7, 14, 27]; and (iii) molecular weight comparison of virion proteins and RNAs [5, 12, 22, 32]. In addition, monoclonal antibody may provide reagent for serotyping of these viruses.

ACKNOWLEDGEMENTS. The authors wish to express our appreciation to Dr. T. Nagabayashi and Dr. M. Higashihara for the supply of viral strains and antiserum. We thank Miss T. Ohmi for preparation of the manuscript. This work was supported by Grant-in-Aid for Co-operative Research No. 57360020 from the Ministry of Edu-

cation, Science and Culture of Japan. Address reprint requests to Dr. Kodama.

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要 約

酵素抗体法 (ELISA) における 5 株の伝染性脾臓壊死症ウイルスの反応性: 服部雅一・児玉 洋・石黒信良¹⁾・石垣克至・見上 彪・伊澤久夫 (北海道大学 獣医学部家畜伝染病学講座, ¹⁾ 共立商事中央研究所) —— 5 株の伝染性脾臓壊死症ウイルス (IPNV) および伝染性フアブリキウス囊病ウイルス (IBDV) 1 株について, 酵素抗体法 (ELISA) における反応パターンの比較を抗 IPNV-VR299 株血清を用いて行なった。5 株の IPNV は反応性により, i) VR299 株, ii) Buhl および Powder Mill 株ならびに iii) d'Honnincthun および Bonnamy 株の 3 群に分けられ, IBDV は抗 IPNV 血清を用いた ELISA では検出されなかった。これら 3 群の IPNV 株ビリオンにはそれぞれ分子量の等しい β 蛋白が存在していた。これらの IPNV 株間の抗原的関連性を免疫拡散法により調べたところ, Buhl 株および Powder Mill 株は VR299 株と類似していたが, これらの株と d'Honnincthun 株および Bonnamy 株は抗原的に異なっていた。また, IBDV と VR299 株との間には交差反応は認められなかった。