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DETECTION OF TROUT HAEMORRHAGIC SEPTICAEMIA RHABDOVIRUS BY CAPTURE WITH MONOCLONAL ANTIBODIES AND AMPLIFICATION WITH PCR

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Introduction

The rhabdovirus causing viral haemorrhagic septicaemia (VHS), produces the most important economic losses in European production of trout (up to 30% of the total production in the heavily affected areas). Fast and highly sensitive detection of VHSV is still essential to detect and control the disease (Sanz & Coll, 1992c), because there is still no commercial vaccine against this virus (Estepa, 1992; Estepa *et al*, 1994). The VHSV genome consists of a negative single-stranded RNA molecule encoding five viral proteins, G (65 kDa, membrane glycoprotein), N (38 kDa nucleoprotein) M1 and M2 (two matrix proteins), and L (RNA polymerase). This paper describes the preliminary application to VHSV of a new method for viral detection previously developed both for plant viruses (Nolasco *et al.*, 1993) and some animal viruses, such as Foot and Mouth disease virus (Rodriguez *et al.*, 1994). This method uses a first step of viral immunocapture with a solid-phase coated with a viral nucleoprotein N specific monoclonal antibody (MAb) of high affinity which recognises a wide range of VHSV isolates (2C9, Sanz and Coll, 1992b) and a second step involving 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 on thermostable DNA polymerases (Taq polymerase).

Materials and Methods

Virus. VHSV 07.71 isolated in France (Le Berre *et al.*, 1977) and obtained from rainbow trout *Oncorhynchus mykiss*, Walb., was grown in Epithelial Papillosum Cyprine cells (EPC) (Fijan *et al.*, 1983, Olesen *et al.*, 1992). The infected supernatants from lysed EPC cultures were used in these experiments.

Immunocapture of VHSV with MAbs.

Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated to dryness with 1 µg/well of protein-A affinity purified MAb 2C9, raised against nucleoprotein N from VSHV, as described previously (Sanz *et al*, 1993). Then, the infected supernatants from lysed EPC cultures were 2-fold serially diluted with dilution buffer (final concentrations, 1M NaCl, 2mM KCl, 8mM Na_2PO_4, 1.4 mM KH_2PO_4, 1M NaCl, 0.24mM Merthiolate, 5 g/l albumin, 0.5 g/l Tween 20, 20mg/ml phenol red, pH 6.8), and 100 µl were pipetted to the MAb coated wells and incubated during 2 hours at room temperature (Estepa *et al.*, 1991). After incubation, the wells were washed 3 times with distilled water. Non-coated wells were also incubated with the supernatants as controls.

Reverse transcriptase (RT)-Polymerase chain reaction (PCR). The RT-PCR reactions were performed in the wells after immunocapture of VHSV. First, 22µl of water were added to each well and heated 5 min at 90°C to break the virus and denature the viral RNA. Both RT and PCR used specific primers from the cDNA sequence of the nucleoprotein N (Bernard *et al.*, 1990) and the glycoprotein G (Thiry *et al.*, 1991). The N gene primers (Table 1) were selected by McAllister *et al.* (1991) and defined a 408 bp region (amino acid 253-371) of the corresponding N gene to differentiate VHSV from IHNV. The G gene primers (Table 1) were selected according to Estepa *et al.*, (1994) and defined

Table 1 Primers used to amplify the N and G genes of the VHSV.

Viral Protein	Product Length, bp		5'sequence3'	Reference
N	408	s	^{bp718}GGAGATAGGAAGGTGATTGTGG	Mac Allister *et al*, 1991
		a	GAGTTTCCTGATGGCTGCCTTG^{bp1126}	
G	379	s	^{bp190}GTCCC**ATG**GAATTTGAAGACATAAAAG	This study
		a	CGA**GTCGAC**TGAAACCCCCTCTATGAA^{bp585}	

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

a 379 bp region (amino acid 64-195) on the corresponding G gene. This fragment of the glycoprotein G was chosen because it encodes the highest hydrophilic region of the glycoprotein G and it probably would contain some of the variability through the isolates. The G primers were designed to contain an initiating codon (ATG) including a NcoI (CCATGG) restriction site in the sense primer and a stop codon (TGA) followed by a SalI site (CTCGAC) in the antisense primer (Estepa *et al.*, 1994). This design allows for the cloning and possible posterior sequencing of the amplified fragment. The sense primers (250 ng/reaction), were used to obtain the cDNA with the Moloney Murine Leukaemia virus reverse transcriptase (RT) (BRL, Gaithersburg, MD, USA). In each case, RT buffer, 10mM dithioerytriol, 500mM 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 50µl. The RT-mixture was incubated during 40 min at 42°C. After addition of the antisense primer (250 ng), water, and 2.5 units of Taq polymerase (Perkin-Elmer, Norwalk Ct, USA) (final reaction volume of 100µl), the samples were transferred to an eppendorf tube and amplified in a thermocycler (Lep Scientific, Milton Keynes, Great Britain). The samples were amplified by 30 successive cycles using the following amplification conditions, 1'20" at 92°C to denature the DNA, 1'30" at 60°C to anneal the primers and 2'20" at 72°C to polymerise the segments. About 15µl of each sample were analysed by electrophoresis in a 1.3% agarose gel containing ethidium bromide (0.35 µg/ml) by electrophoresis. The molecular weights of the G and N sequences amplified were obtained by comparison with the molecular weights of the commercial DNA markers ranging between 2167 and 234 bp.

