Detection of channel catfish virus DNA in latently infected catfish

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Channel catfish virus (CCV) disease is an acute haemorrhagic disease in juvenile channel catfish (*Ictalurus punctatus*). While fish that survive primary CCV infection are suspected of being carriers of CCV, little is known concerning CCV latency. In this report, fingerling catfish were infected with CCV by experimental immersion challenge. Infected fish displayed clinical signs of CCV disease, but 22% of infected fish survived the acute disease. At 140 days post-infection, PCR analysis detected CCV DNA in the blood, brain, intestines, kidney, liver and peripheral blood leukocytes of latently infected fish. Further analysis indicated the CCV genome may exist as circular or concatemeric DNA during virus latency. This study, employing an experimental model of CCV disease, confirms that CCV establishes a latent infection of channel catfish.

Introduction

Channel catfish virus (CCV) causes an acute haemorrhagic disease in channel catfish (*Ictalurus punctatus*) fry and fingerlings (Buck, 1990; Plumb, 1977). Clinical signs of CCV disease include erratic swimming, exopthalmia, distended abdomen and haemorrhage at the fin bases. Histopathological examination reveals necrosis and extensive haemorrhage in vital organs, especially in the liver, kidney and gastrointestinal tract. Epizootics usually involve high mortality and occur sporadically on commercial fish farms in the southern United States during the summer months.

CCV DNA and antibodies have been detected in asymptomatic adult catfish, suggesting that CCV establishes a latent infection in catfish that survive primary disease (Wise *et al.*, 1985; Amend & McDowell, 1984). Reactivation of latent CCV may result in shedding and transmission of infectious virus, leading to disease in susceptible catfish, particularly juvenile catfish. The conditions responsible for virus reactivation are unknown, although stressful conditions including elevated water temperatures ($> 27\ °C$) are associated with CCV outbreaks.

The molecular basis of CCV latency and reactivation is not understood. The DNA sequence of the entire CCV genome has been determined and the 134·5 kbp CCV DNA consists of a 97 kbp unique long component (UL) encoding 65 open reading frames (orfs) bracketed by 18·5 kbp left and right direct repeats (DR_L and DR_R), each encoding 14 orfs (Fig. 1A) (Davison, 1992). Knowledge of the molecular properties of the CCV genome offers opportunities to utilize molecular approaches to investigate CCV pathogenesis.

We recently reported a specific and sensitive PCR based assay for detection of CCV DNA in tissues of acutely infected catfish (Gray *et al.*, 1999). In this study, we utilize an
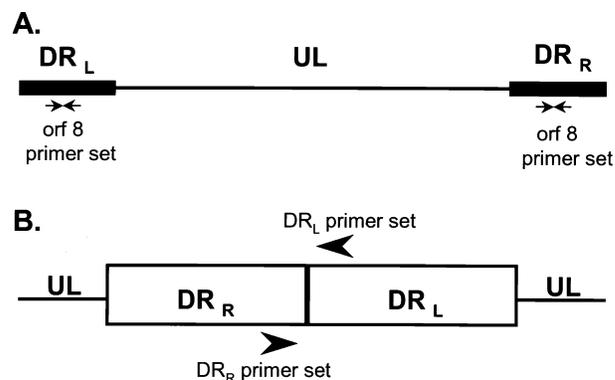
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Fig. 1. The CCV genome. (A) CCV DNA is 134·5 kbp in size and consists of a 97 kbp unique long (UL) region bracketed by 18·5 kbp left and right direct repeats (DR_L and DR_R). The approximate position of the orf 8 primers used in PCR is indicated. (B) Representation of the DR_R–DR_L junction in circular or concatemeric CCV DNA. The approximate location of the PCR primers used to amplify DR_R–DR_L junction DNA is shown.

experimental model for CCV disease and PCR analysis to detect CCV DNA in tissues of catfish which survive primary disease. The results confirm that CCV establishes latent infection in catfish.

Methods

■ **Viruses and cells.** The Auburn strain of CCV (ATCC VR-665) was propagated in Brown Bullhead (BB, ATCC CCL 59) cells cultured in Eagle's minimal essential medium supplemented with 10% foetal bovine serum, penicillin (100 µg/ml) and streptomycin (100 µg/ml).

