In situ HYBRIDISATION: A METHOD FOR THE DIFFERENTIATION OF SPRING VIRAEMIA OF CARP VIRUS FROM SIMILAR FISH RHABDOVIRUSES

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Introduction

Figure 1: SVCV infected carp.

- Spring viraemia of carp virus (SVCV) and pike fry rhabdovirus (PFR) - serologically related rhabdoviruses causing serious haemorrhagic disease in susceptible fish species e.g. carp (Figure 1), pike, tench, bream and grass carp.
- SVCV is notifiable to the OIE and under national legislation of certain countries, but PFR is not. So, correct diagnosis is essential.
- The two viruses can be distinguished in reciprocal serological tests (Ahne *et al.* 1998, Way *et al.* 1998) and by a ribonuclease protection assay (Ahne *et al.* 1998). However, other related rhabdovirus isolates from cyprinid fish are difficult to type by serology, and produce a clinical disease similar to SVC.
- A comparison of SVCV, PFR and non-SVCV-non-PFR isolates based on the nucleotide sequence of a 550bp fragment of the glycoprotein gene divided the isolates into four genogroups (Stone *et al.* 1999) :

I - *Rhabdovirus carpio* group, comprising all isolates classified by serology as SVCV

II - Grass carp rhabdovirus group comprising 1 isolate, previously identified as PFR

III - *Pike fry rhabdovirus* group comprising 2 isolates, one of which was the original PFR isolate

IV - Tench rhabdovirus group comprising the non-SVCV-non-PFR isolates, including some previously identified as PFR by serology

- Isolates from each of the four genogroups can be distinguished by the reverse transcription-polymerase chain reaction.
- These methods have not been widely accepted as the basis for imposing statutory control measures in the case of notifiable diseases (Bernoth 1999) because of concern that the sensitivity of the method may lead to false positive diagnoses.
- We report here the development of alternative methods to distinguish between isolates from the four genogroups.

Materials and methods

Preparation of specimen for ISH. Rhabdoviruses used in this study were cultivated in *Epithelioma papulosum cyprini* (EPC) cells on autoclaved silane-coated slides. 24h post-inoculation the slides were heated at 100°C for 60 seconds and the cells were fixed in 10% neutral buffered formalin.

Probe labelling. Isolates N1-5, V76, PFR R4 and 994591 were selected as reference isolates for the four genogroups and their corresponding 550bp glycoprotein cloned sequences were used as templates for the probe labelling. The digoxigenin-labelled probes were produced by PCR using the primers SVC-F1 (5'-TCTTGGAGCCAAATAGCTCARRTC-3') and PFR-R1 (5'-CCTCAAAGYTGYGGNTGGGC-3'), except for the cloned sequence of N1-5 which was amplified using SVC-F1 and SVC-R4 (5'-CTGGGGTTTCCNCCTCAAAGYTGY-3').

Southern blotting. 100, 10 and 1ng of nested RT-PCR products corresponding to different virus isolates were loaded onto 2% agarose gels. Four blots were prepared in parallel and hybridised, each with a different probe.

***In situ* hybridisation and detection.** Cells were permeabilised with proteinase K and dehydrated. Slides were mounted with 50µl of hybridisation mix: one volume of hybridisation buffer (formamide 50%, dextran sulfate 10%, 4x SSC, yeast tRNA 250µg/ml, 1x Denhart) added to one volume of probe (min. 50ng/µl). After 5 minutes denaturation at 95°C slides were hybridised overnight at 42°C. Up to 10 slides and three probes per slide could be hybridised at a time using the Gene Amp® In Situ PCR System 1000 (Perkin-Elmer). Excess probe was removed by a wash in a large volume of washing buffer WB1 (1x SSC, 6M urea, 0.2% Bovine serum albumin) or washing buffer WB2 (1x SSC, 50% formamide) at 40°C. Specific probe binding was visualised by means of a sandwich of a mouse monoclonal antibody to digoxigenin and an alkaline phosphatase conjugated goat anti-mouse IgG. 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) was used here as substrate for the alkaline phosphatase.

Results

Southern blotting

- Detection limit: 1 to 10ng depending on the probe (Table 1)
- The genogroup II probe is specific (Table 1)
- The genogroup I probe is less specific: hybridised with all the SVCV isolates, but also the isolate 994655 (genogroup IV) - the identification of genogroup I isolates can be achieved by taking into account the detection limit of the probe (Table 1).
- The genogroup III and IV probes did not clearly identify the homologous genogroup isolates (Table 1) under these stringency conditions.

