JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1982, p. 469–472
0095-1137/82/090469-04$02.00/0

Vol. 16, No. 3

Enzyme-Linked Immunosorbent Assay for Identification of Infectious Pancreatic Necrosis Virus

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Received 29 March 1982/Accepted 2 June 1982

The sensitivity, reproducibility, and specificity of an enzyme-linked immunosorbent assay for the identification of infectious pancreatic necrosis virus was determined. It was shown that $10^{5.5}$ tissue culture infectious doses could be assayed by this method. The assay provided significant advantages in terms of time and ease of performance over the routinely employed serological tests. When an antiserum against a single isolate was used, it was possible to identify nine of ten North American isolates, four of six European isolates, and one isolate from Taiwan.

Intensive culture of freshwater and marine fish for food and sport has grown rapidly in recent years. Concomitantly, disease problems, including viral diseases, have also increased. Rapid and accurate identification of the disease agents is required for effective control and an understanding of the epizootiology of the diseases.

One of the most important viral diseases of fishes is infectious pancreatic necrosis (IPN), which results in high levels of mortality in young salmonids (20). Survivors, however, may become lifelong carriers, continually shedding small quantities of the virus (12). Recently, IPN and IPN-like viruses also have been isolated from a variety of non-salmonid fishes and marine invertebrates (13–17).

The IPN virus (IPNV) is the prototype of a newly recognized group of viruses designated birnaviruses (3). The viruses of this group are characterized by a double-stranded RNA genome in two segments and a single icosahedral capsid (2, 7).

The antigenic relationships of the various IPNVs and IPN-like viruses are still not completely understood. There appear to exist at least three serotypes. However, specific serotyping of many isolates has been complicated by conflicting conclusions in studies which used different serological tests (6, 9). Most of the North American isolates previously studied appear to represent a single serotype.

The detection and identification of IPNV depends on the development of characteristic cytopathic effects in susceptible cell cultures and confirmation by specific neutralization tests. The development of more rapid, simpler, and less expensive serological tests for the identification of IPNV is needed. Previously, we (8, 11) have shown that the direct immunofluorescent and immunoperoxidase techniques were sensitive and rapid methods for identifying IPNV in infected cell cultures. Despite the rapidity of these tests, however, the direct immunofluorescent and immunoperoxidase techniques have not gained widespread acceptance as routine diagnostic tools for identifying IPNV, perhaps because of inherent difficulties such as the requirement of expensive equipment for direct immunofluorescence.

The enzyme-linked immunosorbent asssay (ELISA) is another serological method finding wide application in human and veterinary medicine which provides numerous advantages over other tests. This report describes the efficacy of an ELISA test for the identification of IPNV.

MATERIALS AND METHODS

Cell cultures. The continuous rainbow trout gonad (RTG-2) (18) and blue-gill fry (BF-2) (19) cell lines were propagated as described previously (10) at 20°C in a growth medium consisting of Eagle minimal essential medium with Earle balanced salts solution, supplemented with 10% fetal bovine serum.

Viruses. Stock preparations of 17 isolates of IPNVs and IPN-like viruses from fish and oysters from North America, Europe, and Asia were prepared in RTG-2 and BF-2 cell cultures as previously described (10). Isolates from trout in North America have been maintained in our laboratory for many years and included: West Buxton, Maine; Dry Mills, Maine; Berlin, N.H.; ATCC reference strain VR299; Gilbert, Mass.; Reno, Nev.; and Buhl, Idaho. Also from North America were isolates from trout (Vermont) and Atlantic salmon (AS; Quebec, Canada) kindly provided by R. Dexter, Craig Brook National Fish Hatchery, East Orland, Maine, and an isolate from the marine clupeid

menhaden (15), kindly provided by F. Hetrick, University of Maryland, College Park, Md. The antigenic relationships of these latter three viruses had not been studied previously. European isolates from trout maintained in our laboratory included: Bonnamy (France), d'Honninchton (France), Sp (Denmark), and Ab (Denmark). Also included were two viruses from oysters (16), designated OV-2 and OV-7, isolated by B. Hill, Fish Diseases Laboratory, Weymouth, England, and one isolate from trout in Taiwan kindly provided by J. Wu, Academia Sinica, Taipei, Taiwan.