■ **Experimental CCV infection of fish and preparation of clinical samples.** Experimental infection of fingerling channel catfish (5–7 cm) was done at the National Aquaculture Research Center in Stuttgart, AR, USA. Groups of 15–20 fish were either immersion-infected with 1×10^7 or 1×10^6 p.f.u. CCV or mock-infected in 400 ml aerated water at 28 °C for 30 min (Kaneharla & Hanson, 1996). After infection the fish were maintained in 30 gallon aerated tanks at 28 °C. Fish were observed on a daily basis, mortalities recorded and dead fish removed. On day 140 post-infection, surviving fish were sacrificed with 500 mg/l 3-aminobenzoic acid ethyl ester. Peripheral blood and liver, kidney, intestine and brain tissues were collected from infected and mock-infected fish. Leukocytes were isolated from whole blood of latently infected fish as previously described (Bowser et al., 1985). To detect the presence of infectious virus, tissues were homogenized and centrifuged, and virus was quantified by plaque assay on BB cells (Kancharla & Hanson, 1996).

■ **DNA isolation and PCR conditions.** Total cell DNA was isolated from infected and mock-infected BB cells and catfish tissues using a commercial genomic DNA isolation kit (Promega). Sequences of PCR primers were derived from published sequences of CCV orf 8 (Davison, 1992). CCV orf 8 oligonucleotides 5′ ACGTGTATCACGGTCTCACT [CCV genome nucleotides (nt) 12538–12557] and 5′ TTCGAGAAT-CGGGTCTCTGT (nt 12853–12872) were designed to amplify a 335 bp PCR product. For nested set PCR, the internal orf 8 primers 5′ TTCTTCCTCCTCGTCTCTTC (nt 12645–12664) and 5′ AGAACCT-CGGGAGTAGAGCC (nt 12820–12801) were used to generate a 157 bp PCR product.

Nested set PCR assays to detect circular or concatemeric CCV DNA utilized primer sets derived from the CCV DNA DR_R and DR_L (Fig. 1B). The external primers 5′ GGTTCATCCCTCTGAGCCAA (nt 18314–18333) and 5′ GTTCATTCTGACCAGATAGG (nt 422–403) were used in the initial PCR followed by reactions employing the internal primers 5′ AATGTACGTACACCCAGTCC (nt 18353–18372) and 5′ TAACC-TAATTGACCAAGCGG (nt 375–356), which were designed to amply a 579 bp PCR product.

The PCR conditions included 100–500 ng template DNA, 0·25 µg each primer, 10 µM each dNTP, 60 mM Tris–HCl (pH 8·3–10·0), 15 mM $(NH_4)_2SO_4$, 2·0 mM $MgCl_2$ and 1·0 unit Taq DNA polymerase. Fifty amplification cycles were performed, each cycle consisting of a denaturation step at 94 °C for 1 min, annealing at 55 °C for 2 min, extension at 72 °C for 3 min and a final extension at 72 °C for 7 min. For nested set assays, 5 µl of the initial PCR was used as the DNA template for the second PCR which included 30 cycles under the same PCR conditions described above.

Amplification products were analysed by electrophoresis through 1·5% agarose gels and ethidium bromide staining. In some cases, the DNA was transferred to nitrocellulose filters and detected by hybridization with radiolabelled probes and autoradiography as previously described (Gray & Oakes, 1984).

■ **Cloning and DNA sequence analysis of PCR products.** Amplified DNA products of the circular or concatemeric DNA nested set

PCR were ligated into the plasmid pGemT-Easy (Promega). The ligated products were cloned into E. coli JM109 and plasmid DNA was purified. Dideoxy-chain termination sequencing was performed using a Perkin Elmer ABI model 377 DNA Sequencer and Di-terminator FSTaq chemistry. DNA sequence data were analysed using the Sequence Analysis Software Package of the Genetics Computer Group (GCG), University of Wisconsin (Devereux et al., 1984).

