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DIAGNOSTIC AND MOLECULAR EVALUATION OF THREE IRIDOVIRUS-ASSOCIATED SALAMANDER MORTALITY EVENTS

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ABSTRACT: In 1998 viruses were isolated from tiger salamander larvae (*Ambystoma tigrinum diaboli* and *A. tigrinum melanostictum*) involved in North Dakota and Utah (USA) mortality events and spotted salamander (*A. maculatum*) larvae in a third event in Maine (USA). Although sympatric caudates and anurans were present at all three sites only ambystomid larvae appeared to be affected. Mortality at the North Dakota site was in the thousands while at the Utah and Maine sites mortality was in the hundreds. Sick larvae were lethargic and slow moving. They swam in circles with obvious buoyancy problems and were unable to remain upright. On the ventral surface, near the gills and hind limbs, red spots or swollen areas were noted. Necropsy findings included: hemorrhages and ulceration of the skin, subcutaneous and intramuscular edema, swollen and pale livers with multifocal hemorrhage, and distended fluid-filled intestines with areas of hemorrhage. Light microscopy revealed intracytoplasmic inclusions, suggestive of a viral infection, in a variety of organs. Electron microscopy of ultra thin sections of the same tissues revealed iridovirus-like particles within the inclusions. These viruses were isolated from a variety of organs, indicating a systemic infection. Representative viral isolates from the three mortality events were characterized using molecular assays. Characterization confirmed that the viral isolates were iridoviruses and that the two tiger salamander isolates were similar and could be distinguished from the spotted salamander isolate. The spotted salamander isolate was similar to frog virus 3, the type species of the genus *Ranavirus,* while the tiger salamander isolates were not. These data indicate that different species of salamanders can become infected and die in association with different iridoviruses. Challenge assays are required to determine the fish and amphibian host range of these isolates and to assess the susceptibility of tiger and spotted salamanders to heterologous virus isolates.

Key words: Ambystoma maculatum, Ambystoma tigrinum diaboli, Ambystoma tigrinum melanostictum, amphibian decline, amphibian mortality events, iridovirus, molecular characterization, pathology, ranavirus, spotted salamander, tiger salamander.

INTRODUCTION

Members of the family *Iridoviridae* infect a wide-range of invertebrates (genera *Iridovirus* and *Chloriridovirus*) and cold-blooded vertebrates (genera *Ranavirus* and *Lymphocystitivirus*) (Williams, 1996). Members of the genus *Ranavirus* infect fish, reptiles, and amphibians and are associated with clinical illness ranging from unapparent to fulminant. Frog virus 3 (FV3) is the type species of the genus *Ranavirus* (Williams et al., 1999) and the best characterized member of the family *Iridoviridae.* Sequence analysis of a variety of iridoviruses isolated from reptiles, fish, and frogs show them to be very similar to FV3 with amino acid identities ranging from 63–100%, depending upon the specific gene analyzed (Mao et al., 1997, 1999; Chinchar, 2002).

Recently there have been reports of iridovirus isolations from subspecies of tiger salamanders involved in localized mortality events. The first report of a virus isolation from a salamander species was from the larvae of the Sonoran tiger salamander (*Ambystoma tigrinum stebbinsi*) in 1996 (Jancovich et al., 1997). In a second event, a virus was isolated from tiger salamander larvae (*Ambystoma tigrinum diaboli)* near Regina, Saskatchewan (Canada) in 1997 (Bollinger et al., 1999).

In 1998, the U.S. Geological Service, National Wildlife Health Center (NWHC; Madison, Wisconsin, USA) performed diagnostic evaluations of three separate mor-

tality events involving tiger salamander larvae from North Dakota (USA) (*A. tigrinum diaboli*) and Utah (USA) (*A. tigrinum melanostictum*) and spotted salamander (*A. maculatum*) larvae from Maine (USA). This paper reports the first isolation of an iridovirus from the spotted salamander. It also describes the field events, diagnostic evaluation, and the preliminary molecular characterization and comparison of the iridoviruses isolated from tiger salamanders and a spotted salamander.

MATERIALS AND METHODS

Epizootics and sample collection

The first salamander mortality event involved larval tiger salamanders at a Waterfowl Production Area (WPA; 46°48′17″N, 100°35′29″W) managed by the U.S. Fish and Wildlife Service (FWS) in Burleigh County, North Dakota. This event occurred over a 3 wk period in May through June 1998. The second mortality event involved larval spotted salamanders at research study sites (46°40′48″N, 68°00′31″W) near Presque Isle, Aroostook County, Maine. Dead larvae were found for 3 wk during July of 1998. The third salamander mortality event occurred at 2,804 m elevation at Lake Desolation (40°39′33″N, 111°36′05″W) Utah. This die-off involved tiger salamander larvae and occurred from mid-August to early September 1998.

