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Detection of trout haemorrhagic septicaemia rhabdovirus by capture with monoclonal antibodies and amplification with PCR

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This work describes the preliminary application of a new method for viral detection to VHSV. This method was previously developed for plant viruses (Nolasco *et al,* 1993) and some animal viruses, including the virus causing foot and mouth disease. The first step in this method involves viral immunocapture with a solid phase coated with a monoclonal antibody (MAb) specific for viral nucleoprotein N with a high affinity that recognizes a wide range of VHSV isolates (Sanz and Coll, 1992a). The second step involves viral genome amplification with specific VHSV nucleoprotein N (to differentiate rhabdoviruses) or G (to differentiate VHSV isolates) gene primers by the polymerase chain reaction (PCR) based in thermostable DNA polymerases (Taq polymerase).

Microtitre plates (Nunc, Roskilde, Dinamarca) were coated to dryness with 1 µg/well of protein-A affinity purified MAb 2C9, raised against the nucleoprotein N from VHSV, as previously described (Sanz *et al,* 1993). The VHSV infected supernatants from lysed EPC cultures were then 2-fold serially diluted with dilution buffer (final concentrations: 1 M NaCl, 2 mM KCl, 8 mM Na_2PO_4, 1.4 mM KH_2PO_4, 1 M NaCl, 0.24 mM merthiolate, 5 g/l albumin, 0.5 g/l Tween 20, 20 mg/ml phenol red, pH 6.8), and 100 µl was pipetted into the MAb-coated wells and incubated for 2 h at room temperature (Estepa *et al,* 1994). After incubation, the wells were washed 3 times with distilled water.

Water (22 µl) was then added to each well and the whole was heated for 5 min at 90°C. Both RT and PCR used specific primers from the cDNA sequence of the nucleoprotein N and the glycoprotein G. The *N* gene primers (table I) were selected by McAllister *et al* (1991). The *G* gene primers (table I) were selected according to Thiry *et al* (1991) and Estepa *et al* (1994) and defined a 379 bp region (aa 64–195) on the corresponding *G* gene. This fragment of the glycoprotein G was chosen because it encodes the greatest hydrophilic region of the glycoprotein G and would, therefore, probably contain some of the isolate variability.

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Table I. Primers used to amplify the *N* and *G* genes of the VHSV.

Viral protein	*Product length (bp)*		*5'-sequence 3'*	*Reference*
N	408	s	^{bp718}GGAGATAGGAAGGTGATTGTGG	MacAllister
		a	GAGTTTCCTGATGGCTGCCTTG^{bp1126}	*et al,* 1991
G	379	s	^{bp190}GTC**CCATGG**AATTTGAAGACATAAAAG	This work
		a	CGA**GTCGAC**TGAAACCCCCTCTATGAA^{bp585}	

The DNA fragment amplified by the N primers corresponds to the nucleoprotein N amino acids 253–371. It was selected because its conservation among VHSV strains would make this amplification capable of distinguishing all VHSV from other viruses (MacAllister *et al,* 1991). The glycoprotein G primers were selected to differentiate among the different VHSV isolates through the cloning and expressing of one amino-terminal fragment of the glycoprotein G (aa, 64–195) (Estepa, 1992; Estepa *et al,* 1994). s, sense primers. a, antisense primers. NcoI and SalI, restriction sites are noted in bold; ATG and TGA, codons are underlined.

The sense primers (250 ng/reaction) were used to obtain the cDNA with reverse transcriptase (RT) (BRL, Gaithersburg, MD, USA). In each case, RT buffer, 10 mM dithioerytriol, 500 μM each of all four 2'-deoxynucleoside 5'-triphosphates, 5 units human placental ribonuclease inhibitor (Boehringer Manheim, Heidelberg, Germany) and 200 units of RT were added to the denatured immunocaptured VHSV in the wells to a final volume of 50 μl. The RT-mixture was incubated for 40 min at 42°C. After addition of an antisense primer (250 ng), water, and 2.5 units of Taq polymerase (Perkin-Elmer, Norwalkct, USA) (final reaction volume of 100 μl), the samples were transfered to an eppendorf tube and amplified in a thermocycler (Lep Scientific, Milton Keynes, UK). The samples were amplified by 30 successive cycles using the following amplification conditions: 1 min 20 at 92°C to denature the DNA; 1 min 30 s at 60°C to anneal the primers; and 2 min 20 s at 72°C to polymerize the segments. About 15 μl of each sample was analyzed by electrophoresis in a 1.3% agarose gel electrophoresis.

