

Production and shedding of channel catfish virus (CCV) and thymidine kinase negative CCV in immersion exposed channel catfish fingerlings

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ABSTRACT: The progression of channel catfish virus (CCV) and thymidine kinase negative recombinant CCV (CCVTK⁻) infections in immersion challenged channel catfish *Ictalurus punctatus* fingerlings was studied by plaque assay and quantitative polymerase chain reaction (QT-PCR). Infectious CCV was isolated from Day 2 through Day 8 in skin and blood and through Day 10 PI in posterior kidney and gills. Infectious CCVTK⁻ was detected from Day 2 through Day 6 PI in skin and blood and through Day 8 PI in posterior kidney and gills. The kinetics of both infections were similar until Day 5 PI, after which virus concentrations were higher in CCV infected fish than in those exposed to CCVTK⁻. Both viruses were detected in water; however, the CCV concentration was higher than CCVTK⁻ during all time periods and was detected on Days 2 through 5 PI, whereas CCVTK⁻ was detected on Days 3 through 5 PI. The QT-PCR showed higher amounts of viral DNA in the skin and water at early and late periods in infection in CCV challenged groups as opposed to CCVTK⁻ groups. This study indicates that the kinetics of CCV and CCVTK⁻ infections are similar but CCVTK⁻ infections do not persist as long and have reduced shedding ability.

KEY WORDS: *Ictalurid herpesvirus 1* · Quantitative PCR · Thymidine kinase · Vaccines · *Ictalurus punctatus*

INTRODUCTION

Channel catfish virus disease (CCVD) is an acute hemorrhagic infectious disease of catfish *Ictalurus punctatus* fingerlings caused by a herpesvirus with biological characteristics similar to α herpesviruses. During acute CCVD, mortality can be as high as 95%, especially when the fish are exposed to unfavorable environmental conditions (Plumb 1978). Research has demonstrated the potential for vaccination against CCVD but the vaccines have not been well characterized or marketed (Noga & Hartmann 1981, Awad et al. 1989). Recently Zhang & Hanson (1995) constructed a thymidine kinase (TK)-gene-deleted CCV (CCVTK⁻) and showed that this recombinant was less virulent to catfish fingerlings and induced protective immunity

against CCVD. They found that CCVTK⁻ was isolated from posterior kidney for a shorter duration than CCV but in-depth kinetic studies were not performed. In fact, little research has been reported concerning mechanisms of transmission and kinetics of CCV infection. Initial research on kinetics of CCV infection was performed by Plumb (1971) and followed up by Plumb & Gaines (1975) but these studies used intraperitoneally administered virus and shedding was not quantified. Nusbaum & Grizzle (1987) used a radioisotopic tracer methodology to trace the spread of CCV genomes through the catfish host after immersion challenge and implicated the gills as the site of viral entry. To further elucidate the mechanisms involved in the CCVTK⁻ attenuation and relate this to transmission and pathogenesis of CCV, we quantitatively evaluated the progression of CCV and CCVTK⁻ dissemination within the channel catfish fingerling host and the subsequent shedding of virus into the tank water after immersion challenges.

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MATERIALS AND METHODS

Viruses, cells and fish. Channel catfish virus (Auburn clone A) and CCVTK⁻ were propagated in cells of the channel catfish ovary line (CCO) cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 25 mM HEPES, 100 IU ml⁻¹ of penicillin and 100 µg ml⁻¹ of streptomycin. Virus stocks were stored at -70°C until used. Infectious virus were quantified by plaque assay on CCO cells using the method of Buck & Loh (1985). The CCV DNA was extracted from infected CCO cells and quantified as described by Hanson et al. (1994). The 2 groups of channel catfish fingerlings used in these experiments were hatched from single egg masses obtained from stocks with no known history of CCVD and maintained in a flow-through limited-access indoor rearing facility until transferred to experimental tanks. Mississippi normal strain catfish were a gift from William Wolters (USDA-ARS Catfish Genetics Laboratory, Stoneville, MS, USA). MSU-2 strain catfish, obtained as an egg mass from the Mississippi Agricultural and Forestry Experiment Station (Starkville, MS), were hatched and reared in-house.

Virus challenge. Preliminary experiment (Experiment 1): Mississippi normal strain fingerlings (5 to 7 cm) were randomly distributed into 22 groups of 15 fish. Ten groups each were exposed to 250 plaque-forming units (PFU) ml⁻¹ of either CCV or CCVTK⁻ in 600 ml of aerated water for 30 min at 28°C. Two groups of fish were mock infected with DMEM containing water as non-infected controls. After challenge the fish were rinsed in 500 ml of water and transferred to individual polypropylene tanks containing 15 l of dechlorinated municipal tap water supplemented with 100 ppm sodium chloride with continuous aeration. Fish were fed commercial flake food (Tetra min) once a day and the temperature of the room was maintained at 30°C (28°C water temperature). Half of the tanks (n = 5 per treatment) were used for the viral shedding experiment and the other half for virus dissemination assays. The water level in shedding experiment tanks was adjusted throughout the experiment to maintain 1 l fish⁻¹. One fish per replicate (n = 5 per treatment) was sampled at Days 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12 post infection (PI) and euthanized by an overdose of tricaine methane sulfonate (MS 222). Skin scrapings, gills, posterior kidney, liver, spleen and intestine were collected, suspended in 200 µl of DMEM containing 500 IU ml⁻¹ of penicillin and 500 µg ml⁻¹ of streptomycin and frozen at -70°C until processed for virus isolation. Forty ml of water from each of the viral shedding experiment tanks was collected at Days 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10 and 12 PI and centrifuged at 8000 × g for 10 min at 4°C. Supernatants were collected and

centrifuged at 30 000 × g for 2 h at 4°C to pellet the virus. The pellet was resuspended in 500 µl of DMEM containing 500 IU ml⁻¹ of penicillin and 500 µg ml⁻¹ of streptomycin and infectious virus quantified by plaque assay and viral DNA quantified by QT-PCR.