Biologists from the FWS and the University of Maine collected sick larval salamanders from the respective sites. The larvae were sent by overnight delivery to the NWHC to determine the cause of death. The salamanders from North Dakota and Maine died shortly after capture and were shipped overnight chilled or frozen. Larvae from Lake Desolation were collected in lake water, chilled, and shipped alive, overnight to the NWHC.

Diagnostic testing

Larvae were necropsied at the NWHC and gross abnormalities were noted. External examination was performed using an ocular loop or dissecting microscope. Liver, kidney, intestine, spleen, testes, skin, or entire carcasses were submitted for virus isolation. Liver, spleen, and in some cases, entire carcasses were submitted for isolation of bacteria.

Suspensions were prepared separately from the submitted tissues of the tiger salamander larvae from North Dakota (TsND), Utah (TsUT), and spotted salamander larvae from Maine (SsME) as described by Docherty and Slota, 1988. Virus isolation was attempted in fathead minnow (*Pimephales promelas*; FHM; American Type Culture Collection, CCL-42), bullfrog (*Rana catesbeiana*) tongue (American Type Culture Collection, CCL-41), and rainbow trout (*Oncorhyncus mykiss*) gonad (American Type Culture Collection, CCL-55) cell culture. These cultures were incubated for 2 wk at 25 C and checked regularly for viral cytopathic effect (CPE). If CPE was not noted by the end of this 2 wk period a blind passage was performed and incubated as above for another 2 wk.

Samples were cultured on 5% sheep blood agar (BA) and eosin methylene blue agar (Remel, Lenexa, Kansas, USA) and incubated at 35–37 C for 18–24 hr. All bacterial colonies were screened based on typical colony morphology and representative isolates were grown overnight on BA. Isolates were biochemically characterized and identified using an appropriate API of Vite system (BioMerieux, St. Louis, Missouri, USA). Isolates that yielded an uninterpretable identification code were further characterized using Biolog MicroPlates (Biolog, Inc., Hayward, California, USA).

For light microscopy, tissues were placed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm for light microscopy, and stained with hematoxylin and eosin. Spleen and liver were ultrathin sectioned (Bozzola and Russell, 1992) for transmission electron microscopy and viewed at 20,000–30,000× with a Hitachi 600 transmission electron microscope (Hitachi, Brisbane, California). Using the first virus isolate from each salamander mortality event, a virus stock of approximately 10^6 infectious particles/ml was prepared in FHM for electron microscopy. After one freeze-thaw cycle (−80–23 C), the cell suspension was centrifuged at 200×G, for 30 min. The virus-containing supernatant was removed and centrifuged at 80,000×G for 60 min and the pellet was negative-stained with 2% phosphotungstic acid (pH 6.2). The resulting grids were viewed at 20,000–30,000× with a Hitachi 600 transmission electron microscope.

Molecular characterization of virus isolates

Virus propagation: Fathead minnow monolayers, grown in 75 or 150 cm^2 flasks, were infected with FV3, SsME, TsND, TsUT, or Regina ranavirus (RRV; Bollinger et al., 1999) at low multiplicities of infection (MOI) (i.e., <0.01 plaque forming unit [PFU]/ml). Infected cell cultures were incubated at 26 C (FV3 and SsME) or 23 C (TsUT, TsND, and RRV) in Eagles's minimum essential medium with Hank's salts supplemented with 2% fetal calf serum.

At 2–3 days postinfection, when the monolayers were completely disrupted, cultures were frozen at −70 C. Intracellular virus was released by three cycles of freeze-thaw, debris removed by low speed centrifugation, and the clarified supernatant titered by plaque assay (FV3) or 50% tissue culture infectious doses ($TCID_{50}$) assay (RRV, TsUT, TsND, SsME) on FHM monolayers.

Analysis of viral protein synthesis: Confluent monolayers of FHM cells grown in 35 mm tissue culture dishes (Falcon, Washington, Pennsylvania, USA) were mock-infected or infected with FV3 and SsMe at MOIs of 20 PFU/cell and 2 $TCID_{50}$/cell, respectively. Cultures were incubated overnight at 26 C and radiolabeled from 18–20 hr post infection in methionine-free Eagle's minimum essential medium containing Earle's salts (EMEM, Sigma Chemical) containing 20 μCi/ml [^{35}S]methionine (Amersham, 1,000 Ci/mmole). As described by Mao et al. (1997), the cells were lysed in 0.3 ml 125 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 2% 2-mercaptoethanol, and 0.01% bromophenol blue, boiled for 3–5 min, and separated by electrophoresis on 10% SDS-polyacrylamide gels (Laemmli, 1970). Viral proteins were visualized by autoradiography. Analysis of protein synthesis in RRV, TsUT, and TsND infected cells was similar to that described above with the following exceptions: cultures were incubated at 23 C rather than 26 C, and were radiolabeled from 23–27 hr postinfection with 30 μCi/ml [^{35}S]methionine. The change in conditions was prompted by the generally lower titers of RRV, TsUT, and TsND stocks compared to SsME and FV3, and by the inability of the former three viruses to replicate at 26 C. In all cases, cytopathic effect was observed at the time the cells were labeled.