Results

Experimental CCV infection of fingerling catfish

Fingerling catfish (5–7 cm) were immersion-challenged in 400 ml water containing infectious CCV (1×10^7 or 1×10^6 p.f.u.) or were mock-infected for 30 min and then maintained in aerated tanks at 28 °C: 73% of the fish infected with 1×10^6 p.f.u. and 82% of the fish infected with 1×10^7 p.f.u. died between days three and ten post-infection (data not shown). Acutely infected fish exhibited signs of CCV disease including petechial haemorrhage in the fin bases and internal organs and necrosis of infected kidney and liver tissues upon histological analysis (data not shown). In addition, CCV DNA was detected in acutely infected tissues by in situ hybridization using specific CCV DNA probes (data not shown). Approximately 22% of the infected catfish survived the primary CCV disease.

Blood, brain, gastrointestinal, kidney and liver tissues were collected from acutely infected catfish on day three post-infection. In addition, the same tissues were collected on day 140 post-infection from mock-infected fish and from fish which survived the acute disease. The latter fish exhibited no signs of CCV disease. Infectious virus could be detected in tissues of acutely infected fish (day three post-infection), but not in tissues of surviving fish at day 140 post-infection (data not shown).

Detection of CCV DNA in latently infected tissues

Total cell DNA was isolated from the tissues of individual CCV-infected or mock-infected fish and from CCV-infected BB cells. The DNA was used as template for PCR assays employing CCV orf 8 primers. The CCV orf 8 is a 699 bp gene located within each of the CCV DR sequences (nt 12462–13160 and nt 128132–128830) (Fig. 1 A).

Initial experiments were designed to detect CCV DNA in blood of acutely and latently infected catfish (Fig. 2). The CCV orf 8 PCR amplified the expected 335 bp DNA product from CCV-infected BB cells (lane 1), from the blood of two acutely infected catfish (lanes 2 and 3), and from the blood of two fish which survived primary CCV disease (lanes 4 and 5), but not from the blood of two mock-infected fish (lanes 6 and 7). The results demonstrate that CCV DNA can be detected in blood of latently infected catfish.

Nested set PCR assay to detect CCV DNA in latently infected fish

The standard CCV orf 8 PCR is sensitive enough to detect ≈ 350 copies of CCV DNA within a background of 1 µg
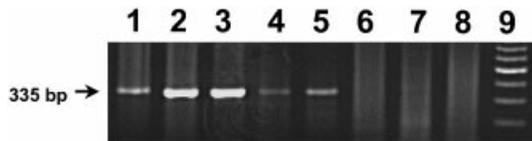
Fig. 2. PCR detection of CCV DNA in latently infected catfish. Reactions included the CCV orf 8 primer sets with 500 ng DNA template derived from CCV-infected BB cells (lane 1), blood from acutely infected catfish (lanes 2 and 3), blood from latently infected catfish (lanes 4 and 5), blood from mock-infected catfish (lanes 6 and 7). No DNA control (lane 8); 100 bp molecular mass standards (lane 9). PCR products and markers were analysed by agarose gel electrophoresis and ethidium bromide staining.
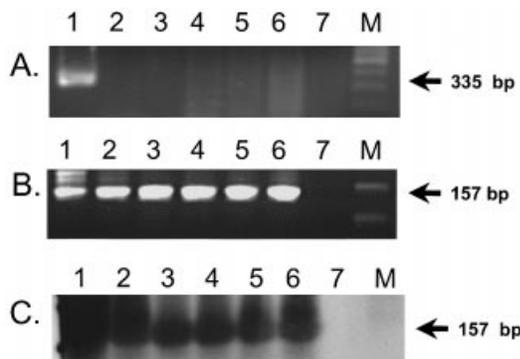

Fig. 3. Standard and nested set PCR detection of CCV DNA in latently infected catfish. (A) Standard orf 8 PCR. Reactions included the CCV orf 8 primer sets with 500 ng DNA template derived from CCV-infected BB cells (lane 1), or blood (lane 2), brain (lane 3), gastrointestinal tract (lane 4), kidney (lane 5) and liver (lane 6) of a latently infected fish, or mock-infected BB cells (lane 7). M, 100 bp molecular mass markers. PCR products and markers were analysed by agarose gel electrophoresis and ethidium bromide staining. (B) Nested set orf 8 PCR including the internal primer set and 5 µl initial PCR reaction. (C) Analysis of PCR products by Southern blot hybridization. Amplified DNA products were transferred to a nitrocellulose filter and hybridized to radiolabelled CCV orf 8 probe. Viral DNA was detected by autoradiography.
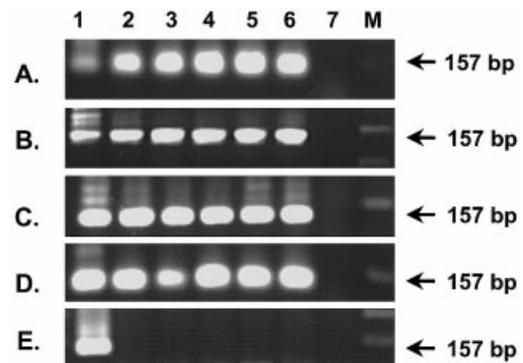