Tissue samples were thawed, weighed, homogenized using an Omni 2000 homogenizer (Omni International Inc., Gainesville, VA, USA) and centrifuged at 10 000 × g for 5 min. The infectious virus and viral DNA in the supernatant were quantified by plaque assay and QT-PCR, respectively. Between the homogenization of tissue samples, the tip of the homogenizer was rinsed serially in 1 M HCl, water, 1 M NaOH, water and DMEM to prevent cross contamination with viral DNA. The efficacy of this procedure was evaluated by homogenizing DMEM containing 1 × 10⁵ PFU ml⁻¹ of CCV. The homogenizer was then decontaminated by the above procedure and 25 µl of DMEM from the final step was tested for the presence of viral DNA by PCR using primers RO1 and RO3 (Table 1). Statistical comparisons of plaque assay and QT-PCR results were performed using the analysis of variance general linear models procedure (SAS Institute, Raleigh, NC, USA). The assay sensitivity limit, given the weight of tissue tested, was used as the value for statistical comparisons involving treatment groups that had a mean value of 0.

Experiment 2: MSU-2 strain channel catfish fingerlings (7 to 10 cm in length) were distributed into 81 groups of 6 individuals. Thirty-six groups were exposed to either CCV or CCVTK⁻ and 8 groups of control fish were mock infected with DMEM as described above. Tanks were aerated and nitrogenous waste metabolized using aquarium box type biological filters that were preconditioned in a tank using biological supplement (Cycle, Mansfield, MA, USA) and fed ammonium chloride daily to maintain a concentration of 10 ppm for 4 wk. Fish were sampled on Days 1–6, 8, 10 and 12 PI. All the fish from 4 tanks of both virus challenge groups and from 1 control tank at every sampling period were euthanized. Four fish from each tank were processed; skin, gills, and posterior kidney samples were taken and blood was collected from the caudal vein from the remaining 2 fish in each tank. Skin scrapings, gills, posterior kidney, and blood samples were suspended in 200 µl of DMEM containing 500 IU ml⁻¹ of penicillin and 500 µg ml⁻¹ of streptomycin and frozen at -70°C until processed for virus isolation as described above.

The viral shedding was evaluated by challenging 5 groups of 15 fish that were held as described above. Two hundred and fifty ml of water was collected from each of 5 tanks per treatment at Days 0.5, 1–8, 10 and 12 PI and centrifuged at 10 000 × g for 10 min at 4°C. Two hundred ml of supernatant was centrifuged at

24 000 × *g* for 150 min to pellet the virus. The viral pellet was resuspended in 1 ml of 1× DMEM containing 500 IU ml⁻¹ of penicillin and 500 µg ml⁻¹ of streptomycin and quantified by plaque assay.

During the experimental period, pH and un-ionized ammonia levels of tank water were monitored daily. If un-ionized ammonia concentrations exceeded 0.1 ppm, two-thirds of the water was replaced with fresh dechlorinated water of the same temperature.

Production of an internal standard for QT-PCR. Most oligonucleotides used in this research were custom synthesized (National Biosciences Inc., Plymouth, MN, USA) (Table 1). Non-biotinylated RO1 and RO3 used in initial cloning work were a gift from John A. Boyle (Department of Biochemistry and Molecular Biology, Mississippi State University).

A PCR target sequence described by Boyle & Blackwell (1991) was used in quantitation. In this QT-PCR procedure, an internal standard (IS) of different size containing identical priming ends as that of target DNA but with a foreign internal sequence was generated. This internal standard was designed such that its amplification product was 15 base pairs (bp) larger than the product from CCV DNA and both the IS and CCV PCR product contained unique unshared sequences. Coamplification of IS and CCV DNA in samples, followed by ELISA based differential quantification of the products, accounted for variations in PCR conditions and sample processing (Lehtovaara et al. 1993). A 152 bp region of CCV genome was amplified using primers RO1 and RO3 (Boyle & Blackwell 1991). PCR was carried out in 50 µl of reaction mixture containing 10× buffer, magnesium chloride (25 mM), 1.5 units of *Taq* DNA polymerase (Promega Corp., Madison, WI, USA), 200 µM of each dNTP, 40 pmol of each primer and 1 pg of purified CCV DNA. Amplification was carried out in a thermal cycler (Precision Scientific Inc., Chicago, IL, USA) for 35 cycles, each consisting of 92°C for 30 s denaturation, 57°C for 30 s annealing and 72°C for 60 s extension. The amplified PCR product was purified; its termini were polished using T₄ DNA polymerase (Sambrook et al. 1989); and it was cloned into the *Hinc* II site of the plasmid, pBlue-script SK⁻ (pBS SK⁻). The ligated plasmid was transformed into the XL-blue strain of *Escherichia coli* (Stratagene, La Jolla, CA, USA) as described by Sambrook et al. (1989). The recombinant plasmid DNA

Table 1 Sequence of oligonucleotides used in generation of internal standard and in quantitative PCR

Oligonucleotide	Sequence
RO1	GCCAAGATCGCGGAGAACG
RO3	CCTCCTCGTCATCATCACC
RO3 ^B	^B TCTCCTCGTCATCATCACC
SK 821	CAGCTATGACCATGATTACG
SK 616	TGAGCGCGGTAATACGACT
RO4 ^F	^F AACATACGGATCGGTCCG
SK 596 ^F	^F AGTCGTATTACGCGCGCT

^B indicates 5' biotinylation, ^F indicates 5' fluoresceination

was purified using a prep-A-gene kit (Bio Rad, Melville, NY, USA), digested with *Mlu* I (a unique site present only in the CCV insert), and then the recessed 3' termini were filled using Klenow fragment of *E. coli* DNA polymerase I (Promega Corp., Madison, WI). Then the product was digested with *Sal* I to delete a

