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Transmission of the *Ambystoma tigrinum* virus to alternative hosts

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ABSTRACT: *Ambystoma tigrinum* virus (ATV) is a lethal virus originally isolated from Sonora tiger salamanders *Ambystoma tigrinum stebbinsi* in the San Rafael Valley in southern Arizona. USA. ATV is implicated in several salamander epizootics. We attempted to transmit ATV experimentally to fish and amphibians by injection, water bath exposure, or feeding to test whether ATV can cause clinical signs of infection or be recovered from exposed individuals that do not show clinical signs. Cell culture and polymerase chain reaction of the viral major capsid protein gene were used for viral detection. Salamanders and newts became infected with ATV and the virus was recovered from these animals, but virus could not be recovered from any of the frogs or fish tested. These results suggest that ATV may only infect urodeles and that fish and frogs may not be susceptible to ATV infection.

KEY WORDS: Ranavirus · Salamander · Frog · Fish · Polymerase chain reaction · Cell culture · Amphibian decline

INTRODUCTION

Disease is among the factors associated with amphibian declines worldwide. Two major pathogens, a chytrid fungus and viruses of the genus *Ranavirus*, are implicated as causative agents for widespread amphibian mortalities and amphibian declines (Cunningham et al. 1996, Berger et al. 1998, Daszak et al. 1999, Hyatt et al. 2000, Collins et al. 2001). *Ambystoma tigrinum* virus (ATV) was isolated from the endangered Sonora tiger salamander *Ambystoma tigrinum stebbinsi* in 1996 after a series of epizootics in the San Rafael Valley (SRV) in southern Arizona, USA (Collins et al. 1988, Jancovich et al. 1997). The morphology, site of replication, and host pathology suggested that ATV is a member of the genus *Ranavirus*, in the family *Iridoviridae*, which was confirmed by molecular tests (Jancovich unpubl. data).

Ranaviruses are associated with diseases of amphibians (Cunningham et al. 1996, Jancovich et al. 1997, Zupanovic et al. 1998, Bollinger et al. 1999, Hyatt et al. 2000) and fish (Moody & Owens 1994, Mao et al. 1997, Mao et al. 1999, Hyatt et al. 2000), and some ranaviruses have broad host ranges. The Bohle iridovirus can infect 3 species of Australian frogs (*Lymnodynastes ornatus*, *L. terraereginae*, *L. lato-palmata*), cane toad *Bufo marinus*, and barramundi fish *Lates calcarifer* (Moody & Owens 1994, Cullen et al. 1995). Mao et al. (1999) isolated an iridovirus originally found in frogs (*Rana aurora*) from a stickleback fish (*Gasterostelus aculeatus*). Iridoviruses are suspected agents of mortalities in frog populations in the USA in Maine and Minnesota, in both frog and salamander populations in Massachusetts, Tennessee, and North Carolina, and in salamander populations in Idaho, Utah, North Dakota, and Wyoming (USGS News Release, available at: www.usgs.gov/public/press/public_affairs/press_release/pr1272m.html) and in Saskatchewan, Canada (Bollinger et al. 1999). It is unknown whether the same virus is responsible when simultaneous die-offs of both frogs and salamanders are observed, such as in Massachusetts, Tennessee, and North Carolina.

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Man-made earthen ponds ('stock tanks') are the sole habitat for the Sonora tiger salamander. Sunfish *Lepomis cyanellus* and mosquito fish *Gambusia affinis*, as well as bullfrogs *Rana catesbeiana*, are repeatedly introduced into these cattle tanks, and rainbow trout *Oncorhynchus mykiss* are introduced into a lake that drains into the SRV. The Chiracahua leopard frog *R. chiracahuensis*, a candidate for listing as a threatened species, inhabits ponds within the area. We examined the hypothesis that one of these organisms may have introduced ATV into salamander populations triggering epizootics. We experimentally infected sympatric amphibian and fish species and examined them for disease symptoms or the presence of ATV. Because of its threatened nature, the Chiracahua leopard frog was replaced by commercially available *R. pipiens* in our experiments. We also examined the susceptibility of another *Ambystoma* species and 1 newt species.

