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Serological techniques for detection of lymphocystis virus in fish

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Abstract

Three serological techniques (indirect immunofluorescence test, flow cytometry, and indirect dot–blot immunoenzymatic assay) have been evaluated for the detection of lymphocystis viral antigens using a gilt-head seabream cell line, SAF-1, and fish leukocytes. Six lymphocystis disease virus (LCDV) isolates from gilt-head seabream, and one reference strain (ATCC VR 342), were tested. Detection of viral LCDV antigens in SAF-1 cells and fish leukocytes by indirect immunofluorescence test occurs at similar periods (5–7 d post-inoculation), and viral antigens were detected as cytoplasmic inclusions located at the periphery of inoculated cells. The percentages of cells with LCDV antigens obtained by flow cytometry were very low, ranging between 0.9% at 5 d post-inoculation and 19.7% at 10 d post-inoculation. The optimal concentration of viral stocks detected by indirect dot–blot immunoenzymatic assay was 0.5 μg ml^{-1}, when purified viral stocks were used as antigens. Inoculated and uninoculated SAF-1 cells could not be distinguished using LCDV antiserum binding. On the basis of these results, indirect immunofluorescence and flow cytometry tests appear to be the best serological methods to detect LCDV antigens in both SAF-1 cells and fish leukocytes.

Resumen

Técnicas serológicas para la detección de virus de linfocistis en peces. Se han evaluado tres técnicas serológicas (inmunofluorescencia indirecta, citometría de flujo y ensayo inmunoenzimático indirecto dot–blot) para la detección de antígenos del virus de linfocistis usando una línea celular procedente de dirada, SAF-1, y leucocitos de peces. En total se han utilizado 6 aislados de virus de linfocistis (LCDV) procedentes de doradas y una cepa de referencia del virus (ATCC VR 342). La detección de los antígenos LCDV en células SAF-1 y leucocitos de peces se producía en períodos similares (a los 5–7 días post-inoculación) usando la técnica de inmunofluorescencia indirecta, y los antígenos virales se detectaban como inclusiones citoplasmáticas localizadas en la periferia de las células inoculadas. Los porcentajes de células con antígenos de LCDV obtenidos por citometría de flujo fueron muy bajos, comprendidos entre el 0,9% a los 5 días post-inoculación y el 19,7% a los 10 días post-inoculación. La concentración óptima de los aislados virales detectados por el ensayo inmunoenzimático indirecto dot–blot fue 0,5 μg ml^{-1}, usando aislados virales purificados como antígenos. No se pudo distinguir células SAF-1 inoculadas y sin inocular usando el antisuero contra el LCDV. En base a los resultados obtenidos, las técnicas de inmunofluorescencia indirecta y citometría de flujo fueron los mejores métodos serológicos para la detección de antígenos de LCDV tanto en células SAF-1 como en leucocitos de peces.

Keywords: Lymphocystis; Immunofluorescence; Dot–blot; Flow cytometry; Viral fish diseases; Detection methods

1. Introduction

Lymphocystis disease (LCD) is a common chronic disease of many salt- and freshwater fish species (Wolf, 1988). Characteristic benign papilloma-like lesions usually develop on the surface of the skin tissue. LCD and its causative agent, *fish lymphocystis disease virus* (LCDV), occur worldwide, and the rate of incidence seems to be increasing (García-Rosado et al., 1999). LCDV can infect, both naturally and experimentally, about 100 different teleost fish species. The skin of the infected specimens is often covered with raspberry-like lesions, sometimes small, and white, pearl-like nodules are formed over the whole body, spreading from the gills to the fins (Hedrick et al., 1992; Sarasquete et al., 1998), which consist of hyper-

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trophic fibroblasts of the skin (Walter and Hill, 1980). Although infected fish appear unsightly, the disease is rarely fatal, but results in an important economic loss in aquaculture systems, because diseased fish cannot be commercialized (Masoero et al., 1986).

The pathology and epidemiology of the disease, in both laboratory experiments and the natural environment, have been well documented (Flügel, 1985; Wolf, 1988; Lorenzen and Dixon, 1991). However, little is known about the process of virus spread (Tidona and Darai, 1997), and the techniques applied for diagnostic purposes (Anders, 1989).