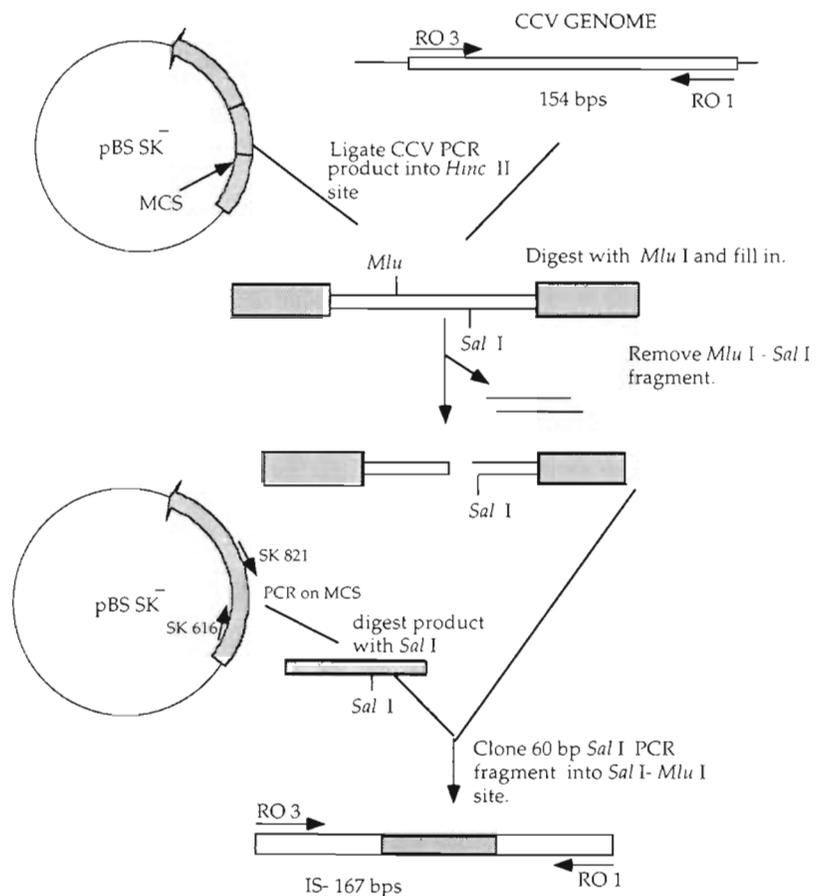


Fig. 1. Schematic diagram of the method used to generate internal standard for quantitative PCR. The resultant product was a cloned target sequence for CCV specific primers RO1 and RO3 in which 45 bp of CCV sequence was replaced by a 60 bp fragment of plasmid DNA, allowing size and sequence specific probe differentiation of CCV and IS PCR products. MCS: multiple cloning site

45 bp fragment from the insert (Fig 1). A fragment of pBS SK⁻ consisting of the multiple cloning site was amplified using flanking primers, SK 821 and SK 616 (Table 1) to obtain a 205 bp PCR product using conditions similar to that used to amplify the CCV fragment. This product was purified, polished and digested with *Sal*I to get a short (60 bp) and a large (145 bp) fragment. The 60 bp fragment was ligated into *Sal*I-*Mlu*I digested plasmid and transformed into XL-blue *E. coli* cells, and positive clones were identified by PCR using primers RO1 and RO3 and confirmed by restriction digestion with *Xba*I and *Xho*I. The recombinant plasmid was linearized by digesting with *Hind*III, purified with a prep-A-gene kit, quantified spectrophotometrically and stored at -20°C until used.

Quantitative PCR. Generation of a standard curve:

A constant number of internal standard molecules were coamplified with increasing levels of CCV DNA molecules in 50 µl reactions as described above except the reaction mixture contained 100 µM of dNTPs and 20 pmol of each primer, RO1 and RO3^B. The DNA samples were denatured at 95 to 100°C for 10 min and immediately placed on ice before adding to the reaction mixture at 57°C. The PCR was carried out for 35 cycles, as described above.

The differential amplification of IS and CCV specific product was determined using ELISA mediated quantification of IS specific and CCV specific oligonucleotide probe hybridization as described by Lehtovaara et al. (1993). Ten µl of each PCR product was diluted in 190 µl of 5 mM Tris-HCl-0.1 mM EDTA buffer (pH 7.8) and transferred equally to 2 parallel wells in a pre-washed, streptavidin coated microtiter plate (Dupont, Boston, MA) (washing buffer contained 25 mM Tris-HCl, pH 7.5, 125 mM NaCl, 2 mM MgCl₂ and 0.3% Tween-20). The plate was incubated at 22°C for 3 h with constant shaking to allow binding of biotinylated PCR product to streptavidin. The solution was poured off and 100 µl of 250 mM NaOH was added to each well and placed on the shaker for 15 min to denature the double stranded PCR product. The plate was then washed 3 times with NaOH and 5 times with washing buffer. Five pmol of fluorescein labeled internal probes (RO 4^F for the CCV product detection well and SK 596^F for the IS product detection well) in 100 µl of 5× saline salt citrate (5× SSC) was added to respective wells. Hybridization of probes to the single stranded PCR product was carried out at 37°C for 2 h with constant shaking. The plate was washed 8 times and 100 µl of 1:1000 dilution of fluorescein specific mouse monoclonal antibody conjugated to alkaline phosphatase (Sigma Chemicals, St. Louis, MO, USA) in antibody dilution buffer [25 mM HEPES, pH 7.5, 125 mM NaCl, 2 mM MgCl₂, 1% BSA (bovine serum albumin) and 0.3% Tween-20] was added to each well and incu-

bated with shaking at 22°C for 1 h. The plate was washed 8 times and 100 µl of 2.5 mg ml⁻¹ p-nitrophenyl phosphate in diethanolamine-MgCl₂ buffer (pH 9.6) was added. The development of color was read spectrophotometrically at 405 nm (Titertek Multiskan Plus, ICN Pharmaceuticals Inc., Costa Mesa, CA) at regular intervals over a 3 h period. A standard curve was constructed by plotting the ratio of CCV/IS values against the number of CCV DNA target molecules in the PCR amplification.