METHODS

ATV culture for transmission experiments. ATV was passed in *epithelioma papilloma cyprini* (EPC; Fijan et al. 1983) cells cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) in 25 cm^2 flasks at 24°C. The virus used for the following experiments was passed in EPC cells fewer than 5 times. Virus titer was determined by plaque assay in EPC cells. ATV multiplies at temperatures from 10 to 31°C in cell culture and infects salamanders from 10 to 28°C (Jancovich et al. 1997, S. Rojas & E. Davidson unpubl.).

Animal husbandry. ***Amphibians:*** *Ambystoma tigrinum* larvae from laboratory colonies were maintained at 28°C in aged tap water, pH 7.5, and fed mealworms twice weekly. Larval *Ambystoma gracile* were field collected from Snohomish County, Washington, USA, while adult red-spotted newts *Notophthalmus viridescens* were collected from the Delaware Watershed, Pennsylvania, USA. All animals shipped to Arizona were housed in 190 l aquaria and fed tubifex worms twice a week. Salamanders were allowed to acclimate in the laboratory for 2 mo before exposure to virus. Adult leopard frogs were obtained from Carolina Biological Supply (Burlington, NC, USA) and were acclimated in the laboratory for 1 mo in 76 l aquaria with sand and water, while being fed laboratory-reared crickets. Bullfrog tadpoles were obtained from the Phoenix Zoo (Phoenix, AZ, USA), housed in 190 l aquaria, and fed frozen spinach twice a week. Adult bullfrogs were collected from the SRV and acclimated in the laboratory for 1 mo in conditions similar to those for the leopard frogs. Water was changed once a week for the duration of the acclimation and experimental periods using aged tap water. Amphibians were held at 25°C over a 10 h light:14 h dark cycle.

Fish: Sunfish *Lepomis cyanellus* and mosquito fish *Gambusia affinis* were collected by seining from cattle tanks in the SRV, held in 190 l circulating aquaria at 18°C, and fed tropical fishfood (Tetramin, TetraWerke, Mille, Germany) twice a week. Rainbow trout, between 10 and 15 cm in length, were obtained from the White Mountain Hatchery, Arizona. Trout were held in circulating 190 l aquaria at 18°C in aged tap water, fed trout pellets (Rangen Inc, Buhl, ID, USA) twice a week, and allowed to acclimate for at least 2 wk before exposure to virus.

Transmission to alternative hosts. *Ambystoma tigrinum* larvae were routinely infected during the course of the experiment by exposure to water in which another larva had died. *Ambystoma gracile* and *Notophthalmus viridescens* were exposed by placing them individually into water in which an *A. tigrinum* larva had died less than 2 d previously. Body wall and liver samples were collected for assay for virus (below).

In feeding trials, sunfish were held in two 76 l aquaria with 15 fish in each aquarium. Mosquito fish were held in two 76 l aquaria with 25 fish in each aquarium. Following acclimation, sunfish and mosquito fish were fed finely chopped body wall, tail, and liver from either infected or uninfected (control) salamanders. Fish were observed for 2 mo for mortality and then euthanized. Body wall samples were assayed for virus.

Cell culture-grown ATV (4×10^5 plaque forming units ml^{-1} in EPC cells [ATV/EPC]) was used as inoculum in injection trials. In the injection trial, 8 sunfish and 16 mosquito fish were each injected with 30 µl of ATV/EPC suspension (approximately 10^3 plaque forming units ml^{-1}) or an equal volume of uninfected EPC cell suspension (control). Approximately 1/3 of experimental and control fish were euthanized at 5, 11, and 32 d postinjection, and body wall samples were processed for virus detection. Rainbow trout were held in 4 aquaria, 6 in each aquarium. Trout in 2 aquaria were fed 2 infected salamanders or uninfected salamanders (control), cut into small pieces, over a 14 d period. Trout were euthanized 15 d after the final feeding, and spleen, body wall, and liver were processed for virus detection.

Rana pipiens were injected with 100 µl of ATV/EPC or EPC cells alone. Half of the frogs were killed 14 d postinjection and the remainder 38 d postinjection. Body wall and spleen samples were taken for virus detection. Bullfrog tadpoles, 5 to 7 cm in length, were fed infected or control salamander body parts 6 times over a period of 2 mo. Tadpoles were euthanized 1 mo later, and liver and body wall samples were taken for virus detection. Nine adult bullfrogs were injected

with 200 µl ATV/EPC and 2 were injected with EPC cells alone. All bullfrogs were euthanized 30 d post-injection and body wall samples were taken for virus detection.

