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Development and Use of a DNA Probe for Confirmation of cDNA from Infectious Salmon Anaemia Virus (ISAV) in PCR Products

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Abstract

Infectious salmon anaemia virus in fish tissues or cultured cells can be detected by polymerase chain reaction amplification of part of genome segment 8. An oligonucleotide probe complementary to sequence within this PCR product was designed. The probe was labelled with digoxigenin and hybridised to PCR products that had been immobilised on a membrane by Southern blotting. The probe provided greater sensitivity than visual examination of PCR products in ethidium bromide stained gels and provides a means to confirm that PCR products of approximately the size expected for infected samples are specific.

Introduction

Infectious Salmon Anaemia (ISA) is a viral disease of Atlantic salmon (Thorud & Djupvik, 1988), which has caused large financial losses in the Norwegian Atlantic salmon farming industry. The virus has since been discovered in Canada (Mullins *et al.,* 1998) and Scotland (Rodger *et al.,* 1998).

The presence of ISAV can be detected by a number of methods including virus isolation in a number of cell lines, histopathology, immunofluorescent antibody tests and polymerase chain reaction (PCR) amplification of part of the viral genome. The first PCR method for amplification of part of segment 8 of ISAV was developed by Mjaaland *et al* (1997). This method is now routinely used in Norway and Scotland for testing samples for ISA. Homogenates of fish tissue and cultured cells inoculated with virus can both be tested in this manner. A positive result is indicated by the presence of a 155 bp product on an agarose gel. Occasionally however, multiple PCR products are evident or the yield of product is very low and difficult to visualise on agarose gels. Therefore, an oligonucleotide probe was designed and a method developed that allows confirmation of PCR tests for the presence of ISAV by revealing the presence of the specific amplification product in agarose gels.

Materials and Methods

PCR

PCR was carried out according to the method of Mjaaland *et al* (1997) using homogenised fish tissue as starting material. A variety of samples were used, from Atlantic salmon (*Salmo salar* L.), rainbow and brown trout (*Oncorhynchus mykiss* Walbaum and *Salmo trutta* L., respectively) and from both fresh and sea water (see Table 1). The majority of these samples had previously given positive results in PCR tests for ISAV. Products were subjected

ISA+

GGCTATCTACCATGAACGAATCACAATGGATACAAAAACATCTACCATGCAT 52

GAGAGAAGCAAACCCCAAACCACGGGAGTTGATCAGACATGCACTGAAGGTG 104

AAGAAGAGGCCAGAAGTAGTCTACGCAATGGGAGTGCTACTTACACTTGGC 155

ISA-

Figure 1. Position of oligonucleotide probe (shaded) within 155bp PCR product. Nucleotides numbered according to EMBL nucleotide database accession number AJ242016; Sequence of ISAV segment 8 from Scotland. Boxes indicate positions of PCR primers ISA+ and ISA- (Mjaaland *et al.*, 1997).

to electrophoresis on a 2 % agarose gel alongside a 50 bp marker ladder (Life Technologies), stained with ethidium bromide and visualised by u.v. illumination.

Seven ten-fold serial dilutions of known positive PCR products with original DNA content of approximately 75 ng were also run on a 1 % agarose gel with digoxigenin (DIG)–labelled markers (Boehringer Mannheim) as well as the 50 bp ladder, and used to determine the sensitivity of the probe.

Southern blot

Aliquots of 10µl of PCR product were subjected to electrophoresis on a 1 % agarose gel and DIG-labelled markers were included alongside the marker ladder. Also included was a positive control PCR product of approximately 75 pg DNA. The gel was photographed with a ruler alongside. The gel was rinsed in dH_2O for 20 min, then soaked twice for 15 min each in denaturing solution (NaCl 1.5M, NaOH 0.5M) (BDH), and two further 15 min soaks in neutralising solution (NaCl 1.5M, TrisHCl 1M, pH 8.0) (BDH), with a rinse in dH_2O between each soak. DNA was transferred to a positively charged membrane using Posiblot™ apparatus (Stratagene). The membrane was then baked for 30 min at 120°C and stored at room temperature.