Highly specific seroepizootiological tests would be advantageous over virus isolation procedures, because several fish viruses may cause similar cytopathic effects (papillomaviruses) and replicate in common cell lines and hosts (e.g. rhabdoviruses, iridoviruses, and birnaviruses) (Pérez-Prieto et al., 1999). In addition, several fish viruses do not replicate in the currently established cell lines (LaPatra, 1997). On the other hand, it has been demonstrated that fish populations previously exposed to a virus could be detected using serological procedures when they were negative by virus isolation (Hattenberger-Baudouy et al., 1995). Another important attribute of serological testing is that it can be accomplished without sacrificing valuable fish. In the case of LCD, rapid and sensitive methods of diagnosis are critical to avoid dissemination of the disease among the fish population, and the serological methods may be important to detect LCDV during the carrier phase of the disease (Sanz and Coll, 1992).

The scope of this work was the design and comparison of three serological techniques, using six LCDV isolates collected from diseased cultured gilt-head seabream (*Sparus aurata* L.), to detect the virus in two fish cells, the cell line SAF-1 and kidney leukocytes obtained from gilt-head seabream.

2. Materials and methods

2.1. Viruses and viral antigens

Six isolates of LCDV obtained from diseased gilt-head seabream (*S. aurata*) cultured in fish farms located in southern Spain were used. Their infectivity characteristics tested in cell lines have been previously described (García-Rosado et al., 1999). LCDV strain Leetown NFH (ATCC VR 342) was used as reference strain.

LCDV isolates were purified following the methodology described by Walter and Hill (1980) and modified by Darai et al. (1983). Briefly, the virus was grown in 75-cm^2 plastic tissue culture flasks (Cultex, S.L., Madrid, Spain). Monolayer cells of SAF-1 (Bejar et al., 1997) were infected and incubated at 20 °C for 15 d. Cultured cells were sonicated (at 40 W for 10 min), and the cell debris was removed by centrifugation at $1200 \times g$ for 10 min at 4 °C. The supernatant was then centrifuged at $30\,000 \times g$ for 60 min at 4 °C (XL-90 Ultracentrifuge, 70 Ti rotor) (Beckman Instruments, Inc., Palo Alto, CA, USA), and the pellet was suspended in 1.5 ml TNE buffer (1 M NaCl, 0.001 M EDTA, 0.05 M Tris, pH 4.0). This suspension of crude virus was layered on a sucrose gradient (20–60% sucrose in TNE) and centrifuged at $70\,000 \times g$ for 2 h at 4 °C. The virus band obtained in the sucrose gradient was withdrawn and diluted in phosphate buffered saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 0.88 mM KH_2PO_4, 7.6 mM $NaHPO_4$, pH 7.2). After centrifugation for 1 h at $40\,000 \times g$, the purified LCDV was suspended in PBS.

2.2. Production of polyclonal rabbit antiserum

Purified LCDV was resuspended in 1 ml of PBS at a protein concentration of 0.31 mg ml^{-1} (Bradford, 1976), and mixed with an equal volume of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO, USA). The solution was subcutaneously inoculated in a New Zealand rabbit following the protocol described previously (Lorenzen and Dixon, 1991). A booster (0.41 mg protein ml^{-1}) was given 3 weeks later using incomplete Freund's adjuvant. The rabbit was bled 1 week after the last injection. The gammaglobulin was partially purified from the rabbit antiserum by ammonium sulfate precipitation, followed by desalting by dialysis in PBS (pH 7.2) (Dumbar, 1987). The purified immune serum was stored at –80 °C. Preimmune and anti-Freund's adjuvant sera were used as negative controls. For further analysis, the anti-LCDV immune serum was immunosorbed onto a monolayer of the SAF-1 cell line.