***In situ* hybridisation (ISH)**

- Intensity: ISH with four probes on homologous virus isolates resulted in specific dark blue cytoplasmic labelling of virus infected cells (Figure 2)
- Specificity: labelling localised to cells showing cytopathic effects (CPE) (Figure 2)
- Cross reactivity: the intensity of staining obtained after ISH was given a score (Figure 2, Table 2) - an objective and repeatable marking was achieved by including reference viruses (isolates N1-5, V76, PFR R4 and 994591) in each assay

Virus putative genogroup and isolates tested	Digoxigenin labelled probe: Genogroup I (N1-5)	Genogroup IV (994591)	Genogroup III (PFR R4)	Genogroup II (V76)
Genogroup I				
10/3	++	+	+	-
M27	++	-	-	-
301/2	+++	-	-	-
B271	+++	++	++	-
88164	+++	++	+	-
30:75	+++	++	++	-
Genogroup IV				
994591	-	++	++	-
950237	-	++	++	-
994655	+	++	++	-
Genogroup III				
PFR-R4	-	-	+++	-
Genogroup II				
V76	-	++	+++	+++

Table 1: 100ng target amplicon were not detected using this method, +: 100ng target amplicon can be detected, ++: 10ng or more target amplicon can be detected, +++: 1ng or more target amplicon can be detected.

2a

Virus putative genogroup and isolates tested	Digoxigenin labelled probe: Genogroup I (N1-5)	Genogroup IV (994591)	Genogroup III (PFR R4)	Genogroup II (V76)	Control (no probe)
Genogroup I: N1-5, M27, Z1, 30:75, 940500 and RHV	+++	-	-	-	-
Genogroup IV: 994591, N.Irl 98-93, 994663 and 994655	Bg	+++	++	+	-
Genogroup III: PFR-R4	Bg	+	+++	+	-
Genogroup II: V76	-	-	+	+++	-
Control: Non-related fish Rhabdoviruses: IHNV and VHSV	-	-	-	-	-
Control: Non-infected EPC cells	-	-	-	-	-

2b

Virus putative genogroup and isolates tested	Digoxigenin labelled probe: Genogroup I (N1-5)	Genogroup IV (994591)	Genogroup III (PFR R4)	Genogroup II (V76)	Control (no probe)
Genogroup I: N1-5, M27, Z1, 30:75, 940500 and RHV	+++	Bg	-	Bg	-
Genogroup IV: 994591, N.Irl 98-93, 994663 and 994655	Bg	+++	+ or Bg	Bg or -	-
Genogroup III: PFR-R4	Bg	+	+++	-	-
Genogroup II: V76	-	Bg	Bg	+++	-
Control: Non-related fish Rhabdoviruses: IHNV and VHSV	-	-	-	-	-
Control: Non-infected EPC cells	-	-	-	-	-

Tables 2a and 2b: Marks were given depending on the intensity of labelling. "+", "++" and "+++" are corresponding respectively to low, fair and intense positive labelling of virus infected cells. "Bg" and "-" correspond to background and negative labelling levels. 2a: Intensity of labelling observed when using the WB I, containing urea. 2b Intensity of labelling observed when using the WB II, containing formamide.