Antiserum. Antiserum to purified IPNV (West Buxton) was prepared in rabbits and guinea pigs as previously described (9). Globulin fractions were obtained by ammonium sulfate precipitation as described by Hebert et al. (5) for rabbit sera and Hebert (4) for guinea pig sera.

ELISA. A solid-phase, double-antibody, antiglobulin method was employed for ELISA. Optimal dilutions of all reagents and reaction times were determined by checkerboard titration. The assay protocol is summarized as follows: (i) Guinea pig anti-IPNV serum globulin, diluted in 0.05 M carbonate buffer, pH 9.6 (3.75 μg/ml), was added to wells (0.2 ml per well) of Immulon I MicroELISA plates (Dynatech Laboratories, Inc.). The plates were incubated for 16 h at 4°C. (ii) The plates were washed four times with 0.2 ml of phosphate-buffered saline (PBS; pH 7.4) containing 0.1% Tween-20 (T-PBS). (iii) Virus antigen (infected cell culture medium either undiluted or diluted in virus-free cell culture medium) was added to duplicate wells (0.2 ml per well). The plates were incubated at room temperature for 2 h. (iv) The plates were washed four times with 0.2 ml of T-PBS. (v) Unlabeled rabbit anti-IPNV serum diluted in T-PBS (50 μg/ml) was added to each well (0.2 ml per well) and incubated at room temperature for 2 h. (vi) The plates were washed four times with 0.2 ml of T-PBS. (vii) Alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Dynatech Diagnostics, Inc.), diluted 1:1000 in T-PBS, was added (0.2 ml per well) to each well and incubated at room temperature for 2 h. (viii) The plates were washed four times with 0.2 ml of T-PBS. (ix) The substrate, *p*-nitrophenyl phosphate, diluted (1 mg/ml) in 10% diethanolamine buffer (pH 9.8), was added (0.2 ml per well) to each well and incubated at room temperature for 45 min. (x) The reaction was stopped by the addition of 3 M NaOH, and the optical densities (OD) at 410 nm of the reactions were measured in a MicroELISA Mini Reader MR 590 (Dynatech Laboratories).

RESULTS

Preliminary experiments which employed a variety of protocols indicated that a solid-phase, double-antibody, antiglobulin method was the most practical and versatile ELISA test for the identification of IPNV. The results of previous serological studies (9) indicated that antiserum prepared against the West Buxton isolate exhibited the greatest amount of cross-reactivity, especially with the North American isolates, and this isolate was therefore chosen for use in the study. Optimal concentrations of reagents and reaction times were determined for the homologous antigen-antibody system by checkerboard titration. The ELISA test was then evaluated for ability to identify 17 different isolates of IPNV and IPN-like viruses from infected cell cultures. These isolates were chosen to represent all three serotypes and the major geographical locations of the disease, North America and Europe. Several isolates, including one from Taiwan, that had not been investigated previously were also included. The results of a representative test are shown in Table 1, where the ODs are listed and also expressed as a positive-to-negative (P/N) ratio. The P/N is the mean OD of replicate wells of the virus test sample divided by the mean OD of the uninfected cell controls. As a result of a series of such tests, a sample was considered definitely positive if the OD was greater than 3 standard deviations above the mean OD of the uninfected cell controls. For practical use by field pathologists, this was equivalent to a P/N of >1.4.

With the West Buxton antiserum (serotype 1), all but one North American and two European isolates were clearly identified (P/N, >2.0). Moreover, with the exception of Buhl, all of the North American isolates previously designated serotype 1 exhibited very high ODs (P/N, >6.0). The virus isolated from marine menhaden in the Chesapeake Bay was also placed in this group.

TABLE 1. Identification of IPNV by ELISA

Isolate	OD[a] at 410 nm	P/N[b]
Vermont	>2.00	>11.8
Gilbert	>2.00	>11.8
Dry Mills	>2.00	>11.8
ATCC VR299	1.82	10.7
West Buxton	1.68	9.9
Berlin	1.59	9.4
Menhaden	1.14	6.7
Reno	1.12	6.6
OV-2	0.52	3.1
Taiwan	0.41	2.4
d'Honninchton	0.40	2.4
SP	0.39	2.3
OV-7	0.37	2.2
Buhl	0.34	2.0
Bonnamy	0.23	1.4
Ab	0.22	1.3
AS	0.20	1.2
Infectious hematopoietic necrosis virus control	0.16	0.9
Uninfected cell controls (±SD; $n = 11$)	0.17 ± 0.02	

[a] Mean OD of four reaction wells.