Hybridisation

An oligonucleotide (5'-CGGGAGTTGATCAGACATGCACTGAAGGTG-3') complementary to a region within the desired 155 bp PCR product was selected from the sequence of ISAV segment 8 (Figure 1). The oligonucleotide was 3'-end labelled with digoxigenin (DIG)-ddUTP and concentration of labelled probe determined according to the manufacturers' instructions (Boehringer Mannheim). Membranes were placed inside a hybridisation roller bottle and approximately 40ml of pre-hybridisation buffer (5x SSC, 1 % blocking reagent (Boehringer Mannheim), 0.1 % N-lauryl sarcosine, 0.02 % lauryl sulphate) was added. The bottle was incubated at 40°C for 1 hour in a rotating hybridisation oven. The hybridisation buffer was removed and 20 ml DIG-labelled probe (10 pmol/ml) added and incubated at 40 °C for 3.5 hours. The probe was then removed and stored at –20 °C for re-use. The membrane was washed twice, for 5 min each, at room temperature in 2x SSC, 0.1 % SDS, followed by two 15 min washes at 40°C in 0.1x SSC, 0.1 % SDS. The membrane was then air-dried and stored for detection. Chemiluminescent detection was carried out according to the manufacturers' instructions (Boehringer Mannheim).

Results

Hybridisation and washing conditions were optimised using replicate membranes containing serial dilutions of positive PCR product obtained from artificially infected salmon. Optimal sensitivity and specificity were obtained using the conditions given above. These conditions enabled detection of 75pg diluted PCR product, thus providing up to 100 times greater sensitivity than visual examination of ethidium bromide stained agarose gels.

Use of positive control DNA provided a better indication of the size of fragments that hybridise to the probe than either DIG labelled markers or correlation of measurements of ethidium bromide stained fragments in agarose gels with fragments detected following hybridisation. Approximately 750pg positive control DNA provided a clear signal without resulting in large blotches on the X-ray film due to excess chemiluminescence, which could obscure positive signals in adjacent lanes of the gel.

The results of hybridisation to test samples are shown in Table 1. PCR amplifications from 110 fish tissue samples from a variety of species, farmed and wild sources in fresh and sea water were used in trials of the DNA probe. Eleven samples did not contain a PCR product of the size expected for ISAV infected fish (155bp). The probe did not hybridise to any of these samples.

Four samples which did contain a PCR product of approximately 155bp did not hybridise to the probe. Two of these samples contained more than one fragment, including one of 155bp. One sample produced a smear of DNA which encompassed a fragment of 155bp. Only one of these samples contained a single PCR product.

In sixteen samples, two or more fragments showed positive hybridisation to the probe. The expected PCR product of 155bp was also present in every case. Estimates of the size of the additional fragments are shown in Table 1. These measurements have a margin of error of approximately +/- 10 bp, due to difficulties in obtaining accurate measurements from blots. The additional fragments were not always visible on agarose gels.

	S.salar (SW)	*S.salar* (FW)	*O. mykiss* (FW)	*S. trutta* (FW)	*S. trutta* (SW)
No. samples tested	25	67	11	6	1
No. samples PCR positive	25	56	11	6	1
No. samples with single fragment probe positive	17	45	10	6	1
No. sample with multiple fragments probe positive	4	11	1	0	0
Sizes of multiple products (±10bp) (additional to 155bp product)	300, >400	300, 700, 500-550	300		

Table 1. Results of probe hybridisation to samples from various fish species used in RT-PCR amplification of 155 nucleotide fragment of ISAV segment 8. FW: fresh water, SW: sea water.

Discussion

PCR amplification from fish sampled outwith farms affected by ISA disease has provided evidence for a wider distribution of ISAV (Raynard, 2000). In this study, the oligonucleotide probe hybridised to PCR products from fish sampled in both fresh and sea water, providing further evidence for the presence of this virus and specificity of the PCR. Selected samples were analysed using the probe and therefore the results reported are not a true reflection of the prevalence of ISAV in Scotland.