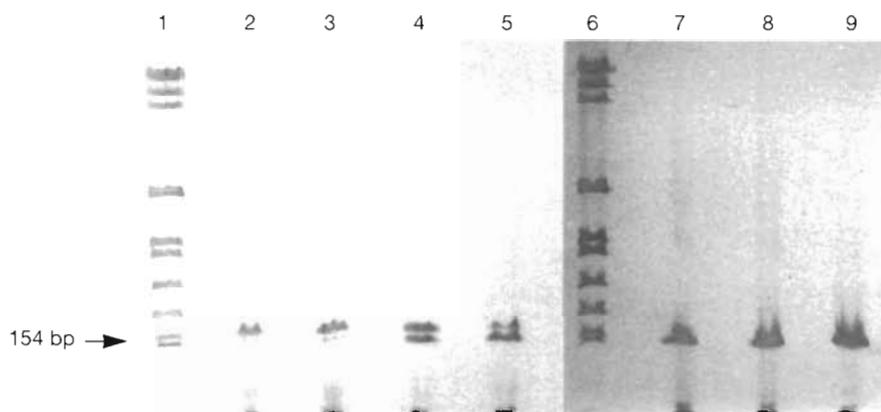
Application of QT-PCR to water and tissue samples:

Concentrated virus from water samples were subjected to QT-PCR. Ten µl of media containing concentrated virus was digested with 2 µl of 10 mg ml⁻¹ proteinase K (Sigma Chemicals, St. Louis, MO) at 55°C for 45 min and then denatured at 95 to 100°C for 10 min. A constant amount of internal standard was added to each sample before coamplification and then the signal ratio of CCV/IS was obtained as described above. The number of viral DNA molecules present in each sample was calculated from the standard curve using the formula, $Y = bX + a$; Y was the signal ratio (\log_{10}) of the sample, X was the number of molecules (\log_{10}) of CCV DNA (unknown), and a and b were constants for the standard curve. The number of molecules of viral DNA shed into the water per fish was calculated for each sample.

Supernatants from homogenates of skin, gills, posterior kidney and blood samples were evaluated by CCV specific QT-PCR as described for the water samples. Ten µl of skin sample supernatants was digested with 4 µl of proteinase K (10 mg ml⁻¹) at 55°C for 1 h and then 14 µl of 50% Chelex-100 (Bio Rad, Hercules, CA) was added to each sample and incubated at 55°C for 30 min. The samples were heated to 95 to 100°C for 10 min and centrifuged at 12 000 × g for 1 min and 14 µl of supernatants was subjected to QT-PCR. Ten µl of supernatants from homogenized gill and posterior kidney samples was digested with 4 µl of proteinase K (10 mg ml⁻¹) at 55°C for 1 h and then denatured at 95 to 100°C for 10 min. For blood samples, 10 µl of supernatants was digested with 6 µl of proteinase K (10 mg ml⁻¹) at 55°C for 1 h and then heat denatured at 100°C for 10 min, centrifuged at 12 000 × g for 2 min, and 14 µl of the supernatant was subjected to QT-PCR.

Trichloroacetic acid precipitation assays. Trichloroacetic acid (TCA) precipitation assays were performed on virus isolated from tissues of fish exposed to CCVTK⁻ and on virus isolated from tank water of the CCVTK⁻ challenge to detect the activity of viral thymidine kinase. The procedure was similar to that described by Piccardo et al. (1979). Briefly, CCO cells deficient in TK activity (CCOBr) (Hanson et al. 1994) were grown to confluency in 6 well plates. The cells were infected with virus samples and 10 µCi ml⁻¹ of

Fig. 2. Photograph of silver stained polyacrylamide electrophoretic gel showing PCR products resulting from the coamplification of a constant amount (10^4 molecules) of internal standard with 10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , and 10^7 molecules of CCV DNA in lanes 2, 3, 4, 5, 7, 8 and 9 respectively. Lanes 1 and 6 are size markers (1 kb ladder, BRL, Gaithersburg, MD, USA)



[³H]thymidine (Amersham, Arlington Heights, IL) was added at the same time. The cells were collected after appearance of plaques and were lysed with 1% sodium dodecyl sulfate. Nucleic acids were precipitated with 10% TCA. The precipitates were collected by filtration on glass-fiber filter papers after several washes with 10% TCA, resuspended in scintillation cocktail and counted using a scintillation counter.

RESULTS

Establishment of QT-PCR

The internal standard produced by deleting a 45 bp fragment from cloned CCV PCR product and inserting a 60 bp fragment from pBS SK⁻ multiple cloning site generated a 167 bp PCR product, 15 bp larger than the PCR product of CCV DNA. The deletion and insertion in the IS was confirmed by digestion with *Xho*I and *Xba*I, which yielded a 160 bp and a 85 bp fragment whereas digestion of negative control (pBS SK⁻) produced only 1 fragment of 60 bp.

Coamplification of a constant (10^3 or 10^4) number of IS molecules and a gradient (10^1 to 10^7) of CCV DNA molecules, using primers RO1 and RO3^B, produced increasing amounts of CCV products and decreasing amounts of IS products (Fig. 2). These results validate the competitive nature of coamplification wherein an excess amount of one template outcompetes the other for primers. The quantitation of PCR products by ELISA demonstrated that speed of development and intensity of color were relative to the amount of PCR product. The ratio of signals from CCV/IS remained stable for 2 to 3 h. The signal ratios were plotted against the number of CCV DNA molecules used in the PCR to produce standard curves with formulas of $Y = 0.359X - 1.234$ with $r = 0.994$ and $Y = 0.315X - 0.774$ with $r = 0.992$ when using 10^4 and 10^3 IS molecules respectively. The curves were linear for 5 orders of magnitude and the sensitivity of detection using 10^4 or

10^3 IS molecules was 1000 molecules (0.15 pg) and 100 molecules (0.015 pg) of CCV DNA, respectively. The signal obtained below the sensitivity level was equal to or less than that of negative controls.