Fig. 4. Nested set PCR detection of CCV DNA in tissues of individual latently infected catfish. (A)–(D) Reactions included the CCV orf 8 primer sets with 500 ng DNA template derived from CCV-infected BB cells (lane 1), or blood (lane 2), brain (lane 3), gastrointestinal tract (lane 4), kidney (lane 5) and liver (lane 6) of each of four individual latently infected fish, or from mock-infected BB cells (lane 7). M, l00 bp molecular mass markers. (E) Corresponding samples from a mock-infected catfish. PCR products and markers were analysed by agarose gel electrophoresis and ethidium bromide staining.
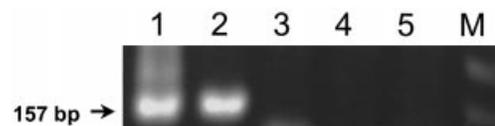

Fig. 5. Nested set PCR detection of CCV DNA in leukocytes of latently infected catfish. Reactions included the CCV orf 8 primer sets with 500 ng DNA template derived from CCV-infected BB cells (lane 1), from leukocytes pooled from two latently infected fish (lane 2), from leukocytes derived from two mock-infected fish (lanes 3 and 4) or from mock-infected BB cells (lane 5). M, 100 bp molecular mass markers. PCR products and markers were analysed by agarose gel electrophoresis and ethidium bromide staining.

catfish DNA (the amount of catfish DNA in ≈ 55 000 cells) (Gray et al., 1999). However, the orf 8 PCR assay could only detect CCV DNA in tissues of 50 % (three of six tested) of fish that survived primary CCV infection and were suspected of being latently infected. Fig. 3 (A) illustrates an example where the standard CCV 8 PCR assay amplified the 335 bp product from CCV-infected BB cell DNA (lane 1), but did not detect CCV DNA in tissues of a fish presumed to be latently infected (lanes 2–7).

A nested set orf 8 PCR, employing orf 8 primers internal to the first set of orf 8 primers, was developed as a more sensitive method to detect CCV DNA in tissues of latently infected catfish. The nested set orf 8 PCR assay amplified the expected 157 bp DNA from CCV-infected BB cell DNA (Fig. 3 B, lane 1), and from DNA derived from the blood (lane 2), brain (lane 3), gastrointestinal tract (lane 4), kidney (lane 5) and liver (lane 6) of a latently infected fish. Southern blot hybridization using a specific radiolabelled CCV orf 8 DNA probe confirmed that the amplified DNA products were authentic (Fig. 3 C, lanes 1–6).

In order to confirm the results, tissues were collected from four additional individual latently infected fish and from three individual mock-infected fish. Fig. 4 (A–D) demonstrates that the CCV orf 8 nested set PCR assay detected CCV DNA in the blood (lane 2), brain (lane 3), gastrointestinal tract (lane 4), kidney (lane 5) and liver (lane 6) of each of the four latently infected fish. Fig. 4 (E) demonstrates that CCV DNA was not detected in tissues of one of the mock-infected fish. Tissues derived from the two other mock-infected fish were also negative for CCV DNA (data not shown).

