Development of an in situ hybridisation procedure for the detection of sole aquabirnavirus in infected fish cell cultures

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Abstract

An in situ hybridisation (ISH) technique has been developed to detect sole aquabirnavirus in infected fish cell lines bluegill fibroblast (BF-2), EPC, and chinook salmon embryo cells (CHSE-214). A 613 bp cDNA probe for viral RNA coding for a fragment of VP2 protein was generated by reverse transcription polymerase chain reaction (RT-PCR) using infectious pancreatic necrosis virus (IPNV) specific DNA primers. Infected cells were strongly labelled, and no non-specific reaction was observed in non-infected cells used as negative controls. The specificity of the probe was examined by testing it against a range of IPNV serotypes such as Ab, Sp and VR-299.

The ISH technique was compared with the immunofluorescence procedure to determine the sensitivity of detection of sole aquabirnavirus in BF-2 cells. The probe used in the ISH technique detected weak positivity at 8 h post-inoculation (p.i.) in the cytoplasm of infected BF-2 cells inoculated with 10³ TCID₅₀/ml, whilst the labelling appears at 24 h p.i. when the immunofluorescence technique was applied. At all other time intervals the results were equivalent.

Keywords: In situ hybridisation; Sole aquabirnavirus; Tissue culture; Immunofluorescence

1. Introduction

Sole aquabirnavirus is the etiological agent of a disease affecting Solea senegalensis characterised by darkening, hyperactivity, erratic swimming, and abnormal behaviour (Rodriguez et al., 1997). The causative agent has been characterised as an aquabirnavirus related serologically to infectious pancreatic necrosis virus (IPNV) Sp (serotype A3) (Perez-Prieto et al., 2001).

The detection of IPNV from fish organs and tissues can be carried out by the use of several techniques, such as immunodot assay and polymerase chain reaction (PCR) test (Babin et al., 1991; Blake et al., 1995). However, the isolation of viruses in cell cultures is still the technique used commonly for the detection of pathogenic fish viruses (Amos, 1985).

In situ hybridisation (ISH) is a technique based on hybridisation of labelled probes complementary to specific viral target sequences in fixed tissues or cells. The probe may be used potentially for diagnostic purposes or for the study of viral pathogenesis and propagation. The adaptation of standard ISH protocol to detect sole aquabirnavirus in cell cultures involves the optimisation of several parameters such as hybridisation temperature and washing buffer stringency, which can affect the ability of the probe to hybridize with RNA (Yang et al., 1999). Cell cultures are evaluated for maximal generation of specific signal and minimal background staining.

This study describes and evaluates an ISH procedure using a digoxigenin (DIG)-labelled probe suitable for the detection and localisation of sole aquabirnavirus genome in infected cell cultures. In addition, ISH and immunofluorescence techniques were compared for their relative sensitivity in detecting sole aquabirnavirus nucleic acid and antigens in tissue culture. Bluegill fibroblast (BF-2) infected cell preparations were used as a tissue model for further work involving formalin-fixed, paraffin-embedded tissues.

2. Materials and methods

2.1. Virus and cell culture

Sole aquabirnavirus isolated from S. senegalensis (Rodriguez et al., 1997), strains of IPNV Sp (American
2.3. ISH procedure

Without further purification.

BF-2, epitheliofoma papulorum cyprini (EPC) and chinoosk salmon embryos cells (CHSE-214) were incubated at 25 °C (20 °C for CHSE-214) in L-15 medium (Gibco) supplemented with 10% foetal bovine serum on silane-treated glass slides (Sigma) until confluence.

Inoculated cells developing cytopathic effects (CPE) were fixed by heating the slides at 100 °C for 1 min, and were immediately placed in 10% buffered neutral formalin (Merck) for 2 h. Finally, they were washed with diethylpyrocarbonate (DEPC)-treated water for 10 min, air dried, and stored at 4 °C until use.

2.2. Probe labelling

In order to obtain the probe, a fragment of the gene coding for VP2 protein in sole aquabirnavirus was amplified by RT-PCR (Rodriguez et al., 2001) using IPV-specific DNA primers V1 (5′-GGA-CCC-CGA-GGA-AAG-3′) and V2 (5′-TCA-TGG-GTC-TGA-GCA-GGC-3′). Amplified sequences were then purified from agarose gels (Gene-clean, Biorad), linked to pGEM-T plasmid, cloned in Escherichia coli DH5α, and labelled by incorporation of DIG-11-dUTP (Roche) during a PCR reaction. DIG-labelling PCR was carried out in a 100 μl reaction volume containing 1×PCR buffer (Roche), 2.5 mM MgCl2, 500 ng V1 primer, 500 ng V2 primer, 5 U Taq-polymerase (Roche) and 200 μM DIG labelled nucleotides. These primers generate a 613 bp probe.

