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Experimental Channel Catfish Virus Infection Mimics Natural Infection of Channel Catfish

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Abstract

Channel catfish virus (CCV) causes a severe hemorrhagic disease in channel catfish fry and fingerlings. CCV epizootics are associated with elevated water temperatures and high mortality rates. Survivors of acute disease are latently infected with the virus. In this study, we investigated conditions effecting CCV pathogenesis and latency utilizing an experimental immersion model to simulate natural infection and a population of Arkansas catfish verified to have no prior CCV exposure. The results indicate that the Auburn-1 laboratory strain is comparable to CCV field isolates in virulence and ability to establish latent infection. The study confirms that water temperature and fish age effect susceptibility to acute infection. Twenty-four week old fish were more susceptible to acute CCV infection at 28°C than at 24°C. Eight week old fish were susceptible to disease at 24°C and 28°C. Yearling catfish, although more resistant to acute disease, were susceptible to latent CCV infection. CCV latency was established as early as 27 days following experimental infection and maintained for at least one year post infection. The CCV infection model described in this report is useful for further investigation of CCV pathogenesis and latency and for evaluation of potential antiviral therapies.

Introduction

Commercial catfish farming is a major aquaculture industry in the southern United States contributing especially to the economies of Arkansas, Mississippi, Alabama, and Louisiana. In 2002, nearly 200 Arkansas catfish operations covering 38,000 water surface acres generated over $65 million in sales (Anonymous, 2003). However, several factors, including disease, may hamper production and cause significant economic loss (Anonymous, 1997).

Channel catfish virus (CCV) disease is the leading viral disease in catfish. This herpesvirus causes an acute hemorrhagic disease in juvenile channel catfish, Ictalurus punctatus (Buck, 1990; Plumb, 1977). Clinical signs of the disease include erratic swimming followed by lethargy, punctate hemorrhaging at the base of fins, abdominal swelling, and exophthalmia. Internally, visceral organs such as the kidneys, intestines, and liver often exhibit hemorrhagic lesions (Wolf et al., 1972). Infectious CCV and viral DNA may be detected in kidney, intestine, liver, brain, and muscle tissues of acutely infected fish (Plumb, 1971; Gray et al., 1999a). CCV epizootics usually occur during summer months, when water temperatures exceed 25°C and channel catfish fry and fingerlings are abundant (Plumb, 1977; 1978). Outbreaks in hatchery stocks may cause high mortality of fish in infected ponds (Plumb, 1978).

Survivors of acute CCV infection are latently infected, a characteristic of herpesviruses (Plumb et al., 1981; Wise et al., 1985; Gray et al., 1999b). Viral DNA may be detected in blood, brain, intestines, kidney, liver, and peripheral blood leukocytes of latently infected fish by PCR analysis using CCV specific primers (Gray et al., 1999b).

In addition to the ability to establish latent infection in the host, CCV shares many characteristics with other herpesviruses. Morphologically, CCV is similar to other herpesviruses in size and structure (Wolf and Darlington, 1971). The overall organization of the double-stranded CCV DNA genome into unique and repeat regions is typical of herpesvirus genomes (Davison, 1992). In addition, CCV gene expression is temporally regulated in the same manner as that of other herpesviruses (Dixon and Farber, 1980; Silverstein et al., 1995, 1998; Huang and Hanson, 1998; Stingley and Gray, 2000).

CCV infection of channel catfish provides an excellent natural model to investigate herpesvirus pathogenesis and latency. Previous experimental studies of CCV pathogenesis were limited because of the inability to confirm that fish were not already latently infected. We have recently verified that catfish stocks at the Stuttgart National Aquaculture
Research Center in Stuttgart, Arkansas are negative for CCV latency, indicating that these fish have no prior exposure to CCV (Gray et al., 1999b). In this study, we have used this CCV negative catfish population to develop an experimental model of CCV infection of channel catfish that mimics natural infection.

### Materials and Methods

**CCV strains and propagation.**—Channel catfish ovary (CCO) cells were maintained at 27° C in Eagle’s minimum essential medium (EMEM) supplemented with penicillin (5,000 U/ml), streptomycin (5,000 U/ml) and 5% fetal bovine serum (FBS). The laboratory strain, CCV Auburn-1 (American Type Culture Collection VR-665), and three CCV isolates from epizootics in Mississippi were propagated in CCO cells. Viral stocks were generated by infection of confluent CCO monolayers and viral titers were determined by plaque assay on CCO cells (Stingley and Gray, 2000).