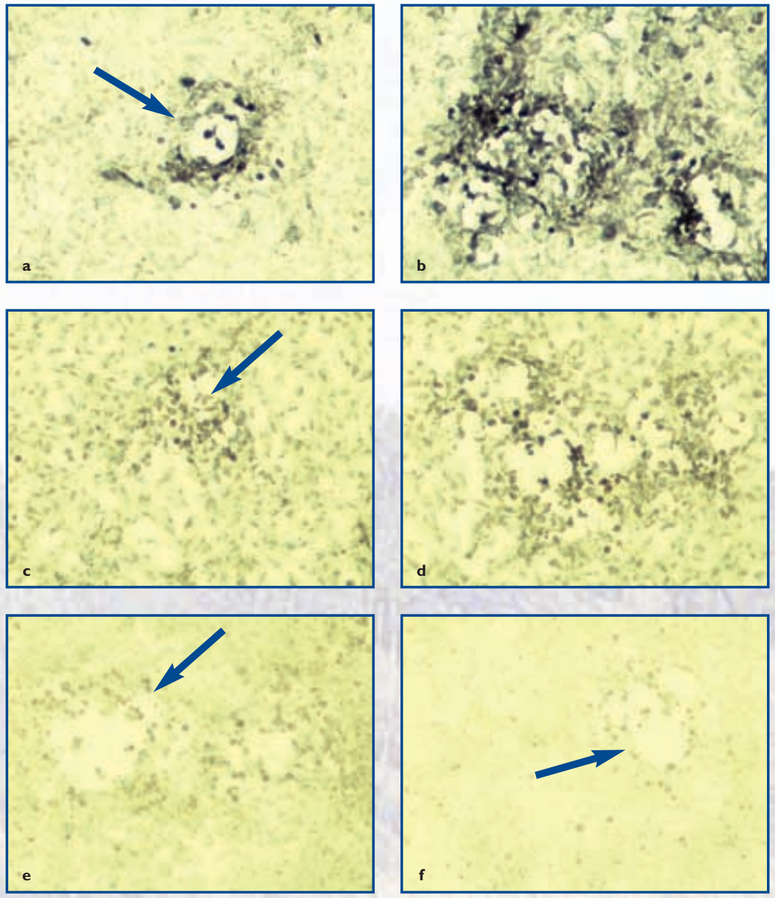

Figure 2: Micrographs of virus infected cells presenting different levels of labelling intensity after ISH. a and b correspond to a "+++" intensity, c and d to "+", e to "Bg" and f to "-". Arrows indicate virus related CPE.

Discussion

Southern blotting

- Good sensitivity for all probes tested.
- Inadequate specificity under those conditions.
- Further optimisation required before it can be used to identify virus isolates from field samples.

***In situ* hybridisation**

- Gene Amp® In Situ PCR System 1000 (Perkin-Elmer): three probes can be tested at a time on different areas of a single slide - reduced number of slides to process and associated cost.
- Enhanced cell adhesion: use of silane rather than poly-L-lysin coated slides - heating the slides at 100°C before fixation in formaldehyde.
- No significant loss of cells was observed: suitable for slow growing isolates producing very few virus-infected cells after 24h of culture.
- Reference slides can be prepared and preserved for at least one month without significant difference in the labelling intensity.
- ISH procedure saves time compared to immunoassay & neutralisation tests that require a high virus titre in the culture supernatant.
- BCIP/NBT substrate: non-toxic, intense blue labelling and very little background so that rapid reading of results at low magnification is possible.
- Use of a scoring system based on the signal intensity: demonstrates the suitability of the technique for allocation of an isolate to a genogroup.
- Two washing buffers that contained either urea (WB I) or formamide (WB II) were tested. WB II resulted in a reduction of the cross-reactivity of some probes (Table 2) - but as a routine procedure use of WB I (less toxic) is advised.
- RT-PCR and sequencing of amplicons can be achieved in less than 1.5 days and could be retained for routine identification. The ISH procedure takes 3.5 days, including culture of cells and virus on the silane treated slides.
- Little equipment required for ISH: and is a molecular complement to other immunological reference techniques applicable in most laboratories involved in fish disease diagnosis.

Alternative approach

- Reverse hybridisation on membrane
- Described in a linked poster: Sheppard *et al.* "The detection and differentiation of piscine vesiculo-like viruses using an RT-PCR reverse hybridisation technique".

References

Ahne, W., Kurath, G. and Winton, J. (1998). A ribonuclease protection assay can distinguish spring viremia of carp virus from pike fry rhabdovirus. Bull. Eur. Ass. Fish Pathol. 18: 220-224.

Bernoth, E.M. (1999). Application of DNA-based molecular diagnostic techniques in fish disease diagnosis - Opportunities and constraints from a government officer's point of view. Bull. Eur. Ass. Fish Pathol. 19: 235-239.

Stone, D.M., Sheppard, A., Lui, C.T.Y., Taylor, G.R., Denham, K., Dixon, P.F. & Way, K. (1999). Nucleotide sequence analysis of the glycoprotein gene of putative spring viraemia of carp virus and fry rhabdovirus isolates reveals four distinct piscine vesiculovirus groups. XI International Congress of Virology, Sydney. Abstract VP24.04, p 351.

Way, K., Banyard, A.R., Dixon, P.F., Schmidt, N.T. & Stone, D.M. (1998). Is PFR virus simply SVC virus disguised in 'pike clothing'?: comparative studies on rhabdoviruses from cyprinid fish. Poster presentation at IV International Symposium on Viruses of Lower Vertebrates, Weymouth. Abstract PP-06..

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