Amplification was performed using 35 cycles of 1 min at 95 °C, 1 min at 53 °C and 1 min at 72 °C, followed by a final elongation step consisting in 10 min at 72 °C. A final step of 5 min at 25 °C completed the reaction. DIG-labelled PCR products were stored at −20 °C, and used directly as a probe without further purification.

2.3.ISH procedure

Cells on slides were permeabilised with proteinase-K (10 μg/ml final concentration) for 30 min at 37 °C in a humid chamber for optimum probe penetration, and were then rinsed with Tris buffer prior to hybridisation. Target viral genome and probe were simultaneously denatured at 95 °C for 5 min, and hybridisation performed overnight.

Unbound probe was removed by washing for 10 min in a shaking incubator at hybridisation temperature. The detection of the probe was carried out by incubating with an anti-DIG monoclonal antibody (Roche) conjugated to alkaline phosphatase for 1 h.

The colorimetric detection of hybridisation signal was carried out with a freshly prepared solution of the chromogenic substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylyphosphate (NBT/BCIP) (Roche) according to the manufacturer’s protocol. Hybridisation signals were observed microscopically as dark purple precipitates in target cells. To enhance the contrast, cell cultures were counter-stained with 0.5% Bismarck Brown Y solution (Sigma). Slides were then rinsed with distilled water and subsequently dehydrated in 95 and 100% ethanol, treated with xylene twice, and finally mounted with a cover-slip by using a permanent mounting medium. All solutions were prepared using DEPC-treated water, and incubations performed at room temperature in a humid chamber.

In addition to the preservation of cell structure and optimum permeabilisation of cells for labelling, it is very important to determine the appropriate hybridisation and washing protocols. A series of experiments, in which a range of hybridisation temperatures and stringency of washing buffer have been tested, were carried out in order to establish the optimum conditions.

Hybridisation was carried out at 40, 50 and 60 °C overnight, and post-hybridisation washes with 1×, 2× and 4× standard saline sodium citrate (SSC) concentration (1× SSC contains 50 mM NaCl and 15 mM sodium citrate, pH 7).

2.4. Immunofluorescence assay

Polyclonal antiserum was developed in female New Zealand rabbit inoculated with purified sole aquabirnavirus. Briefly, purified virus was mixed with an equal volume of Freund’s complete adjuvant (Sigma) and subcutaneously injected in the rabbit following the protocol previously described by Garcia-Rosado et al. (2002). The IgG fraction of the rabbit antiserum was separated and purified by affinity chromatography onto a column of protein A-sepharose CL-4B (Amersham Pharmacia Biotech).

Preliminary assays were undertaken with serial dilutions of the antibody in order to determine the appropriate concentration allowing viral antigen detection without background. BF-2 cells grown on silane-treated slides were fixed in buffered neutral formalin solution for 30 min and permeabilised with Triton X-100 (0.3% in Tris buffer) for 10 min at room temperature. Viral antigens in cells were detected using the indirect fluorescence antibody technique (IFAT) (Rodriguez et al., 2001).

Staining was carried out with the antibody against sole aquabirnavirus (1/500 in phosphate buffered saline (PBS) with 0.3% skimmed milk), and an anti-rabbit monoclonal antibody (Sigma) conjugated to fluorescein-isothiocyanate (FITC) (1/160 in PBS with 0.3% skimmed milk) for 30 min at room temperature in the dark. Slides were mounted with 90% glycerol in Tris buffer (pH 9.5), and immediately examined with an epifluorescence microscope.