**Fish infections.**—Channel catfish were maintained at the Stuttgart National Aquaculture Research Center, ARS-USDA, in Stuttgart, AR. Immersion infection of 8-week-old fish in 400 ml of water and immersion infection of 24-week-old fish in 600 ml of water were performed with 10^7, 5 x 10^7, or 10^8 pfu of CCV for 30 min. Immersion infection of 1-year-old fish was carried out in 2 liters of water with 4.5 x 10^7 pfu of CCV for 30 min. After initial immersion, fish were maintained in 30-gallon tanks at either 24° or 28° C. Fish that survived infection at 24-week-old were maintained at the Stuttgart facility for more than 2 years post infection (p. i.).

Intraperitoneal (i. p.) injection was used to infect additional 1-year-old fish. The fish were anesthetized in 100 mg/l tricaine (3-aminobenzoic acid ethyl ester, Sigma Chemical Co.) prior to injection. Each fish was injected with 10^7, 10^8, or 10^9 pfu CCV in 100 ìl EMEM, 2% FBS. Mock-infected fish were each injected with 100 ìl EMEM, 2% FBS. Following infection, the fish were transferred to 30-gallon tanks and maintained at 28° C for seven to ten days.

**Infectious virus assay.**—Tissues (kidney and liver) were harvested and pooled in one ml EMEM, 2% FBS. The tissues were homogenized in the media and centrifuged to pellet cellular debris. Blood samples were not homogenized. Ten-fold serial dilutions of samples were applied to CCO monolayers. The cells were observed for ten days, and plaque formation, if any, was recorded.

**CCV DNA detection in latently infected fish.**—CCV latency in fish was confirmed by the ability to detect CCV DNA, but not infectious virus, in tissues. Total cell DNA was isolated from infected and mock-infected catfish tissues using the Wizard Genomic DNA Purification kit (Promega Corp.). Standard PCR conditions were generally used to detect CCV DNA in tissues of latently infected fish. CCV open reading frame (ORF) 3 primers (CCV genome nucleotides [nt] 4881 - 4899 and 5117 - 5136 (Davison, 1992) were designed to amplify a 256 bp PCR product from 0.5 - 1.0 ìg DNA template in the 50-cycle reaction. Each cycle consisted 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. Amplification products were analyzed by UV illumination following electrophoresis through 1.0% agarose gels and ethidium bromide staining. To confirm the results, the DNA sequence of the PCR products was determined and demonstrated to be specific to CCV ORF 3.

Nested set PCR was required to detect CCV DNA in blood derived from latently infected fish at one year p. i. (Gray et al., 1999b). The nested-set PCR assay utilized primers within CCV open reading frame (ORF) 8. Initially “external” primers were used to amplify a 335 base pair (bp) product from 0.5 - 1.0 ìg DNA template in a 50-cycle reaction using conditions as described above. Nested primers (nt 12646 - 12664 and 12801 - 12820) were employed in a subsequent reaction to generate a 175 bp product using 5 ìl of the external reaction product as the template. For the nested-set PCR, 30 amplification cycles were used, each consisting of a denaturation step at 94° C for 1 min, annealing at 53° C for 3 min, extension at 72° C for 3 min, and a final extension at 72° C for 7 min. Amplification products were analyzed as described above.

**Statistical analyses.**—To compare the virulence of the Auburn-1 CCV strain to that of field isolates, the Kaplan-Meier estimates of the survivor distribution for each group were obtained and compared using the log rank Chi-square test. For all other survival experiments, the proportion surviving at the end of each experiment, rather than the entire curve, was used in statistical comparisons with the Fisher’s Exact Test. A significance level of 5% was used to declare differences.

### Results

**Infection with CCV Auburn-1 strain and CCV field isolates.**—The CCV Auburn-1 strain is the prototype laboratory CCV strain and has been passaged in culture for several years. To determine whether the CCV Auburn-1 is as virulent as three CCV field isolates, we compared survival rates of infected 24-week old catfish. In each tank, 20 fish were infected by immersion with 10^8 pfu of CCV and maintained at 28° C. Fish infected with the Auburn-1 strain and the CCV isolates exhibited clinical signs of CCV disease including aberrant swimming patterns and hemorrhagic lesions. The Auburn-1 strain was virulent with a 10 day survival rate (35%) that was not statistically different from the survival rates of the CCV isolates (15 to 30%) (Fig. 1).