When clinical signs of an ATV infection (skin pustule formation, anorexia, hemorrhaging, or lethargy; Jancovich et al. 1997) were observed, or at the conclusion of an experiment, animals were euthanized in 1% tricane methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA). Animals were dissected and tissues collected using sterile instruments. Healthy, laboratory-reared *Ambystoma tigrinum* were exposed to ATV/EPC by water bath during each experiment to document infectivity of the inoculum and to provide infected tissues for exposure of fish and frogs and infectious water for exposure of *A. gracile* and *Notophthalmus viridescens*.

Virus isolation. Tissues were homogenized separately in MEM with 2% FBS using glass tissue grinders and centrifuged at 3000 × *g* for 10 min, and the supernatant was filtered through 0.45 µm filters. EPC cells were infected at 24°C with 100 to 200 µl of tissue homogenates, and flasks were rocked every 10 min for 1 h. After 1 h, MEM with 10% FBS was added to the flasks, and the cells were incubated at 24°C and observed daily for cytopathic effects. Both positive and negative controls were included in each assay. When the cell monolayer had completely lysed, virus and infected cells were harvested and stored at –70°C.

DNA isolation and polymerase chain reaction (PCR). DNA was isolated from lysed cell culture extracts, or from healthy control or diseased animal tissue. Samples were homogenized in TES buffer (10 mM Tris, 0.1 M NaCl, 1 mM EDTA) with 1 mg ml^{-1} Proteinase K and 1% sodium dodecyl sulfate. Preparations were incubated at 50°C for 1 h and then incubated at 37°C overnight. DNA was isolated by phenol:chloroform:isoamyl alcohol extraction followed by ethanol precipitation. An approximately 500 base pair region of the 5′ end of the major capsid protein (MCP) gene was amplified by PCR using primers described in Mao et al. (1997). PCRs were performed (94°C 1 min, 92°C 1 min, 50°C 2 min, 72°C 3 min, cycled 25 times) and the products were visualized by agarose gel electrophoresis. Both positive and negative controls were included in each assay.

RESULTS

Over 20 *Ambystoma tigrinum* used in this study developed disease symptoms within 14 d after exposure and died in less than 30 d, as did 2 *A. gracile* and 2 *Notophthalmus viridescens* exposed to water in which an *A. tigrinum* had died. Signs of disease in *A. gracile* and *N. viridescens* included white polyp formation on the skin, lethargy, anorexia, and hemorrhages similar to those reported previously for *A. tigrinum* (Jancovich et al. 1997). All experimentally infected salamanders displayed mottled livers, hemorrhages in stomach and intestines, and accumulation of fluid in the body cavity. Virus was detected by cell culture and PCR from all tested tissues in each case (Table 1).

None of the frogs or fish developed any signs of ATV infection. Internal organs and tissues of control and experimental animals did not differ, and none of the symptoms observed in exposed salamanders was observed in frogs or fish. Cell culture and PCR were negative for all frogs, fish, and controls exposed by injection or feeding (Table 1).

Using primers to the MCP gene we were able to use PCR along with cell culture to detect ATV in tissue

Table 1. Detection and transmission of *Ambystoma tigrinum* virus. +: positive results; –: negative results; PCR: polymerase chain reaction; SRV: San Rafael Valley, Arizona, USA

Species	Source	Method of exposure	Disease symptoms	Cell culture/ PCR	Control: survivors/total	Experimental: survivors/total
Ambystoma gracile[a]	Wild caught (WA)	Water bath	+	+/+	1/2	0/2
Notophthalmus viridescens[b]	Wild caught (PA)	Water bath	+	+/+		0/2
Rana pipiens[b]	Carolina Biological Supply	Injection	–	–/–	8/11	10/12
R. catesbeiana[a,b]	Tadpoles: Phoenix Zoo (AZ)	Water bath/ feeding	–	–/–	10/10	10/10
	Adults: SRV	Injection	–	–/–	2/2	9/9
Gambusia affinis	SRV	Injection,	–	–/–	16/16	16/16
		feeding	–	–/–	25/25	24/25
Lepomis cyanellus	SRV	Injection,	–	–/–	8/8	8/8
		feeding	–	–/–	12/15	12/15
Oncorhynchus mykiss	White Mountain Hatchery (AZ)	Feeding	–	–/–	9/12	12/12