The majority of samples produced a single positive fragment of 155 bp on the x-ray film. There was a varying degree of chemiluminescent intensity, probably due to varying DNA concentrations between samples. In most of the cases where more than one positive signal was observed, hybridisation of the probe to a fragment of approximately 300bp occurred. This was probably due to the presence of a dimer of the 155 bp target which is occasionally present at concentrations high enough to be observed on ethidium bromide stained gels (Cunningham & Snow, 2000). In this study, the 300bp product was not visible on the gel. The other positive signals had sizes ranging from 350 to 500 bp and one of 700 bp. These products were not visible on the gel. It is possible that some of these also arise from multimers of the specific PCR product. However, it could also be the case that non-specific hybridisation of the probe has occurred. If so, there could be hybridisation to cellular cDNA, the genetic material of other microorganisms present in the sample, or even to cDNA derived from another, as yet unidentified, aquatic Orthomyxovirus. It may be possible to reduce non-specific hybridisation by the addition of extra blocking agents such as yeast RNA (A. Gregory, FRS Marine Laboratory, personal communication). However, as they are not of the size expected for ISAV infected material and do not interfere with the signals from the specific product, the occasional presence of additional signals does not interfere with the conclusions drawn from this process.

Four samples produced a PCR product of the size expected for ISAV infected fish on the agarose gel yet did not hybridise with the DNA probe. These samples could potentially give rise to a false positive result if PCR diagnosis is used in isolation. In practical applications of the ISAV PCR method on material from fish farms in Scotland, PCR results are always considered alongside the results from other tests such as immunofluorescent antibodies, culture, and histopathology to provide as much information as possible before making a diagnosis. Therefore the risk of false positive results is minimised.

One of the samples that was apparently PCR positive but probe negative produced a smear of DNA, and two others contained multiple products. Such results should be treated with caution and ideally, other tests would be undertaken to provide additional indications of the presence of ISAV.

The results presented here have demonstrated that probes can be useful to confirm the specificity of PCR products seen on gels, and that the PCR method has a low risk of producing false positive results. The converse situation, where ISAV is present at very low levels in the sample and PCR product yield is

too low to be visualised on agarose gels but can be detected by DNA probe, was not found. Therefore, the PCR method used appears to have a low risk of false negative results. The use of the DNA probe may therefore be of greatest use in cases where there are multiple PCR products or doubt as to whether a product of 155bp really has arisen from amplification of ISAV.

Southern blotting and probe hybridisation is a relatively straightforward procedure that is faster, cheaper and more convenient than sequencing a 155bp PCR product. In this way, the presence and identity of PCR products resulting from amplification of the ISAV genome can be confirmed, even in cases where multiple or low concentration PCR products occur.

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References

Cunningham, C. O. & Snow, M. (2000). Genetic analysis of infectious salmon anaemia virus (ISAV) from Scotland. Dis. Aquat. Org. 41: 1-8.

Mjaaland, S., Rimstad, E., Falk, K., & Dannevig, B.H. (1997). Genomic characterisation of the virus causing Infectious Salmon Anemia in Atlantic Salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. J. Virol., 71: 7681-7686.

Mullins, J. E., Groman, D., Wadowska, D (1998). Infectious salmon anaemia in salt water Atlantic salmon (*Salmo salar* L.) in New Brunswick, Canada. Bull. Eur. Ass. Fish Pathol., 18, 110-114.

Raynard, R. S. (2000) A survey of wild fish in Scotland for evidence of infectious salmon anaemia virus - report for the period May 1998 to December 1999. Marine Laboratory Aberdeen Report 05/00. 17pp. Fisheries Research Services, Aberdeen.

Rodger, H. D., Turnbull, T., Muir, F., Millar, S. & Richards, R. H. (1998). Infectious salmon anaemia (ISA) in the United Kingdom. Bull. Eur. Ass. Fish Pathol., 18, 115-116.

Thorud, K. & Djupvik, H. O. (1988). Infectious anaemia in Atlantic salmon (*Salmo salar* L.). Bull. Eur. Ass. Fish Pathol., 8, 109-111.