Dissemination and shedding of CCV and CCVTK⁻

Preliminary experiment (Experiment 1)

The overall mean cumulative mortality in virus dissemination experiments was 37 and 16% in CCV and CCVTK⁻ challenges, respectively, and 54 and 41% in CCV and CCVTK⁻ challenges of the shedding experimental tanks, respectively. No deaths occurred in the control tanks and no infectious virus was isolated from sampled control fish. Infectious virus was first isolated in skin, gills, posterior kidney and intestines at Day 1 PI from fish challenged with CCV and by Day 2 PI it could be isolated from all the tissues (Fig. 3). In the fish challenged with CCVTK⁻, infectious virus was first isolated at Day 2 PI in all tissues. In general, virus reached peak levels in tissues by Day 3 in CCV challenged fish and by Day 4 in CCVTK⁻ challenged fish. Based on the amount of virus isolated, the posterior kidney was the principal target tissue followed by skin, gills, spleen, liver and intestine. This was also confirmed by QT-PCR on skin, gills, posterior kidney and water samples. The ratio of total DNA molecules over infectious virus was about 500 in most of the tissue samples. The procedure used for decontamination of the homogenizer between samples was effective in preventing cross contamination with viral DNA (data not shown).

Due to variations in virus levels per fish no consistent differences were noted in infectious virus levels in tissues in this experiment. However, early in the infection (Days 0.5 and 1) there were significantly higher levels of DNA in the skin in CCV infected fish than in CCVTK⁻ infected fish (Fig. 4). Also, only infectious CCV was isolated from water samples, on Days 2 to 4 PI, which corresponds to peak virus titers obtained

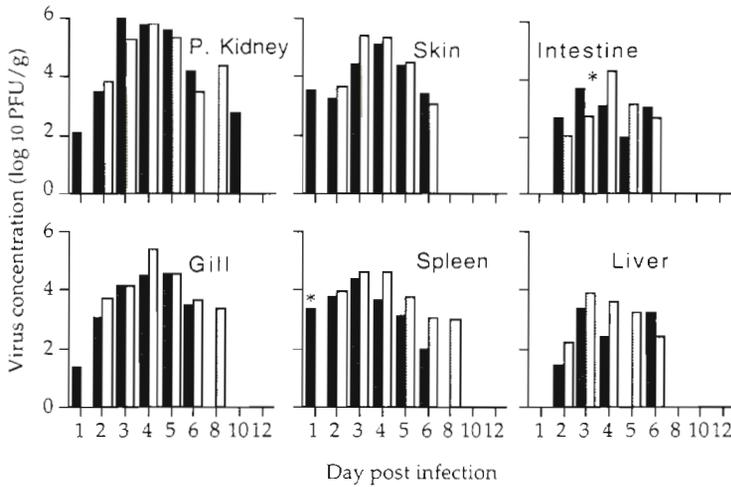


Fig. 3. Mean infectious virus concentrations isolated from posterior kidney, gills, skin, spleen, intestine and liver of CCV (dark bars) and CCVTK⁻ (light bars) challenged fish (*Ictalurus punctatus*) when sampled at various times PI in Experiment 1. An asterisk indicates that the values of the 2 treatments are significantly different (p < 0.05). Standard errors of least squared means for log transformed data were 0.41, 0.34, 0.35, 0.35, 0.27 and 0.26 for posterior kidney, gills, skin, spleen, intestine and liver, respectively (n = 5 observations per mean)

from tissue samples. The DNA of both CCV and CCVTK⁻ was detected in water throughout the experimental period; however, the amount of CCV DNA detected was significantly higher than CCVTK⁻ at Days 1, 3, 6, 8 and 12 (Fig. 5). When infectious virus could be detected, the number of genomes detected was about 10⁴ to 10⁵ times higher than the plaque forming units.

The un-ionized ammonia levels reached stressful levels (>0.1 ppm) in all tanks by Day 5 PI and exceeded 0.3 ppm by Day 8 PI. Two-thirds of the water in all the tanks was changed with fresh water at Day 8 and at Day 10 to reduce ammonia stress.

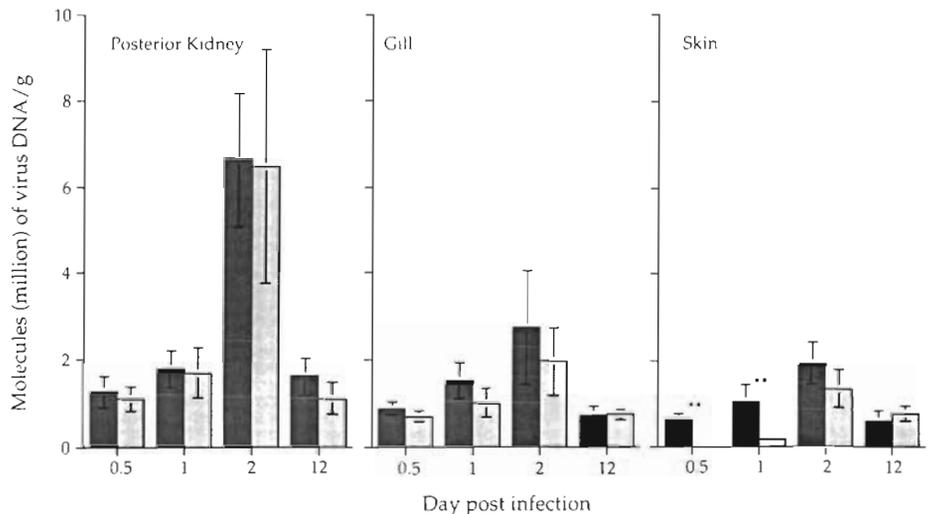


Fig. 4. Concentration of virus DNA (mean ± SD) in posterior kidney, gills and skin of CCV (dark bars) and CCVTK⁻ (light bars) challenged fish (*Ictalurus punctatus*) at early and late times PI in Experiment 1. A double asterisk indicates that the values are significantly different at p < 0.01