Detection of CCV DNA in peripheral blood leukocytes of latently infected fish

The finding that CCV DNA was present in the blood and several other tissues of latently infected fish suggested that CCV may establish latent infection of the fish leukocytes. Therefore, total cell DNA was isolated from purified peripheral blood leukocytes and the nested set orf 8 PCR was used to detect CCV DNA. The results indicated that CCV DNA was present in pooled leukocytes derived from two latently infected fish (Fig. 5, lane 2), but not in leukocytes derived from two individual mock-infected fish (lanes 3 and 4).
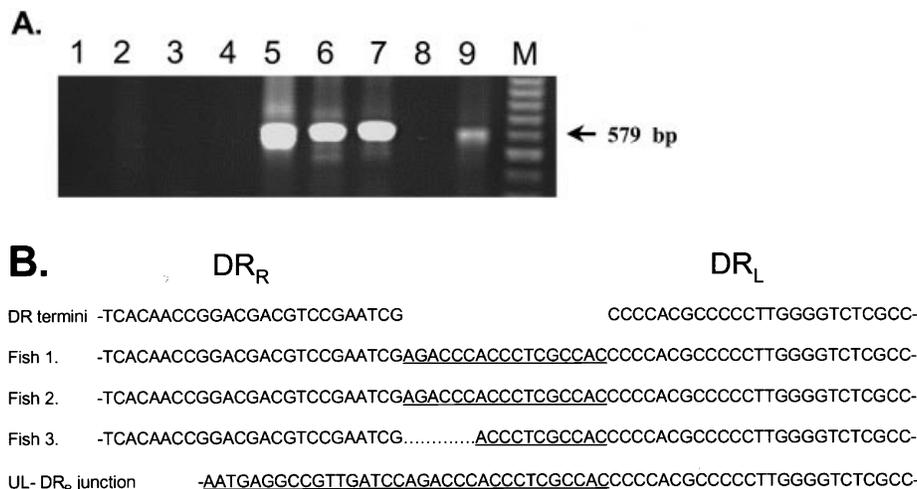
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Fig. 6. Nested set PCR to detect circular or concatemeric DNA in blood of latently infected catfish. (A) Reactions included the CCV DR_R and DR_L primer sets (Fig. 1B) with 500 ng DNA template derived from mock-infected BB cells (lane 1), from blood derived from three individual mock-infected fish (lanes 2–4), from blood of four individual latently infected fish (lanes 5–8) or from CCV-infected BB cells (lane 9). M, 100 bp molecular mass markers. PCR products and markers were analysed by agarose gel electrophoresis and ethidium bromide staining. (B) DNA sequence of PCR products amplified from blood of three latently infected fish. The DNA sequence of the DR_R and DR_L termini and also the UL–DR_R junction of genomic CCV DNA are from Davison (1992). Fish 1, 2 and 3, sequence of the DR_R–DR_L junction in PCR amplified DNA derived from blood of three individual latently infected catfish.

Circular/concatemeric CCV DNA in blood of latently infected fish

A study was undertaken to determine whether the CCV genome can exist in a circular or concatemeric form in latently infected fish. Nested set PCR primers were designed so that amplified products could only be derived from circularized CCV DNA or adjacent genomes arranged in head to tail (DR_R to DR_L) concatemeric intermediates (Fig. 1B). PCR products, approximately the same size as the expected 579 bp DNA, were amplified from blood of three of four latently infected fish (Fig. 6A, lanes 5–8), and from CCV-infected BB cells (lane 9). However, PCR products were not amplified from blood derived from three mock-infected fish (lanes 2–4) or from mock-infected BB cells (lane 1). This result confirms that CCV DNA persists in blood of latently infected fish and indicates that the latent CCV DNA may exist in a circular or concatemeric form.

The amplified DNA products were cloned into plasmid vectors and the DNA sequence was determined (Fig. 6B). The results confirmed that the PCR products amplified from DNA derived from latently infected catfish do indeed contain DR_R-to-DR_L sequences arranged in a head-to-tail fashion. Precisely at the DR_R–DR_L junction each of the cloned PCR products contained an additional 11 or 17 bp. Analysis of the DNA sequence indicated that the additional base pairs were derived from UL sequences at the UL–DR_R junction of the CCV genome. DNA derived from latent fish #1 and #2 contained 17 bp derived from UL sequences (nt 115554–115670) and the DNA derived from latent fish #3 included 11 bp derived from

UL sequences (nt 15660–115670). Analysis of additional DNA clones derived from the three latently infected fish revealed similar results, although the precise number of UL sequences varied somewhat from clone to clone. The unusual nature of these junctional clones is not understood. However, previous studies investigating the DNA structure of human herpesvirus type 6 (HHV-6) in infected cells also describe aberrant junctional clones generated by PCR amplification of the HHV-6 DR_R-to-DR_L junction (Gompels & Macaulay, 1995; Thomson et al., 1994). The studies offer no explanation for the anomalous junctions other than attributing them to possible PCR artefacts. However, it is of interest that HHV-6 and CCV DNAs have similar structures, suggesting that genomic structure may be related to the altered DR_R-to-DR_L junction products generated by PCR.