About 50 μl of the VHSV-infected EPC cell supernatants, containing 10^5–10^6 $TCID_{50}$ of VHSV, was incubated with solid phase coated with MAb 2C9 anti-N (sensitivity of about 10^2 $TCID_{50}$/well) (Sanz and Coll, 1992a). MAb 2C9 was selected because of its high titre (Sanz and Coll, 1992a,b) and its capacity to recognize all the VHSV strains (Sanz *et al,* 1993).

The fragments of the G or N DNA sequences of VHSV defined by the primers used in the RT and PCR reactions (table I) were only amplified in the wells coated with MAb 2C9 and incubated with the VHSV-infected supernatants (fig 1, lines 3 and 4). The band corresponding to the G DNA was more intense than the one corresponding to the N (fig 1, lines 3 and 4).

The amplified bands corresponded to 390 bp (257 kDa) in the case of N protein and 347 bp (230 kDa) in the case of G protein. When non-coated wells were used, the amplified bands could not be detected even when they were incubated with VHSV-infected supernatants (fig 1, lines 5 and 6). Similarly, controls of MAb 2C9-coated wells incubated with supernatants from non-infected EPC cells did not show any amplified bands of the appropiate molecular weight (fig 1, lines 1 and 2).

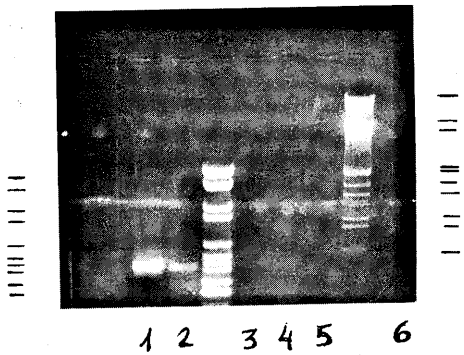

Fig 1. Agarose gel electrophoresis of PCR amplified VHSV captured by solid-phase anti-VHSV MAb. Lines 1 and 2: amplifications of controls using the N (line 1) or the G (line 2) primers without VHSV-infected supernatants and MAb coated wells. Line 3: amplification using N primers containing VHSV-infected supernatants and MAb coated wells. Line 4: amplification using G specific primers containing VHSV-infected supernatants and MAb-coated wells. Lines 5 and 6: amplifications using N or G primers containing VHSV-infected supernatants but non-coated wells. M, markers: left markers from top to botton, 2 176, 1 766, 1 230, 1 033, 653, 517, 453, 394, 288 and 234 bp; right markers from top to bottom, 21 276, 5 148, 3 530, 2 027, 1 904, 1 709, 1 375, 947, 831 and 564 bp.

This method for VHSV detection by ELISA and PCR, which combines speed, high sensitivity and double-checked specificity (MAb and primers), would allow automation for the processing of a large number of samples (the gel electrophoresis step is currently being substituted for by spectrophometric estimations and 96-well plates for PCR amplifications are already on the market). The method shows promising potential for use in routine diagnosis of the virus. The amplification based on the N primers would allow a first diagnosis of VHSV. In addition, the amplification of short stretches of glycoprotein G with primers containing target sequences for restriction enzymes, would allow rapid cloning and sequencing for the complete identification of the strain/isolate causing the infection (Sanz and Coll, 1992b). More work needs to be done in order to select the correct MAb and/or primer sets. It is important that both the N and G primer sets be tested with a large panel of VHSV isolates in order to investigate their potential limitations before this method can be used as a universal assay for VHSV.

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