[a]Larval morphs; [b]adult morphs

samples of experimentally infected salamanders. Sequencing the approximately 500 base pair PCR product of the MCP gene confirmed the presence of ATV. ATV preparations were infectious to laboratory-reared tiger salamanders exposed to the virus as positive verification controls during each experiment. PCR and cell culture methods accurately detected ATV in tissues of salamanders experimentally infected with ATV and did not detect virus in healthy control animals. In all situations in which ATV was present, both cell culture and PCR confirmed the presence of virus. In no situation was one method positive while the other was negative.

DISCUSSION

Our goal was to test whether ATV could be maintained or transmitted by species sympatric to the endangered tiger salamander in the SRV, particularly those introduced into the region by humans. Sudden salamander die-offs apparently related to ATV suggested that virus might be introduced periodically into the salamander populations by another species. Nonnative fish were first introduced into the SRV in the 1950s and bullfrogs in the 1970s. Fish and bullfrog adults are very efficient predators on salamander eggs and larvae (Collins et al. 1988, Snyder 1998).

Some of the amphibians and fish tested in this study have been previously associated with ranavirus infections. Cultured rainbow trout is host to epizootic hematopoetic necrosis virus (Langdon 1989), bullfrogs are host to frog erythrocytic virus (Gruia-Grey et al. 1989, Gruia-Gray & Desser 1992), and ranaviruses have been isolated from leopard frogs and passed in newts (Clark et al. 1968). However, we did not observe symptoms of ATV in tested sympatric frogs or fish, and were unable to detect virus in animals exposed by injection, water bath, or ingestion of infected salamanders. Even though bullfrogs, sunfish, and mosquito fish are predators of salamanders in the SRV, ingestion of multiply infected salamanders by these animals did not lead to detectable infection in our laboratory experiments.

Our assay techniques did not rule out the possibility that ATV could have been present in the digestive tract or excretory system of the tested animals. These tissues proved difficult to assay on cell culture, as control as well as treated specimens led to lysis of the cells. Facilities for histological studies were not available. We tested only species that are routinely found in the *Ambystoma tigrinum stebbensi* habitat in southern Arizona as possible alternative hosts for ATV. Other species of fish or frogs may be capable of maintaining this virus. Viruses genetically similar to ATV have been isolated from diseased salamanders in 6 other locations in Arizona, at 2 sites in Colorado, and in Saskatchewan (Bollinger et al. 1999, Jancovich et al. unpubl.), where different species of amphibians and fish are sympatric. Infectivity of the Bohle iridovirus for barramundi, but not for rainbow trout (Moody & Owens 1994), and of epizootic hematopoetic necrosis virus for rainbow trout, but not for barramundi (Langdon et al. 1988), illustrates the specificity of some ranaviruses for particular fish species.

Most ranaviruses crossreact immunologically (Hyatt et al. 2000). Previous exposure to a ranavirus and the ability of the host to clear a viral infection may therefore have played a role in our inability to recover virus from some of the frogs and fish we examined. Bullfrogs, mosquito fish, and sunfish were obtained from the SRV, where salamanders are periodically infected with ATV. It is therefore possible that these animals had been previously exposed to the virus and had acquired an immune response that allowed them to clear the infection. The rainbow trout and leopard frogs, however, were obtained from commercial sources and to our knowledge had not been exposed to a ranavirus. Techniques were not available at the time of these experiments to determine the immune status of these animals.

Our data suggest that ATV may not be transmitted by frogs or fish in the SRV. The host range of ATV may be restricted to salamanders and newts. We suggest that another agent, such as insects, birds, humans, or salamanders introduced as bait, may be responsible for introducing the virus in the SRV leading to epizootics. Alternatively, sublethally infected salamanders may maintain the virus and reinfect the population during periods of high density, temperature fluctuation, or other stress.

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