Viral latency was examined in fish which survived...
experimental infection with Auburn-1 and the CCV isolates. At 27 days p. i., blood, kidney, and liver tissues were harvested and pooled from 3 fish infected with 10⁷ pfu of the Auburn-1 strain or with each CCV isolate. Surviving fish were confirmed to have resolved the acute infection by the inability to detect infectious virus in the kidney and liver tissues by infectious virus assay. PCR analysis employing an ORF 3 primer set detected CCV DNA in blood samples of fish originally infected with the Auburn-1 strain and the CCV isolates (Fig. 2). The expected 256 bp PCR products were gel-purified and sequenced to confirm CCV specificity. Control PCR reactions using template DNA from the blood of mock-infected fish or no DNA template were negative (Fig. 2). These results demonstrated that the Auburn-1 laboratory strain and CCV field isolates cause acute disease in experimentally infected catfish and that survivors are latently infected by 27 days p. i. Subsequent experiments in this study used the Auburn-1 strain.

**Parameters of experimental CCV infection.**--To examine experimental parameters for CCV infection of channel catfish, we determined the effects of viral dose, fish age, and water temperature on survival rates of infected fish. In an effort to mimic natural infection as closely as possible, fish were infected by immersion in water containing specific doses of Auburn-1 CCV. Two sets of fish, 8-week old and 24-week old, were immersion-infected with 10⁷, or 5 x 10⁷ pfu of CCV at 24°C or 28°C and monitored daily. Fish that died exhibited signs of CCV disease.

**Fig. 1.** Infection with CCV Auburn-1 strain is similar to that of field isolates. Survival rates are shown for channel catfish infected with Auburn-1 (diamonds), MS-1 (squares), MS-2 (triangles), MS-3 (circles) and mock-infected (*). Twenty fish in each group were infected with 10⁸ pfu CCV or mock-infected by immersion in 400 ml of water for 30 min, then transferred to 30 gallon tanks and maintained at 28°C. Survival was monitored daily and mortalities were recorded. Employing Kaplan-Meier analysis, the test of equality had a p-value = 0.4417, indicating that there are no differences in the survival curves among the CCV strains.

**Fig. 2.** Detection of CCV DNA in the blood of fish latently infected with Auburn-1 and three CCV field isolates (MS-1, MS-2, and MS-3). DNA was isolated and pooled from three infected fish and from three mock-infected fish at 27 days p. i. CCV IE 3 gene primers were used in 50-cycle PCR to detect the expected 256 bp CCV DNA amplification product upon agarose gel electrophoresis and ethidium bromide staining. Control reactions employing DNA isolated from blood of mock-infected fish and reactions which included no DNA template were included.
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Fig. 3. Effects of age and temperature on the survival rate of CCV-infected channel catfish. Survival rates are shown 24-week old (A) and 8-week old (B) channel catfish infected with $10^7$ (triangles) and $5 \times 10^7$ (circles) pfu CCV or mock-infected (diamonds). Each group included at least 32 fish. Infections were conducted at 24°C (open symbols) or 28°C (solid symbols). Fish were monitored and mortalities were recorded on the various days post-infection.

Water temperature had a dramatic effect on the survival rate of 24-week old fish. At 24°C, 100% and 86% of 24-week old fish survived infection at $10^7$ and $5 \times 10^7$ pfu, respectively, whereas only 17% and 7% survived infection with similar doses at 28°C (Fig. 3A). The temperature effect at both viral doses was highly significant ($p<0.0001$) in 24-week old fish.

In contrast, this temperature effect was not observed with 8-week old fish. At 24°C, only 1% and 15% of 8-week old fish survived infection at $10^7$ and $5 \times 10^7$ pfu, respectively, whereas at 28°C, 8% and 6% of 8-week old fish survived similar infection (Fig. 3B). No significant differences between survival rates at the two temperatures were observed at either viral dose ($p<0.05$). The results indicate that 24-week old fingerlings were susceptible to CCV disease only at elevated water temperatures, but the younger 8-week old fish were susceptible at either 24°C or 28°C.

**CCV infection of 1-year old fish.**—During CCV epizootics, juvenile channel catfish exhibit symptoms of CCV disease; however, adult fish are more resistant to acute disease (Plumb, 1978; Hedrick et al., 1987). To determine whether 1-year old fish could be acutely infected with CCV, we compared survival rates in fish infected by immersion with fish infected by intraperitoneal (i.p.) injection.

Twelve 1-year old fish were i.p. injected with 100 μl EMEM, 2% FBS alone (mock-infected), or containing $10^3$, $10^4$, or $10^5$ pfu CCV. Mock-infected fish had a 92% survival rate, whereas fish infected with $10^3$, $10^4$, and $10^5$ pfu CCV had survival rates of only 40%, 25%, and 0%, respectively (Fig. 4). As expected, yearling fish infected via immersion with $4.5 \times 10^7$ pfu CCV had a significantly higher survival rate. Only two of the eight fish infected by immersion did not survive (Fig. 4).

