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NOTE

# Recovery of ranavirus dsDNA from formalin-fixed archival material

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ABSTRACT: The extraction and amplification of nucleic acid from formalin-fixed and paraffin-embedded tissues has become an important exercise in the collection of retrospective epidemiological data. A protocol is described that enables the extraction and amplification of dsDNA from fixed tissues within paraffin blocks and from specimens stored in 10% (aq) formalin. The procedure can be used for the examination of ranavirus DNA within archival tissues thereby providing valuable data for identifying the origin and tracing the spread of ranaviruses.

KEY WORDS: Iridoviruses · Ranaviruses · Polymerase chain reaction

Ranaviruses are a group of viruses (genus *Ranavirus*) that contain a linear dsDNA genome and belong to the family *Iridoviridae*. Viruses belonging to this genus continue to be identified with an increasing frequency from fish, amphibians and reptiles (refer to Hyatt et al. 1999). Within the past 2 yr, viruses have been isolated from frogs (e.g. *Rana temporaria*; Drury et al. 1995), toads (e.g. *Bufo marinus*; Zupanovich et al. 1998a), the tiger salamander *Ambystoma tigrinum stebbinsi* (Jancovich et al. 1998), and tortoises (e.g. Westhouse et al. 1996, Mao et al. 1997).

Ranaviruses cause a systemic infection within their host (Ahne et al. 1997). The infections, as illustrated by epizootic haematopoietic necrosis virus (EHNV), are manifested in the haematopoietic tissues of the kidneys, the liver and the spleen. In these organs the haematopoietic cells are selectively infected and destroyed (Langdon et al. 1986); it is the necrosis of these vital organs that leads to large-scale deaths (e.g. Langdon et al. 1986, Whittington et al. 1996). Whilst the significance of ranaviruses is known in fish health e.g. epizootic haematopoietic necrosis is an OIE (Office international des epizooties) reportable disease, their significance in relation to the health, conservation and international trade of herpetofauna is only now being recognised (e.g. Henstberger et al. 1993, Zupanovich et al. 1998b, Daszak et al. 1999). Due to the potential for ranaviruses to infect a broad range of hosts (refer to Hyatt et al. 1999 and Daszak et al. 1999), it is becoming increasingly important to examine archival specimens for the presence of these viruses. Only with this information will it be possible to obtain data to determine the geographical origin (Mao et al. 1997, Hyatt et al. 1999), time of emergence (endemic) or introduction (exotic) spread, and impact of ranaviruses on the piscine and herpetofauna within any one geographical region.

In earlier studies we (Gould et al. 1995) showed that 2 regions of the EHNV genome could be used for ranavirus identification. Specifically, regions of the major coat protein (MCP) provided data that could differentiate EHNV from Bohle iridovirus. Mao et al. (1997) also demonstrated that the coding region for the MCP could be used for ranavirus identification. We extended this initial work by analysing a large number of ranaviruses from Australia, Europe, North America and South America by the sequencing of polymerase chain reaction (PCR) products (Hyatt et al. 1999). The data, together with that presented by Mao et al. (1997), supported the original findings that sequence data of the MCP and other regions of the ranavirus genome could be used to differentiate known ranaviruses. It is therefore important to have the capacity to amplify nucleic acid from archived specimens that are suspected, or known, to be infected with ranaviruses.

Within scientific literature there are numerous protocols for extracting viral nucleic acid from fixed tissue (e.g. Rogers et al. 1990, Kallio et al. 1991, Crissan & Mattson 1993, Mohan et al. 1995, Frank et al. 1996, Mancuso et al. 1997, Crawford & O'Toole 1999); however none of these protocols describe an effective procedure for the extraction of nucleic acid from ranaviruses. At a recent workshop on amphibian diseases and immunity held at the San Diego Zoo (July, 1998;

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funded by the National Science Foundation), some scientists brought to our attention that they could not extract ranavirus dsDNS from formalin-fixed tissues. In this short communication we describe a method for recovering and amplifying the dsDNA of ranaviruses from formalin-fixed archival specimens.