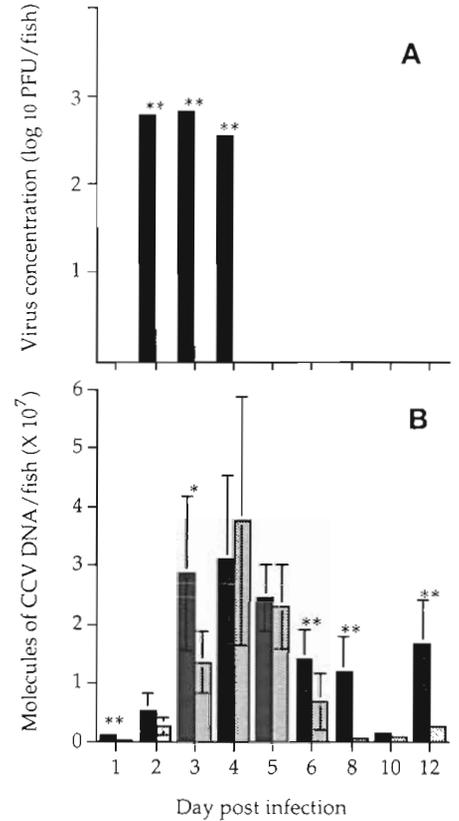


Fig. 5. Infectious virus (A) and viral DNA (B) levels detected in tank water of either CCV (dark bars) or CCVTK⁻ (light bars) challenged fish (*Ictalurus punctatus*) in Experiment 1. A single or double asterisk indicates that the values are significantly different at p < 0.05 and p < 0.01 respectively

The activity of thymidine kinase in virus isolated from fish exposed to CCVTK⁻ was tested by TCA precipitation assay. The results demonstrated detectable TK activity of many of the isolates, especially late in the infection (Table 2).

Table 2. Results of trichloroacetic acid precipitation assays on virus isolates from *Ictalurus punctatus* tissues and water samples of CCVTK⁻ challenged groups from Experiments 1 and 2. Values represent the number of TK positive isolates/ the number of isolates tested. A culture was considered TK positive if it incorporated more than 2-fold the amount of ³H-thymidine as did cultures infected with CCVTK⁻

Virus sample	Experiment 1	Experiment 2
Day 3	0/1	ND ^a
Day 4	1/4	0/2
Day 5	1/4	0/3
Day 6	4/4	0/3
Day 8	1/1	0/3
Day 4 water	ND	0/1
Day 5 water	ND	0/2

^aND: not done

Experiment 2

The experimental design was modified to improve on weaknesses noted in the preliminary trial. To reduce the variability in results caused by sampling bias, all the fish in each tank per replicate were taken. Because lower levels of virus were detected in spleen, liver, and intestine and no unusual trends in virus replication were noted in these organs, only posterior kidney, skin and gill tissues were taken. Also blood samples were evaluated for virus levels, to differentiate tissue-associated virus from that in circulation in the highly perfused organs. Biological filters were used to reduce the ammonia stress and a different stock of fish was used to reduce the chances of latent virus reactivation. In the shedding assay, an increased water sample volume was taken to improve sensitivity. The overall cumulative mortality in the virus dissemination experiments was 35, 10 and 0% in CCV, CCVTK⁻ and

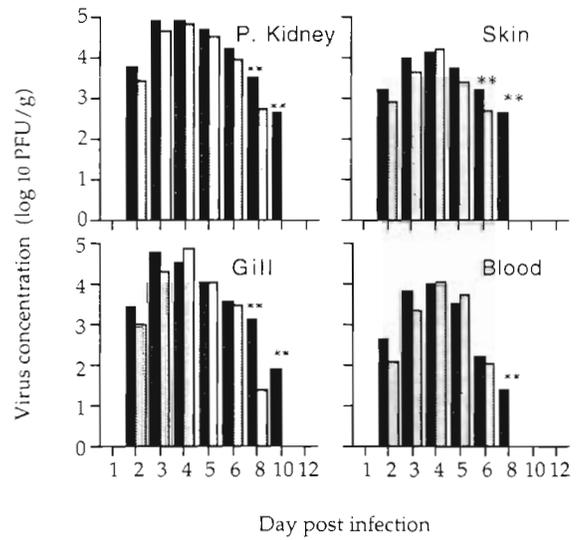
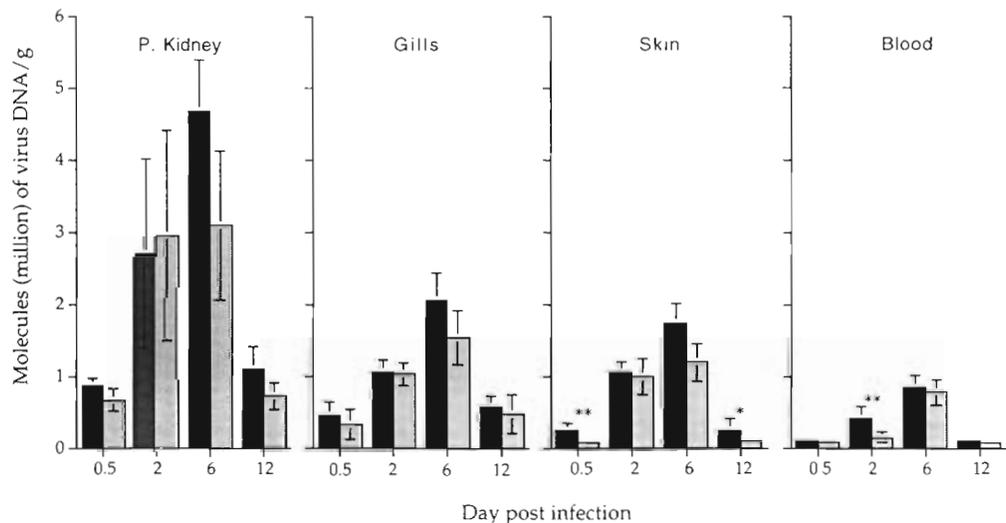