2.3. Collection and purification of fish leukocytes

Head kidney tissue was removed from anesthetized adult gilt-head seabream. Tissue was suspended in Leibovitz medium (L-15) (Gibco, Renfrewshire, Scotland, UK) with 2% fetal bovine serum (FBS) (Gibco), 1% antibiotic solution (100 IU penicillin and 100 μg ml^{-1} streptomycin) (Gibco), and 2% heparin (Sigma). Tissue suspensions were filtered through a 100-μm nylon membrane. The filtrate was layered on a gradient of Percoll (34–51%) and centrifuged for 30 min at $400 \times g$. Leukocytes were isolated from the corresponding band of the Percoll gradient and resuspended in a wash buffer (L-15 supplemented with 0.1% FBS and 1% of the antibiotic suspension), and then centrifuged for 30 min at $400 \times g$. Leukocytes were resuspended in L-15 medium supplemented with 5% FBS, 1% antibiotic solution, and 10 μg ml^{-1} concanavalin A (Sigma), and maintained for 3 d in 12.5 cm^2 flasks. Leukocytes were inoculated with LCDV when they were adherent to the flasks. Cultured leukocytes were later processed by flow cytometry and indirect immunofluorescence assay at 7 and 18 days post-inoculation (p.i.).

2.4. Flow cytometry analysis

Monolayers of SAF-1 cell line or kidney leukocytes in 24-well plates (Cultex) were inoculated with each LCDV isolate in triplicate and incubated for 1 h at 20 °C to allow adsorption of the virus to the cells. The monolayers were overlaid with L-15 medium supplemented with 2% FBS and 1% antibiotic solution, and incubated for 5, 10, and 18 d p.i. After each incubation period, the cells were scraped and centrifuged at $300 \times g$ for 20 min at 4 °C. The pellets were resuspended in a small volume of PBS, gently mixed with 1 ml cold formaldehyde (3.7%) at 4 °C for 15 min, and centrifuged again at $300 \times g$ for 20 min. Afterwards, the pellet was mixed with a 0.1% PBS–Triton X100 solution for 5 min, and washed twice with 30% PBS–skimmed milk. Then, the primary anti-LCDV antibody was added to the cells and incubated for 30 min at 4 °C with gentle agitation. After washing three times by centrifugation in PBS, the second antibody, goat-anti-rabbit-IgG FITC conjugate (Sigma) (1:200 in PBS), was added and incubated at room temperature for 30 min. Two further centrifugation cycles in PBS were carried out to remove non-specific binding, and the final pellet was resuspended in 0.5 ml of PBS to determine the fluorescence of the cellular suspensions by flow cytometry using a cytometer, EPICS XL (Coulter, Mostoles, Madrid, Spain), equipped with an argon ion laser (200 mW at 448 nm excitation).

2.5. Indirect immunofluorescence (IFT) and indirect dot–blot immunoenzymatic tests

SAF-1 cells or kidney leukocytes were cultured in 24-well plates, in which 12-mm diameter coverslips treated with 0.01% gelatin in PBS were previously deposited. Cell monolayers were inoculated with the different LCDV isolates and incubated for 5 d. After the incubation period, the cells were processed as mentioned above for flow cytometry analysis, and the coverslips were examined under confocal microscopy (Leica, Solms, Germany).

The dot–blot assay was conducted following the protocol described previously (Castro et al., 1995). Briefly, 50 μl of purified LCDV virus were filtered through Immobilon-P sheets (Millipore Co., Bedford, MA, USA) using the MilliBlot-D microfiltration apparatus (Millipore). The sheets were treated three times with 5% skimmed milk–PBS solution (10 min each), and then washed with PBS for 3–5 min. Anti-LCDV serum was used as the primary antibody at 1:1000 to 1:40 000 dilutions. Anti-rabbit IgG serum raised in sheep (kindly supplied by Dr. J. Mateos, Laboratory of Animal Physiology, University of Malaga, Spain) was used as the second antibody at a 1:40 dilution. A peroxidase–antiperoxidase rabbit complex (PAP) (Sigma) was used as the labeled antibody. PBS with carrageenan (0.7%) and sodium azide (0.01%) was used as dilution buffer for the first antibody, and PBS for the second antibody and for PAP. The revealing substrate used was 0.6 mg ml^{-1} of 4-chloro-1-naphtol (Sigma) in PBS with 0.3% (vol/vol) of 30% H_2O_2 solution.