**Materials and methods.** ***Tissues:*** Tissues (kidney, liver and/or spleen) from redfin perch *Perca fluviatilis* and from 2 chondra pythons *Chondrapython viridis* were used in this study. The redfin perch were infected with epizootic haematopoietic necrosis virus (EHNV, accession number 96-512) and the 2 chondra pythons were infected with a ranavirus (CRV#1, accession number 98-0804 and CRV#2, accession number 98-0814) (unpubl.). The tissues had been stored as either formalin-fixed (10% [v/v] neutral buffered formalin) tissue within paraffin embedded blocks (CRV#1a, CRV#2a, EHNV) or as 'wet' specimens (10% [v/v] neutral buffered formalin; for a minimum of 10 mo) (CRV#1b, CRV#2b). Note: the nomenclature of the ranavirus from *Chondrapython viridis* as CRV is temporary.

***Extraction of dsDNA from paraffin blocks and non-embedded material:*** Paraffin sections were cut from EHNV-infected tissues and CRV-infected tissues. If sections were adhered to glass slides, the sections were de-paraffinised by warming them in an oven (60°C) for 10 min and then rinsing in xylene (100%, 5 min) followed by 100% ethanol (5 min). These 2 washes were repeated (× 2) and the slides were dried at room temperature (24°C) for 10 to 15 min. For paraffin-embedded tissues (not on glass slides), 1 to 2 sections were placed in an Eppendorf tube and the wax was removed with washes (× 3) of xylene (100%). The contents were then briefly centrifuged (5 min, 11 340 × *g*) in a Sigma (Model 112) microfuge to pellet the released tissue; the supernatant was discarded. The xylene was removed with washes (× 3) of ethanol (100%), followed by centrifugation (11 340 × *g*, 5 min) to remove traces of ethanol. The samples were processed as described below.

Formalin-fixed non-paraffin-embedded tissues of approximately 200 mg were cut into 1 $mm^2$ tissue blocks with a sterile disposable scalpel blade. The tissue samples were digested (without washing) in 500 µl with proteinase K (50 µg $ml^{-1}$) in the presence of sodium dodecyl sulfate (SDS, 0.1 %) for 3 h (37°C), vortexing on a regular basis. If the sample was to be heat-treated, then the digestion mixture was heated at 100°C in a heating block for 10 min and then cooled on ice.

The digested tissue was extracted twice with phenol/chloroform followed by a single ether extraction. 1/10 the volume of 3 M NaOAc was added to the supernatant and mixed with 2.5 volumes of ethanol (–20°C) and the nucleic acids were precipitated at –20°C overnight. Nucleic acids were collected by centrifugation (11 340 × *g*, 10 to 15 min) and washed (× 1) in 70% ethanol.

***Control tissues:*** Prior to sectioning, a paraffin block containing samples of liver, kidney and spleen from an uninfected redfin perch was sectioned; this sample served as the negative control. These sections were also de-paraffinised and treated as described above. In protocols involving sectioning, it is critically important to demonstrate that the microtome knife and associated equipment are not contaminated with ranavirus DNA. The control serves this purpose in addition to demonstrating the specificity of the PCR primers.

***Amplification of DNA:*** The PCR was performed in a Perkin Elmer Cetus Thermal Cycler (Perkin-Elmer, Norwalk, Connecticut, USA). The reaction mixture (in 25 µl, total volume) contained 50 to 100 pmol of primers, 1 × PCR buffer (50 mM KCl, 10 mM TRIS-HCl, 3 mM $MgCl_2$, pH 9 at 25°C) 0.2 mM of dATP, dCTP, dGTP, and dTTP, 0.625 to 1.25 U of *Taq* polymerase (Promega Corporation, Madison, USA) and the template. Primers used for PCR amplification of MCP DNA fragments were as follows. Primary PCR reactions were done for 35 cycles using 50°C annealing for 2 min, a 72°C extension for 2 min followed by a 1 min strand-dissociation step at 94°C; the primers were FV3-991 (5'-CGCAGTCAAGGCCTTGATGT) and FV3-1571R (5'-AAAGACCCGTTTTGCAGCAAAC).