Fig. 6. Mean infectious virus concentrations isolated from posterior kidney, gills, skin, and blood of CCV (dark bars) and CCVTK⁻ (light bars) challenged fish (*Ictalurus punctatus*) when sampled at various times PI in Experiment 2. A double asterisk indicates that the values are significantly different at $p < 0.01$. The standard errors of least squared means for log transformed data were 0.16, 0.16, 0.13 and 0.17 for posterior kidney, gills, skin, and blood respectively ($n = 5$ pooled observations per mean)

mock challenges, respectively, and 42 and 15% in CCV and CCVTK⁻ challenges, respectively, in the shedding experiment. Both types of viruses were isolated from skin scrapings, gills, posterior kidney and blood samples beginning at Day 2 PI. Once again the posterior kidney demonstrated the highest amount of virus production of the organs assayed (Fig. 6). During the early infection, virus DNA levels in CCV infected fish were higher than in CCVTK⁻ infected fish (Fig. 7). Also, shedding assays indicated higher amounts of

Fig. 7. Concentration of virus DNA (mean ± SD) in posterior kidney, gills and skin of CCV (dark bars) and CCVTK⁻ (light bars) challenged fish (*Ictalurus punctatus*) at early and late times PI in Experiment 2. A single or double asterisk indicates that the values are significantly different at $p < 0.05$ and $p < 0.01$ respectively



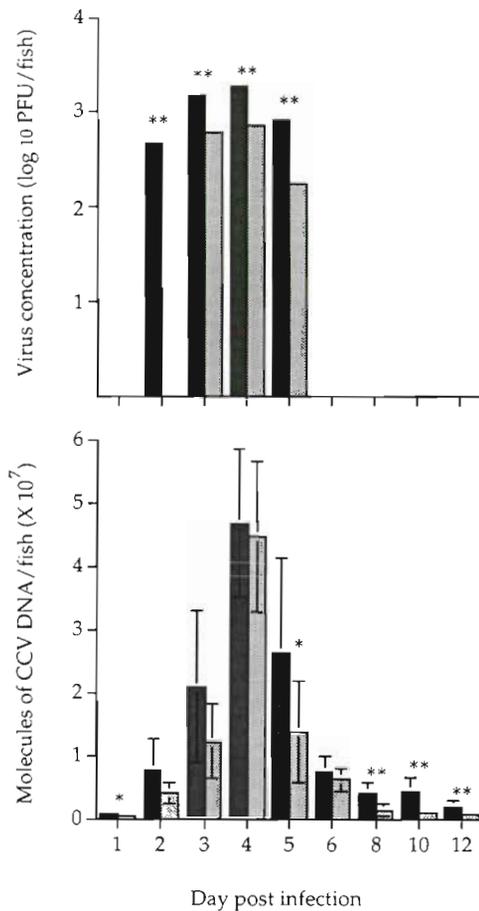


Fig. 8. Infectious virus (A) and viral DNA (B) levels detected in water of either CCV (dark bars) or CCVTK⁻ (light bars) challenged fish (*Ictalurus punctatus*) from Experiment 2. A single or double asterisk indicates that the values are significantly different at $p < 0.05$ and $p < 0.01$ respectively

infectious virus and virus DNA in CCV infected tanks than in CCVTK⁻ tanks (Fig. 8). During later stages of the infections, significantly higher amounts of infectious virus were found in tissues of CCV infected fish than in fish infected with CCVTK⁻. No infectious virus was isolated from the sampled control fish.

TCA assays revealed no apparent increase in thymidine kinase from virus isolates of CCVTK⁻ infected fish during the course of the experiment (Table 2).

DISCUSSION

In this investigation of the progression of CCV and CCVTK⁻ infections in waterborne-virus-exposed channel catfish fingerlings, the replicative patterns were similar for both types of viruses. Peak systemic levels occurred on Days 3 to 4 PI. Posterior kidney was the principal target organ for viral production followed by gills, skin and spleen. The amounts of virus isolated

from tissues were higher than previously reported (Plumb 1978, Zhang & Hanson 1995). Both CCV and CCVTK⁻ were isolated from the skin and gills at high concentrations. This is the first report on the shedding pattern of CCV. In Experiment 1, only CCV was isolated from tank water during the peak viral production in the tissues; however, both type of viruses were detected in the Experiment 2.

The use of QT-PCR in detecting viral DNA molecules was sensitive and very effective. The ELISA-based analysis of the product allowed the detection and quantitation of very low amounts of virus in tissues and in water. The use of an IS and quantitation based on the ratio of competitive product accounted for variability in sample chemistry. In general, viral DNA was detected in most of the tissues by 12 h PI and remained detectable through the last day of sampling (12 d PI). Infectious virus levels during these early and late time points were below detection limits. The number of virus DNA molecules detected by QT-PCR was over 500 times more than that detected by plaque assay. QT-PCR was also very sensitive in detecting viral DNA shed into tank water throughout the experimental period.