3. Results

3.1. Immunodetection by indirect immunofluorescence test (IFT)

The detection of LCD viral antigens was performed by IFT in virus-infected SAF-1 and in infected leukocytes from kidney fish (Figs. 1 and 2), employing anti-LCDV immune serum ranging from 1:100 to 1:5000. A dilution of 1:2500 was chosen on the basis of the optimal staining on LCDV-infected cells and due to the lower non-specific fluorescence background.

All the LCDV isolates were detected in SAF-1 infected cells at 5, 10, and 18 d p.i. and in inoculated leukocytes at 7 and 16 d p.i. as cytoplasmic inclusions located at the periphery of the inoculated cells using confocal microscope (Figs. 1 and 2, respectively).

3.2. Immunodetection by flow cytometry

Flow cytometry was used to quantify the degree of viral infection in the inoculated cells at different dilutions of the anti-LCDV serum (1:100 to 1:2500). The dilution of the polyclonal antibody for optimal immunodetection of viral antigens by flow cytometry was 1:100 (data not shown).

Inoculated SAF-1 cells were examined at 5, 10, and 18 d p.i. Cells showing viral antigens were detected at 5 d p.i. only in one isolate (LCDV-6). The percentage of cells with viral antigens at 10 d p.i. increased significantly ($P < 0.001$, Student's *t*-test) in all virus isolates, ranging from 8.6% (isolate LCDV-5) to 19.7% (isolate LCDV-1). These percentages decreased significantly ($P < 0.001$) at 18 d p.i., ranging between 5.8% (isolate LCDV-1) and 7.7% (isolate LCDV-5).

At 7 d p.i., the percentages of leukocytes showing viral antigens were low, ranging from 1.5% (isolate LCDV-1) to 2.8% (isolate LCDV-6). These values increased significantly ($P < 0.001$) at 16 d p.i., reaching 6.3% in leukocytes inoculated with the isolate LCDV-3 and 18.6% in leukocytes inoculated with LCDV-6.

3.3. Indirect dot–blot immunoenzymatic assay

The results obtained in the dot–blot assay show that the best routine dilution of the polyclonal antibody was 1:10 000 (Fig. 3). The optimal concentration of viral proteins, when purified viral stocks were used as antigens, was 0.5 μg ml^{-1} (Fig. 4). However, when crude virus from infected SAF-1 cells was used as antigen, uninoculated control cells showed cross-reaction with anti-LCDV serum, despite the fact that the serum was properly immunoadsorbed over SAF-1 cells (Fig. 3).