Hemi-nested PCR reactions were performed for 25 cycles using the same temperature and time conditions but with the primers P1050N (5'-TCAAGAGCGC-CACGCTGGTGTA) and FV3-1571R. 1 µl of the primary PCR reaction mix was added to a 50 µl nested PCR reaction mix prior to amplification.

**Results and discussion.** The reaction protocols (Gould et al. 1995) used to amplify the MCP coding region failed to work with formalin-fixed, paraffin-embedded material, whilst 'wet' formalin-fixed tissues (that had been stored in 10% [v/v] neutral buffered formalin) produced variable results (Fig. 1). By boiling the digestion mixture for 10 min, PCR products were consistently present in hemi-nested PCR reactions (Fig. 1). The validity of the amplified DNA products was verified by sequence analysis (data not shown). Primers used in the amplification process were used in the dye-termination sequencing reactions and each fragment was sequenced twice. Sequences derived from formalin-fixed or 'wet' tissue were identical (not shown), indicating that the preparation procedure resulted in no spurious sequence alterations.

The variation in the results may be attributed to the amount of virus present within the samples. The sensitivity of the protocol is not known and can only be determined from experimentally infected tissues that have been stored for different periods of time. The pro-

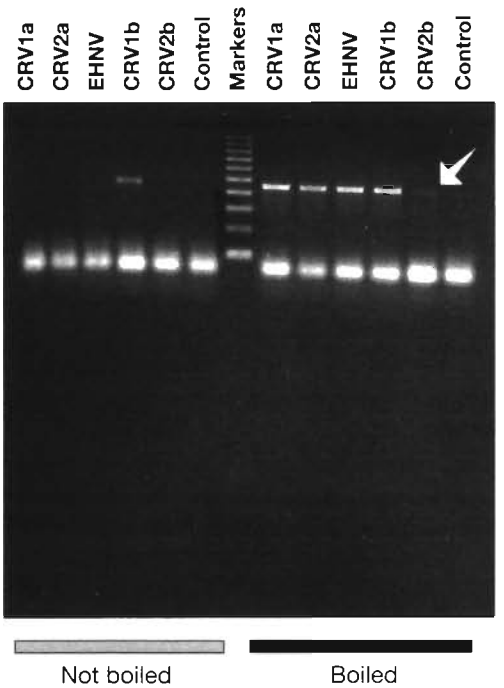


Fig. 1. DNA products from the polymerase chain reaction (PCR) amplification of template DNA using primers P1050N and FV3-1571R are shown by ethidium bromide staining of a 2% agarose gel. Samples designated CRV#1a and CRV#2a and EHNV represent formalin-fixed tissues within paraffin sections. Samples CRV#1b and CRV#2b were 'wet' tissues (refer to text) that had been stored in 10% neutral buffered formalin for 10 mo. The arrow indicates the presence of a weak band. Control tissue was sections containing uninfected tissues (liver, spleen, kidney) from redfin perch *Perca fluviatilis*. Markers were 100 to 1000 bp markers (DMW-100L) from GeneWorks (Adelaide, South Australia)

tocol described in this short communication is similar to others (e.g. Jackson et al. 1990, Kallio et al. 1991, Sepp et al. 1994) where boiling was found to be an effective step in retrieving DNA from formalin-fixed tissues. Whether modifications to the protocol such as deleting the protein K digestion step or increasing the times for digestion and/or boiling of the tissues will increase the effective yield of DNA remains to be tested.

Irrespective of the times of digestion and/or boiling, the described procedure appears to 'retrieve' sufficient DNA whereby a PCR product can be produced without a decrease in the fidelity of the DNA sequences.

The method described in this communication enables the successful amplification of a 450 bp DNA fragment containing a portion of the MCP region of the dsDNA genome of ranaviruses. This simple procedure should prove very useful to field biologists and molecular epidemiologists who are interested in understanding the origin, distribution and spread of ranaviruses throughout the world.

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