In the preliminary experiment, infectious CCV was isolated as early as Day 1 PI from posterior kidney, skin, gills and spleen. These results are in agreement with the findings of Nusbaum & Grizzle (1987) implicating the gills as a likely portal of entry for CCV in immersion challenges. Similar observations were reported regarding portal of entry of infectious hematopoietic necrosis virus (IHNV), a rhabdovirus infecting salmonids. The skin and gills were identified as important sites of (IHNV) entry and replication in the initial stages of infection (Yamamoto & Clermont 1990). From Day 2 PI, CCV and CCVTK⁻ were isolated from all the tissues, indicating a generalized viremia. This is supported by the finding of virus in blood samples by Day 2 in Experiment 2. In both experiments the progression of CCVTK⁻ infection was comparable to that of CCV through Day 6 PI. Thereafter, Experiment 2 demonstrated a marked decrease in CCVTK⁻ levels. Similar findings were reported previously by Zhang & Hanson (1995). Experiment 1 did not support these findings. There are 3 possible reasons for this discrepancy. Firstly, stressful un-ionized ammonia levels experienced late in Experiment 1 compromised the study. Also, the results of TCA precipitation assays showed an increase in viral associated thymidine kinase activity, demonstrating probable recrudescence of latent CCV with progression of the disease. In Experiment 1, only 1 fish per replicate ($n = 5$) was sampled at each time point, which contributed to the high variation. Variability between replicates was reduced considerably in Experiment 2 by increasing the sam-

pling size and taking all the fish per tank, thus eliminating sampling bias. Also, ammonia levels were kept below stressful levels by using biological filters.

In Experiment 2, CCV was consistently isolated until Day 10 PI from posterior kidney and gills and until Day 8 PI from skin and blood. On the other hand, less CCVTK⁻ was isolated after Day 6 from all tissues and was lacking after Day 8. Reasons for the decline in concentrations of CCVTK⁻, when compared to CCV, may be related to lack of TK activity. This enzyme is essential for viral replication in differentiated, nonproliferating cells (Wilcox et al. 1992, Slater et al. 1993, Mulder et al. 1995). The TK gene of neurotropic herpesviruses plays an important role in neuropathology and, hence, contributes to the virulence of the virus (Field & Wildy 1978, Kit 1990). Although our study did not include the effect of CCV on the nervous system, it is possible that late viral production is due to more persistent replication in nonproliferating cells and the wild-type virus is able to replicate because of TK activity. This effect has been demonstrated in non-neuronal tissue as well. *In vitro* assays studying the replication of pseudorabies virus, *Suid herpesvirus-1*, in peripheral blood mononuclear cells demonstrated that TK⁻ viruses had impaired growth (Mulder et al. 1995). It is interesting to note that CCV replicates well in cultured channel catfish mononuclear cells (Chinchar et al. 1993), however CCV and CCVTK⁻ replicative abilities have not been compared in these cells. The results of QT-PCR indicated that there were no significant differences between CCV and CCVTK⁻ DNA concentrations in most tissues throughout the infection. However, virus DNA in the skin of CCV infected fish was higher than that of CCVTK⁻ infected fish during early periods in the infection in both experiments.

The shedding patterns of CCV and CCVTK⁻ from infected fish were distinct. In Experiment 1, only infectious CCV was detected in water and in Experiment 2 its levels were significantly higher than CCVTK⁻. The duration of shedding correlates to the peak virus titers isolated from tissues. Although there were no significant differences between CCV and CCVTK⁻ isolated from tissues during peak production, their shedding patterns were significantly different. It is interesting to note that during the period of most intense increase in shedding the CCV levels in the skin were higher than the CCVTK⁻ levels, providing circumstantial evidence to support the skin as a site of shedding. The reason for the reduced shedding ability of CCVTK⁻ was not clear. Similar observations have been reported for other herpesviruses indicating that recombinant viruses are shed in lesser amounts and for a shorter duration (Cornick et al. 1990, Moormann et al. 1990, Slater et al. 1993, Kimman et al. 1994). Typically, the amount of virus DNA detected in the water was 10³ to 10⁴ times

greater than that detected by plaque assays. This difference was probably due to the difficulties in isolating infectious virus without damaging the envelope. However, this may also represent differences in export of properly processed virus (infectious) and viral DNA or the identification of virus that has been inactivated by environmental factors. It is interesting to note that levels of viral DNA in the water of CCVTK⁻ infected fish were not different from those of CCV infected fish on Day 4 PI for both experiments. This indicates a strong disparity between shed infectious virus concentration and viral DNA.

This study provides insight into several aspects of CCV pathogenesis including tissue types involved, possible sites of viral entry and shedding, and the period of shedding into water. A more complete understanding of the pathogenesis of these viruses requires sequential histopathological studies on the tissues to precisely locate cells and tissues involved in the viral entry, dissemination and shedding. Also the demonstrated reduction in persistence and shedding ability of CCVTK⁻ is encouraging for recombinant CCV vaccine and vaccine vector development (Zhang & Hanson 1996), as containment of recombinant viruses may not pose a serious problem. The presence of wild type CCV in CCVTK⁻ challenged fish late in the preliminary experiment, as indicated by TCA precipitation assays, where the controls showed no sign of productive infection implies a recrudescence of latent CCV and not tank to tank contamination. The quantitative PCR used in these experiments showed no detectable latent CCV in pre-infection assays. However, subsequent to this study, nested PCR analysis performed using 2 sets of primers directed to the TK region of CCV demonstrated the presence of CCV in 4 out of 5 fish from the stock used in Experiment 1 and none of the 5 fish sampled from the stock used in Experiment 2. The safety and efficacy implications of the potential of recombinant CCV to induce recrudescence of latent CCV warrants further investigation. Furthermore, whether the CCVTK⁻, or a combination of CCVTK⁻ and ammonia-related stress, induced the recrudescence remains to be determined. The QT-PCR was very useful in quantifying low levels of viral DNA, but the biological significance of the findings is unclear since it detects viral DNA that may or may not be from infectious virions.

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