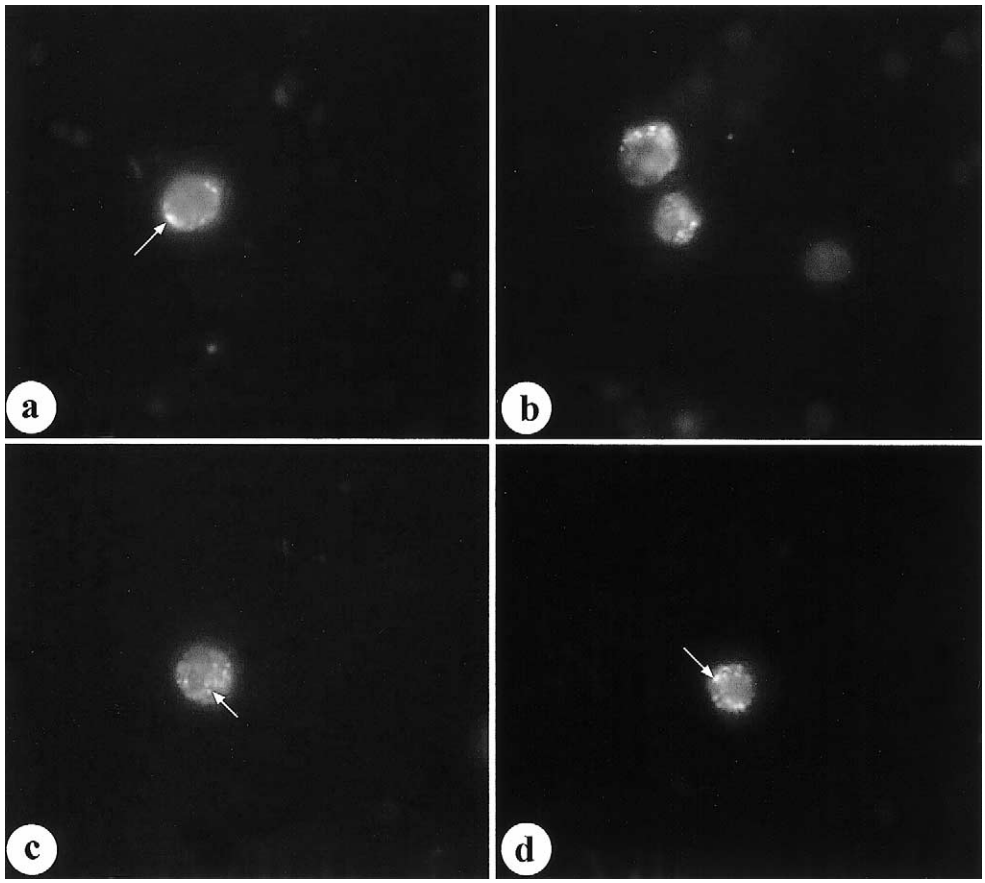

Fig. 1. Detection of lymphocystis disease, LCD, viral antigens in SAF-1 cells by indirect immunofluorescence assay at 5 d post-inoculation: (a) LCDV-1, (b) LCDV-2, (c) LCDV-4, and (d) LCDV-5.

4. Discussion

In disease management of homeothermic and poikilothermic animals, serological analyses are a primary epizootiological and diagnostic tool (LaPatra, 1997). However, the use of serological procedures in virus surveillance and certification of finfish has not been included in the guidelines for either national or international fish health inspection authorities (Hattenberger-Baudouy et al., 1995). This is partly due to the belief that virus isolation procedures are the most sensitive diagnostic method, but also because of the lack of information about the immune responses of finfish to viruses. Most of the various techniques used for the diagnosis of viral infections in fish require inoculation of cell cultures with fish tissue homogenates. However, most fish cell lines currently used in fish virology are derived from freshwater species. This fact could be a limiting factor when virological assays are performed from marine fish (Fernandez et al., 1993).

Several serological techniques have been applied to detect the humoral immune response to different fish pathogenic viruses, such as viral hemorrhagic septicemia virus (VHSV) (Olesen et al., 1991), infectious hematopoeitic necrosis virus (IHNV) (LaPatra et al., 1989; Alonso et al., 1999), and birnaviruses (Hsu et al., 1989; Novoa et al., 1995). In addition, immune identification and detection of fish viruses have been widely applied (McAllister and Schill, 1986; McAllister and Owens, 1987; Evensen and Rimstad, 1990; Hyatt et al., 1991; Rodriguez et al., 1995).

Lymphocystis disease is frequently found in marine cultured fish of the Mediterranean region. The presence of antibodies to LCDV in a large number of apparently healthy fish suggests that LCDV is common in the fish population (Lorenzen and Dixon, 1991). For this reason, a quick diagnosis of LCDV in asymptomatic fish, without the sacrifice of the animal, may help in the prevention of disease outbreaks in the farmed fish population. In the present study, three serological techniques have been evaluated for the detection of FLD viral antigens using six LCDV isolates and one reference strain (ATCC VR 342). All the LCDV tested showed similar serological characteristics (García-Rosado et al., 1999), probably due to the presence of conserved group-specific antigens, a feature common in different iridoviruses (Hedrick et al., 1992). In addition, all the LCDV isolates used in this study presented the same viral structural proteins revealed by western-blot analysis (unpublished data).