We next determined whether or not 1-year old fish infected by immersion develop latent infection despite the lack of acute disease development. Blood, kidney, and liver samples were harvested from 3 individual fish at 107 days post-infection. Infectious virus assays performed on CCO monolayers confirmed that infectious CCV could not be detected in the...
kidney or blood of these fish. Total cell DNA isolated from the tissues served as template for PCR analysis utilizing the ORF 3 primer set. CCV DNA was detected in the blood, kidney, and liver tissues from the infected fish, but not in blood from mock-infected fish or reactions omitting the DNA template (Fig. 5). The results demonstrate that viral latency can be established in fish infected as adults.

**Long-term persistence of latency following experimental CCV infection.**—CCV latency is established as early as 27 days p. i. (Fig. 2). In this study, we determined whether or not viral latency is maintained for as long as one year following experimental CCV infection. Twenty-four week old fish were infected with 1 x 10^7 pfu CCV at 24° or 28° C (Fig. 3A). At one year p. i., total cell DNA was isolated from blood of surviving fish. Standard PCR employing ORF 8 external primers did not detect CCV DNA in the blood samples (Fig. 6). However, CCV DNA was detected in the blood of each of five latently infected fish examined using ORF 8 nested set PCR. CCV DNA was not detected in blood of mock-infected fish.

**Discussion**

This study describes an experimental model of CCV infection of channel catfish, focusing on immersion-infection to simulate conditions of natural infection. A particular advantage of this study is the use of channel catfish that were confirmed to be uninfected prior to experimental infection. Previous studies employing experimental infection of channel catfish have used fish populations with no history of CCV (Plumb et al., 1981; Hedrick et al., 1987; Nusbaum and Grizzle, 1987). However, CCV DNA has been detected in fish populations with no known exposure to CCV, indicating latent CCV infection in these fish (Wise et al., 1985). The channel catfish population used in this study has been maintained at the Stuttgart National Aquaculture Research Center without exposure to CCV. This fish population is not latently infected as confirmed by PCR analysis (Gray et al., 1999b).

The Auburn-1 strain is the prototype laboratory strain commonly used in experimental analysis of CCV. This

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Fig. 5. Detection of CCV DNA in tissues of one year-old fish infected by immersion. DNA was isolated and pooled from the blood, kidney, and liver of three infected fish at 107 days p. i. and from the blood of three mock-infected fish. CCV IE 3 gene primers were used in 50-cycle PCR to amplify the expected 256 bp product upon agarose gel electrophoresis and ethidium bromide staining. Controls reactions using no DNA template were included.

Fig. 6. Detection of CCV DNA in fish blood at one year p. i. 24-week old fish were experimentally infected by immersion with CCV. At one year p. i., total cell DNA was isolated from the blood of 5 surviving infected fish (1-5) and from mock-infected fish. PCR employing CCV ORF 8 external primers did not reveal the expected 335 bp product upon agarose gel electrophoresis. Nested-set PCR employing CCV ORF 8 internal primers amplified the expected 175 bp CCV DNA. Control reactions using DNA template from mock-infected fish or using no DNA template were included.
study confirmed that the laboratory strain is useful in mimicking natural infection. Similar to CCV field isolates, the Auburn-1 strain induced acute disease in infected fish and established latent infection in survivors. CCV DNA could be detected in the blood of latently infected fish as early as 27 days p. i. and as late as one year p. i., indicating rapid establishment and long-term persistence of viral latency.

The age of fish and water temperature effect the susceptibility of channel catfish to natural CCV infection, as evident by the fact that CCV outbreaks cause disease in juvenile catfish and occur when water temperatures exceed 25°C (Plumb, 1977, 1978). Our results support these findings and indicate that these factors also affect susceptibility during experimental infections. The 24-week old catfish were dramatically more susceptible to disease at the higher water temperature (28°C). In contrast, the 8-week old fish were susceptible to disease when infected at 24°C or 28°C. It is possible that a temperature effect on disease in 8-week old fish might be revealed by exposure to lower viral doses.

Adult catfish are more resistant to CCV infection than fry and fingerlings and do not generally develop acute CCV disease during epizootics. In our experimental model, yearling catfish were less susceptible to CCV disease than fingerlings upon immersion infection. Yearling fish are not innately resistant, as acute CCV disease could be induced by i.p. infection at relatively low doses. In the absence of acute disease following immersion infection, one-year old fish developed latent infection, as demonstrated by detection of viral DNA in the tissues by 107 days p. i. The implication is that during CCV outbreaks older fish may not die, but become latently infected and serve as a source for long term viral persistence in the population.

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Literature Cited