Discussion

This is the first study to utilize an experimental model to confirm that CCV establishes latent infection in channel catfish. A CCV immersion infection model was utilized to induce channel catfish disease in fingerling catfish. The presence of CCV DNA in specific tissues of latently infected catfish which survived primary CCV infection was detected by PCR analysis.

These results provide support for previous studies reporting detection of CCV DNA in catfish suspected of harbouring latent virus. Wise et al. (1985) used DNA hybridization to detect CCV DNA in tissues of adult fish with no history of CCV infection and also from adult fish which had survived a CCV outbreak 4 years earlier. Boyle & Blackwell (1991) utilized PCR to detect CCV DNA in an undefined tissue

of a single catfish reported to be a latent carrier of CCV. Baek & Boyle (1996) used nested set PCR to detect CCV DNA in the blood of two of five healthy broodstock fish with no history of CCV infection. An advantage of the present study of CCV latency is that a specific catfish stock, confirmed to be CCV negative, was experimentally infected under controlled conditions permitting a defined clinical history of the latently infected fish.

CCV DNA was detected in the blood and several other tissues of latently infected catfish. Wise *et al.* (1985) also detected CCV DNA in a variety of tissues derived from asymptomatic catfish. The results suggest the possibility that CCV may establish latent infection in a common cell type such as the lymphocyte. Infectious CCV can be co-cultured from leukocytes of asymptomatic adult catfish which are immuno-suppressed with dexamethazone (Bowser *et al.*, 1985). In the present study, CCV DNA was detected in peripheral blood leukocytes isolated from latently infected catfish. Other herpesviruses including HHV-6, human cytomegalovirus and Epstein–Barr virus (EBV) also establish latent infection in leukocytes (Kondo & Mocarski, 1995; Kondo *et al.*, 1991; Kieff, 1996).

Initial experiments were done to investigate the structure of CCV DNA in latently infected fish. A previous report demonstrated that CCV DNA exists in an 'endless' (circular or concatemeric) configuration in acutely infected BB cells (Cebrian *et al.*, 1983). In the present study, we detected the presence of circular or concatemeric DNA in blood of latently infected fish by PCR utilizing DR_R-to-DR_L primer sets. The assay cannot differentiate between circular or concatemeric DNA forms. However, the circular DNA form may be favoured since concatemeric DNA would be expected to exist as a replicative intermediate during active viral DNA synthesis and not during virus latency. Other herpesviruses, including herpes simplex virus, varicella-zoster virus and EBV exist in a circular DNA form during virus latency (Meier & Straus, 1992; Kieff, 1996). The results of this study do not rule out the possibility that CCV DNA may also exist in a linear state or integrated into the cellular genome during virus latency.

Previous studies reporting CCV DNA or CCV antibodies in asymptomatic catfish suggest that CCV latency may be prevalent in catfish populations (Wise *et al.*, 1985; Plumb, 1978; Amend & McDowell, 1984). However, the incidence of latent CCV infection in wild and commercial catfish populations has not been sufficiently investigated. Serological assays, such as serum neutralization, may not be reliable for identifying latently infected fish in catfish populations (Amend & McDowell, 1983, 1984). The standard and nested set CCV PCR assays described in this and our previous study (Gray *et al.*, 1999) are specific and sensitive and will be useful in epidemiological studies to screen catfish populations for CCV DNA detection.

Experimental CCV infection of channel catfish offers a model to investigate the molecular basis of herpesvirus pathogenesis and latency. Bird *et al.* (1988) reported that CCV RNA may be detected in tissues of asymptomatic catfish suggesting that the CCV genome is transcriptionally active during virus latency. Further studies will investigate CCV gene expression in tissues of latently infected fish and will identify latency associated transcripts.

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