Detection of viral LCD antigens in SAF-1 cells and fish leukocytes by IFT occurs at similar post-inoculation time (5–7 d). This diagnostic technique has been applied to detect infectious pancreatic necrosis virus (IPNV) and antibodies in rainbow trout IPNV-carrier (Ahne and Thom-

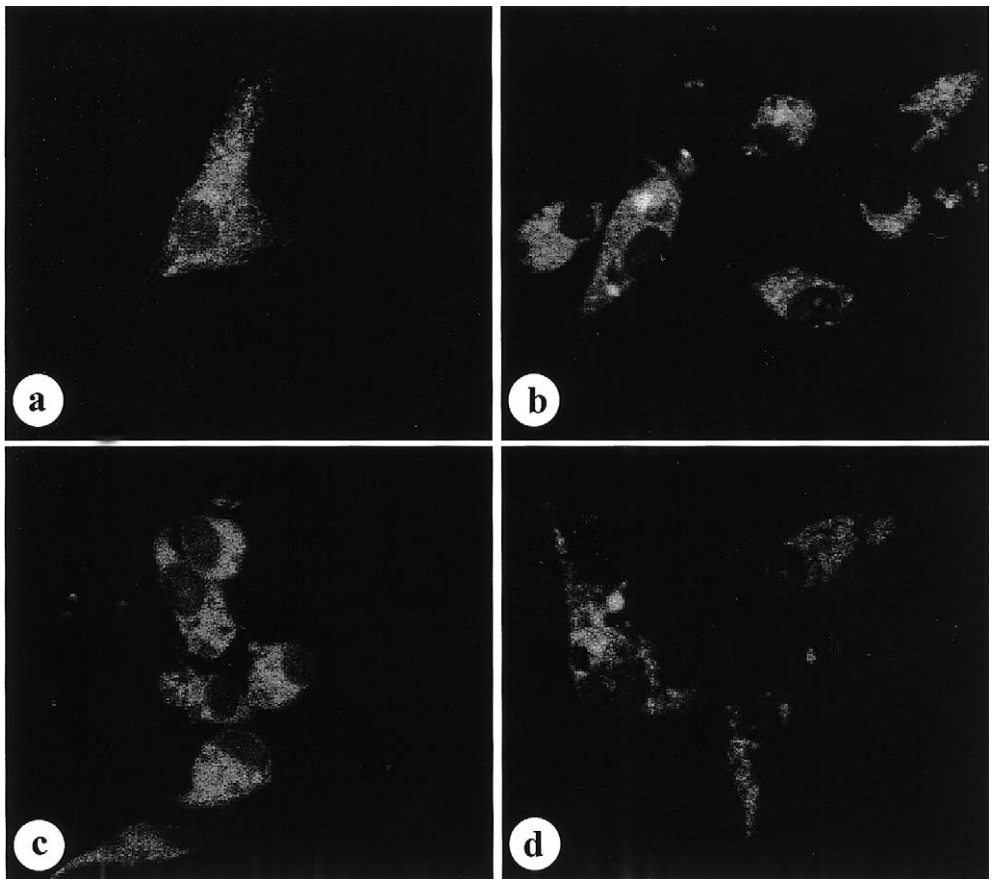

Fig. 2. Detection of LCDV antigens in inoculated fish leukocytes at different post-inoculation (p.i.) periods. (a), (b) Inoculated leukocytes with LCDV-5 at 8 and 16 d p.i., respectively; (c), (d) inoculated leukocytes with LCDV ATCC VR 342 at 8 and 16 d p.i., respectively. Viral antigens were detected as cytoplasmic inclusions located at the periphery of inoculated cells (arrows).

sen, 1986). These authors detected IPNV in the peripheral blood leukocytes of all infected fish and in the mucus of the skin of IPNV-infected fish. In addition, IFT was used to detect antigens from different rhabdoviruses (Lu et al., 1994). The results reported by these authors are similar to those obtained in the present study using the SAF-1 cell line, since the number of fluorescing cells increased progressively with time of infection until 10 d p.i.

The percentages of cells (SAF-1 and leukocytes) with LCD viral antigens obtained by flow cytometry were very low, ranging between 0.9% at 5 d p.i. and 19.7% at 10 d p.i.. Flow cytometry has been successfully applied to detect and quantify IPN viral antigens from different infected fish cells, including fish sperm (Rodriguez et al., 1992), CHSE-214 cells (Rodriguez et al., 1995), and salmonid infected leukocytes (Rodriguez et al., 1991; Pérez-Prieto et al., 1999; Johansen and Sommer, 1995). The results obtained by these authors showed that more than 90% of the leukocytes from infected salmonids presented specific IPNV antigens. On the contrary, low percentages of viral antigens were detected by flow cytometry in viral coinfection with IPNV and IHNV in salmonids (Alonso et al., 1999). These authors suggested that the low antigen detection could be due to autointerference by defective particles of IHNV. Multiple passage of LCDV in cell cultures resulted in loss of viral infectivity,