[b] P/N, Mean OD of test samples/mean OD of uninfected cell controls.

Most of the European isolates, including the oyster virus, and the isolate from Taiwan exhibited intermediate values (P/N, ≥2.0 to <6.0). Three viruses gave P/N values of 1.35 or less, below the minimum positive value. These included Bonnamy, Ab, and the AS isolate from Canada. The AS isolate had not previously been serotyped. The specificity of the test for IPNV was confirmed with a totally unrelated virus, infectious hematopoietic necrosis virus, for which the P/N ratio did not differ from that of the controls. Similar results were obtained in a series of trials with different preparations of the same virus isolates.

Whereas the OD values measured in the ELISA reader provided numerical confirmation of IPNV identification, the yellow color in wells exhibiting P/N values ≥2.0 was clearly more intense than it was in control wells and easily permitted positive identification by visual inspection.

The sensitivity of the ELISA test was determined by testing dilutions of stock West Buxton IPNV (Table 2). In this test, levels of $10^{5.5}$ tissue culture infective doses per ml ($TCID_{50}$/ml) were easily identified. This level of sensitivity makes the test very practical, since the usual virus titers from IPNV-infected cell cultures range from $10^{7.0}$ to $10^{9.0}$ $TCID_{50}$/ml. Furthermore, in our hands, cultures with moderate or marked cytopathic effects invariably exceeded the minimum virus titers required.

DISCUSSION

The results presented in this report show that IPNV can be identified by this ELISA. The test as described here permitted positive identification in approximately 7 h with a sensitivity of $10^{5.5}$ $TCID_{50}$/ml. Additional experiments (data not shown) indicated that the duration of reaction time for each reagent could be shortened to 1 h, thereby providing identification in as little as 3 to 4 h. Thus, in terms of the time required for identifying IPNV isolated from cell cultures, the ELISA provided a significant advantage over the routinely used neutralization test which requires a minimum of several days and can take as long as 7 days. Although the use of a simple ELISA reader provided quantitative values, the test as performed here is also suitable for use by pathologists in the field without access to such equipment. Positive wells were clearly identified by their yellow color.

TABLE 2. Sensitivity of identification of IPNV by ELISA

Virus[a] concn ($TCID_{50}$/ML)	OD[b] at 410 nm	P/N[c]
$10^{6.7}$	1.34	6.4
$10^{5.5}$	0.40	1.9
$10^{4.7}$	0.22	1.1
$10^{3.5}$	0.19	0.9
Uninfected cell control (±SD)	0.21 ± 0.01	

[a] West Buxton isolate of IPNV.
[b] Mean OD of two reaction wells.
[c] P/N, mean OD of test samples/mean OD of uninfected cell controls.

The antiserum used in this investigation was prepared against a serotype 1 isolate from North America (West Buxton). This antiserum appeared to distinguish among several groups of IPNV. With one exception (Buhl), all of the North American isolates from trout previously designated serotype 1 (6) exhibited very high P/N values (>6.0). The previously untyped isolate from the marine clupeid menhaden and the trout isolate from Vermont also had P/N values >6.0 and thus appeared to be serotype 1 viruses. The Buhl isolate did not fit the pattern for serotype 1 viruses but nevertheless was clearly identified as IPNV. The serological relationship of this isolate with the other IPNV viruses should be investigated further. The AS isolate from Atlantic salmon in Canada appeared to be negative in this test. This is in agreement with our previous studies which employed virus neutralization kinetics (9), which indicated that the AS isolate is an IPNV but serologically distinct from the other North American isolates.

Thus, the West Buxton IPNV antiserum used in this ELISA clearly identified all but one North American isolate. This test with a single antiserum should, therefore, have wide applicability in North America. In addition, most of the other isolates were also identified, although the P/N values were lower, clustered in the range of >2 to ≤3. Only the Bonnany, Ab, and AS isolates were not identified. However, the use of a polyvalent antiserum combining antisera to these viruses should make this ELISA universally applicable.

ACKNOWLEDGMENTS

This research was supported by research grants from the Animal Health Special Grants Program of the United States Department of Agriculture and the Maine Life Sciences and Agriculture Experiment Station.

The technical assistance of K. Janney is gratefully acknowledged.

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