2.5. Sensitivity assay

BF-2 cells were cultured on silane-treated slides until confluence and then they were inoculated with sole
Table 1
Detection of sole aquabirnavirus in highly infected BF-2 cells under different hybridisation conditions

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Washing buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1× SSC</td>
</tr>
<tr>
<td>40</td>
<td>++</td>
</tr>
<tr>
<td>50</td>
<td>++</td>
</tr>
<tr>
<td>60</td>
<td>+</td>
</tr>
</tbody>
</table>

- Intensity of labelling: (+) weak positive; (+++) moderate positive; (++++) strong positive.

aquabirnavirus ranging from 10² to 10⁶ TCID₅₀/ml. At 4, 8, 24 and 48 h post-inoculation (p.i.) slides were fixed with buffered neutral formalin and finally processed for ISH and IFAT following the protocols mentioned above.

3. Results

3.1. ISH protocol

Sole aquabirnavirus-infected and non-infected BF-2 cell cultures were used as the starting material for optimisation of ISH protocol. After simultaneous denaturation of target viral genome and probe, hybridisation was carried out overnight. Sole aquabirnavirus detection in cells by ISH at temperatures ranging from 40 to 60 °C yielded different results (Table 1). The hybridisation at 50 °C resulted in a strong positive reaction with numerous labelled cells, and low background in comparison with that carried out at 40 °C, positive cells showed dark purple staining confined to the cytoplasm (Fig. 1). The non-specific signal was lower at 60 °C than at 50 °C, but the intensity of the specific signal was also weaker at the highest temperature tested.

After the hybridisation procedure, cell cultures were washed and blocked. The stringency of the washing procedure was increased by lowering the salt concentration. The unbound probe was removed by washing the slides with buffer containing 4×, 2×, and 1× standard SSC concentration. The labelling at 40 and 60 °C was higher using the washing buffer supplemented with 4× SSC, but non-specific signal was also observed. The decrease of SSC concentration in washing buffer result in the disappearance of this background, and no differences in the intensity of the signal either in control or in inoculated cells was observed using 1× SSC or 2× SSC washing buffer, regardless of the temperature tested.

The conditions selected for sensitivity and specificity determination of the ISH procedure have been hybridisation at 50 °C and the use of a washing buffer supplemented with 1× SSC.

3.2. ISH sensitivity

Viral sole aquabirnavirus infection was detected directly in inoculated cells by the use of both IFAT and ISH. Both techniques were tested and compared for their sensitivity to detect sole aquabirnavirus on BF-2 cells inoculated with different viral titres (Table 2). The results showed that ISH seems to be more sensitive than IFAT, since at higher viral titres (10⁵ TCID₅₀/ml), ISH technique detected earlier (4 h p.i.) cells showing positive signals.

At low viral titres (10¹ TCID₅₀/ml) IFAT method detected less positive cells at 24 h p.i. but, in contrast to the ISH method, positive signal was not observed at 8 h p.i. Cells
Table 2
Comparison of ISH and IFAT for sole aquabirnavirus detection in formalin-fixed BF-2 cells inoculated with different viral titres

<table>
<thead>
<tr>
<th>TCID50/ml</th>
<th>ISH time p.i. (h)</th>
<th>IFAT Time p.i. (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>10^4</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>10^3</td>
<td>−+</td>
<td>−+</td>
</tr>
<tr>
<td>10^2</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>10^1</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

* Intensity of labelling: (−) negative labelling; (−+) weak; (+) low, (+++) medium; (++++) high; (NT) not tested.

inoculated with 10^4 to 10^5 TCID50/ml showed positive binding when were examined 8 h p.i. by both methods.

3.3. Probe specificity

The specificity of the probe was demonstrated by hybridisation to viral RNA from IPNV Sp and Ab (strains representative for European isolates), VR-299 (representative for North American isolates), and VHSV (used as negative control). The reactivity was specific for IPNV, detecting all strains of IPNV tested. No hybridisation was detected on RNA from VHSV used as negative control.

Fig. 2. In situ hybridisation of BF-2 (A), EPC (B), and CHSE-214 (C) cells infected with 10^6 TCID50/ml sole aquabirnavirus. Cells were processed at 8 h p.i. Negative control cells are shown on the left.
3.4. Effect of tissue culture on hybridisation signal

ISH was carried out using EPC and CHSE-214 cells grown on silane-treated slides. Cells were inoculated with \(10^8\) TCID\(_{50}\)/ml and fixed at 8 h p.i. The number of cells showing labelling and the intensity of the hybridisation signal were quite similar when hybridisation was carried out on BF-2 and EPC cells (Fig. 2). BF-2 cells with positive signal were distributed randomly in the preparation, in contrast infected EPC cells appeared forming clumps.