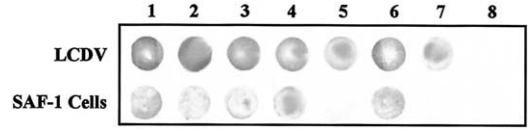

Fig. 3. Detection of 0.5 μg of LCDV and SAF-1 proteins using the immunodot–blot test at several dilutions of the antiserum anti-LCDV: (1) 1:1000; (2) 1:2000; (3) 1:2500; (4) 1:5000; (5) 1:10 000; (6) 1:15 000; (7) 1:20 000; and (8) 1:30 000.

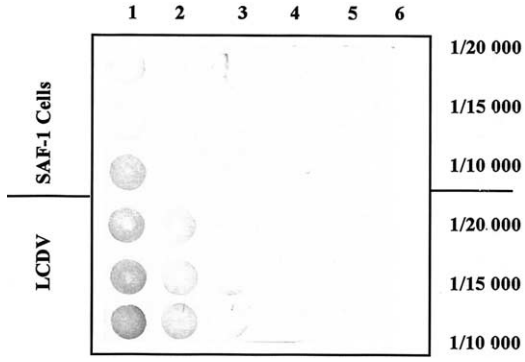

Fig. 4. The immunodot–blot test: detection of several amounts of LCDV and SAF-1 proteins: (1) 0.5 μg, (2) 0.25 μg, (3) 0.12 μg, (4) 0.06 μg, (5) 0.03 μg, (6) 0.01 μg, using dilutions of the antiserum anti-LCDV: 1:10 000, 1:15 000, and 1:20 000.

and it is possible that the in vitro replication of LCDV would be an inefficient process due to the presence of defective interfering (DI) LCDV particles (Walter and Hill, 1980). For this reason, the low percentage of LCDV antigens by flow cytometry may be due to the autointerference produced by these DI particles.

Immunofluorescence tests have been extensively used for the detection of viral antigens in cell cultures and fish tissues, because these methods are rapid and sensitive, and, in addition, the observation of cytopathic effects is not required (LaPatra et al., 1989). However, the need for fresh fish tissues is a disadvantage of these techniques. Furthermore, autofluorescence of fish tissues can interfere with the interpretation of the results (Ahne, 1981). In the present study, the adsorption of the polyclonal antiserum (anti-LCDV) onto SAF-1 cells has reduced most of the non-specific staining.

The immunodot–blot technique has been used in fish virology on the basis of its simplicity, speed, and sensitivity. In the assay, the viral proteins are bound to a solid-phase matrix of high binding capacity, and are detected by their immunoenzymatic reactivity. The immunodot assay proved to detect 0.85 ng of IPNV and 4 ng of VHSV and IHNV, which represented an amount of 10^3 $TCID_{50}$ (McAllister and Schill, 1986). Other authors, using this technique, detected amounts of 2.5 ng of purified IPNV proteins (10^4–10^5 $TCID_{50}$) (Hsu et al., 1989). However, in the present study using LCDV, the presence of false positive samples (cross-reaction with the uninfected cells) constitutes an important limitation for the application of this technique as a diagnostic tool. Similarly, detection of birnavirus directly from homogenates has not been possible using the immunodot assay (Novoa et al., 1995). These authors found false positive reactions in organs from uninfected fish. Other authors have pointed out the presence of non-specific reactions with fish tissues (Hattori et al., 1984), probably due to matrix saturation by the high content of protein in fish homogenates. In the present study, several dilutions of the samples were assayed without significant improvement in the results (Fig. 4).

In short, the fluorescence tests, IFT and flow cytometry, appear to be the best methods to detect LCDV antigen in SAF-1 cells and fish leukocytes. However, future efforts should be directed to develop and design alternative molecular techniques to improve the speed and specificity of lymphocystis disease virus detection, such as PCR and hybridization with DNA probes.

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