Results

About 50µl of the VHSV-infected EPC cell supernatants, containing 10^5-10^6 $TCID_{50}$ of VHSV, were incubated with solid-phase

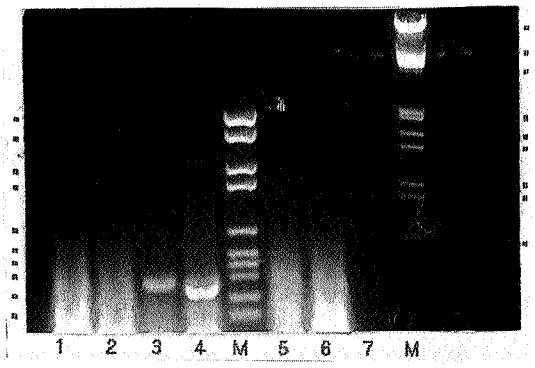

Figure 1.- Agarose gel electrophoresis of PCR amplified VHSV-infected supernatants. Lines 1 and 2, amplifications of controls using the N (line 1) or the G (line 2) primers without VHSV-infected supernatants and MAb coated wells. Line 3, amplification using N primers containing VHSV-infected supernatants and MAb coated wells; Line 4, amplification using G specifics 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. Line 7, empty well. M, markers; left markers from top to bottom, 2176, 1766, 1230, 1033, 653, 517, 453, 394, 288 and 234 bp; right markers from top to bottom, 21276, 5148, 3530, 2027, 1904, 1709, 1375, 947, 831 and 564 bp.

coated with MAb 2C9 anti-N (sensitivity of about 10^2 $TCID_{50}$/well) (Sanz and Coll, 1992a). MAb 2C9 has been selected mainly because of its high titre (Sanz and Coll, 1992a,b) and its capacity to recognise all the VHSV strains (Sanz *et al.*, 1993).

The fragments of the G or N DNA sequences of VHSV defined by the primers used (Table 1) in the RT and PCR reactions were amplified only in the wells coated with the 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. By using the non coated wells, amplified bands could not be detected even when incubated with VHSV-infected supernatants, (Fig.1, Line 5, 6). Similarly, controls of MAb 2C9 coated wells incubated with supernatants from non-infected EPC cells did no show any amplified bands in the appropriate molecular weight (Fig.1, lines 1 and 2).

Discussion

The technique of solid-phase immunocapture of VHSV and the posterior amplification of part of its genome, amplified efficiently and specifically the N and G gene primer-defined sequences from VHSV-infected cell culture supernatants. The different intensity between the bands obtained with the N and the G primers could be due to the amplification conditions (Fig. 1), which were different from the ones that Mac Allister *et al.*, (1991) choose for the N gene. In our conditions, heating, annealing and polymerisation times are higher and we did not allowed for a final polymerisation time. For instance, our annealing temperature is 15°C higher than Mac Allister's, to minimise non specific hybridisations and to increase specificity. By the VHSV immunocapture we obtained high enough amount of virus for the PCR, making unnecessary a viral RNA extraction step. Comparing this technique with the MAb based ELISA described before (Sanz and Coll, 1992a), the ELISA has one step less but with a lower specificity and sensibility. To increase its detection limit, it is necessary the PCR step. This method for VHSV detection by ELISA and PCR which combines speed, high sensitivity and double checked specificity (MAb and

primers) could allow automation of the processing of a large number of samples (the gel electrophoresis step is currently being substituted by spectrophotometric stimations and 96-well plates for PCR amplifications are already in the market), being a promising candidate for routine diagnosis of the virus (Rodriguez *et al.*, 1994). The amplification based on the N primers would allow a first diagnostic of VHSV and in addition, the amplification of short stretches of the glycoprotein G with primers containing target sequences for restriction enzymes would allow its rapid cloning and sequencing for the complete identification of the strain/isolate causing the infection. More work is needed, however, to select the correct MAb and/or primer sets since it is important that both the N and G primer sets would be tested with a larger panel of VHSV isolates to investigate their potential limitations in an universal assay for VHSV.

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Summary

A new technique for detection and preliminary characterisation of Viral Haemorrhagic Septicaemia Virus (VHSV) uses a first step of viral immunocapture with solid-phase coated with anti-N viral-specific monoclonal antibodies (MAbs) and a second step involving viral genome amplification (either N or G genes) by RT-PCR reactions. The N primers could be used to detect and differentiate VHSV from other rhabdoviruses whereas de G primers may allow the differences/relationships between isolates to be further characterised at a later date. This method, makes unnecessary a viral RNA extraction step, it is more specific and sensible than the ELISA assay and, in a near future, it should allow sensible processing of a large number of samples for routine diagnosis of VHSV.

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