There was no hybridisation on infected CHSE-214 at 8 h p.i. When these cells were fixed 24 h p.i. the cytoplasm showed strong labelling.

4. Discussion

Nuovo (1994) reported that the threshold for ISH detection is 10–20 copies of the target sequence per cell, making ISH less sensitive than other methods based on gene amplification. In this sense, polymerase chain reaction (PCR) represents the most sensitive method for detecting viruses, although it does not give any information about which specific cells in a tissue are infected (Kim, 2003). By means of ISH and IFAT techniques it is possible to detect infected cells in tissues and to evaluate morphological lesions simultaneously, representing a valuable tool not only for viral diagnosis but also for pathogenesis studies.

Although viral detection by ISH has been reported previously in marine organisms (Sano et al., 1993; Bruce et al., 1994; Gregory, 2002), studies performed for in situ detection of IPNV are currently carried out by means of immunohistochemistry (IHK) (Evensen and Rimstad, 1990). IPNV has been diagnosed using DNA probes on blots (Rimstad et al., 1990; Dopazo et al., 1994), but only one study using ISH in Atlantic halibut, yolk-sac larvae has been reported (Biering and Bergh, 1996). This kind of study can provide information about which organs to target for diagnosis of clinical diseases and for viral detection in carrier fish. IPNV virological diagnosis is normally based on cell isolation and further confirmation by means of serological or molecular techniques (Rodriguez et al., in press).

The theoretical aspects of ISH are poorly understood, and many steps in the procedure have to be determined empirically to avoid non-specific hybridisation of the probe. Hybridisation must be performed under stringent conditions. In this study hybridisation temperature and stringency in washing buffer are the parameters that have been modified to optimise ISH protocol. The aim of this study was to assess the use of ISH for the detection of sole aquabirnavirus, a virus isolated from \(S.\) senegalensis that belongs to the \textit{Aquabirnavirus} genus (family Birnaviridae), and to compare it with IFAT for their relative sensitivity.

BF-2 infected cells showed good labelling when hybridisation was performed at 50°C overnight, and post-hybridisation washes with 1× SSC-supplemented washing buffer. These are more stringent conditions that those applied during hybridisation for IPNV detection by Biering and Bergh (1996), thereby demonstrating the higher specificity of the probe used in this study.

The results obtained indicate that ISH technique is potentially slightly more sensitive than IFAT for the detection of sole aquabirnavirus from inoculated cells (Table 2). These findings may be explained by the fact that viral nucleic acid may be detected in inoculated cells in absence of viral antigens (Kim and Chae, 2002). Previous studies have also demonstrated that ISH is more sensitive than immunohistochemistry techniques for the in situ detection of different viruses (Allan et al., 1989, 1993). Although Biering and Bergh (1996) found both methods have approximately equal sensitivity.

In addition to its higher sensitivity, ISH has another advantages such as less susceptibility to structural alteration caused by fixation, less background (tissues normally have auto-fluorescence), the signal does not fade, and simultaneous observation of histological lesions is easier by ISH than by IFAT technique.

The probe used was specific for IPNV, being able to recognise all strains tested in this study. Positive hybridisation signal was strong regardless of the cell used for hybridisation, in contrast infected EPC cells appeared forming clumps.

These findings may be explained by the fact that viral nucleic acid may be detected in inoculated cells in absence of viral antigens (Kim and Chae, 2002). Previous studies have also demonstrated that ISH is more sensitive than immunohistochemistry techniques for the in situ detection of different viruses (Allan et al., 1989, 1993). Although Biering and Bergh (1996) found both methods have approximately equal sensitivity.

Briefly, an ISH procedure for sole aquabirnavirus detection in cell tissues has been established. Maximum signal and minimum non-specific labelling was seen when hybridisation was carried out at 50°C and post-hybridisation washes with buffer supplemented with 1× SSC concentration. Comparison between IFAT and ISH demonstrated that the latter is potentially slightly more sensitive, making possible the detection of viral RNA as soon as 8 h p.i. in cell cultures infected at low titres. This probe recognises all three IPNV strains tested proliferating either in BF-2, EPC, or CHSE-214, and has the potential to be used for diagnostic purposes.

On the other hand, ISH would provide information about sole aquabirnavirus pathogenesis, which is important to determine the portal of entry of the virus, which are the target organs involved in viral replication, and which tissues or organs should be considered for viral detection in asymptomatic carriers.
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References