Analysis of restriction enzyme fragment length polymorphisms (RFLP) of viral DNA: Confluent monolayers of FHM cells, grown on 60 mm tissue culture dishes, were mock-infected or infected with FV3 and SsME at 20 PFU/cell and 2 $TCID_{50}$/cell respectively. Frog virus 3 cultures were incubated at 26 C overnight while the SsME-infected cultures were incubated at 23 C. Mock and virus-infected cells were radiolabeled from 20–23 hr post infection in Dulbecco's Modified Eagle's medium containing 4% fetal calf serum (D4) and 25 μCi/ml [^{3}H] methyl-thymidine. Following labeling, the cells were lysed in 0.6 ml of 100 mM NaCl, 10 mM Tris-HCL, pH 8.0 25 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% SDS. Proteinase K was added to a final concentration of 166 ug/ml and the samples digested overnight at 37 C. The next day radiolabeled viral DNA was isolated by phenol-chloroform extraction. Viral DNA was resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (TE) and digested with *Hind* III and *Xba* I according to the manufacturer's (Promega, Madison, Wisconsin) recommendations. Viral DNA was separated by electrophoresis on 0.7% agarose gels and visualized by fluorography using Enhance (New England Nuclear). Analysis of radiolabeled viral DNA from TsUT, TsND, and RRV infected cells was performed essentially as described above with the following exceptions: MOIs for TsND, TsUT, and RRV were 1–5 $TCID_{50}$/cell; cultures were incubated at 23 C and radiolabeled from ~24–32 hr postinfection (TsUT and RRV) or 46–51 hr postinfection (TsND). As with the labeling of viral proteins in infected cells, conditions for radiolabeling with [^{3}H] thymidine were modified to reflect the input multiplicities of the different virus isolates and the temperature of incubation. In all cases, cells were labeled at times when cytopathic effect (cell rounding) was evident.

Sequence analysis of the major capsid protein gene: Total DNA was extracted from virus-infected cells essentially as described above and viral DNA, corresponding to the 5′ end of the major capsid protein gene, was amplified by PCR using primers (forward primer: 5′ GACTTGGCCACTTATGAC 3′; reverse primer: 5′ GTCTCTGGAGAAGAAGAA 3′) targeted to conserved regions within the coding region (Mao et al., 1997). Using these primers, an approximately 500 bp fragment was generated and cloned into pCRII as described by the manufacturer (Invitrogen, Carlsbad, California). Plasmid DNA bearing the viral insert was introduced into *Escherichia coli* strain INVαF′ by transformation and colonies bearing the viral insert selected by blue-white screening. Plasmid DNA was prepared by SDS-alkaline lysis of infected bacterial cells and the resulting plasmid DNA sequenced on a LiCor 4000 (Lincoln, Nebraska, USA) automated sequencer using primers targeted to flanking vector sequences. Initial processing of the raw sequence data was performed using DNAsis (Hitachi), whereas the final multiple alignments were performed using DNASTAR (Madison, Wisconsin).

RESULTS

Field observations and clinical signs

Field observations were made by biologists over a relatively short period of time and do not represent an in-depth analysis of all site conditions. During the mortality event at the WPA in North Dakota, thousands of dead larval tiger salamanders

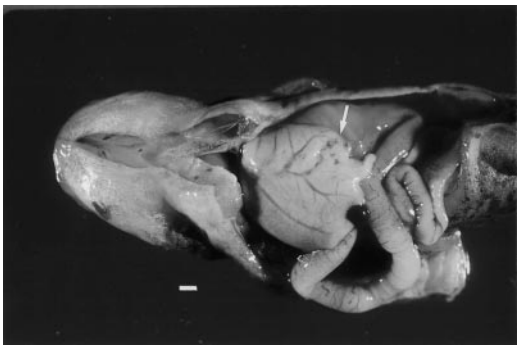

FIGURE 1. Iridovirus infected tiger salamander larva with severe gular edema and a large pale liver. Petechial hemorrhages are present in the subcutaneous tissue and liver (arrow). Bar=0.5 cm.

were observed along the shoreline and in the water. Aquatic vegetation, macro-invertebrates, minnows, northern leopard frogs (*Rana pipiens*), turtles, and water birds, appeared to be healthy.

Spotted salamander larvae at six separate sites in Maine suffered mortalities estimated in the hundreds.

In Utah, total mortality was unknown, however on a single visit in September, hundreds of dead tiger salamander larvae were seen along the shoreline and on the lake bottom. Apparently healthy birds and mammals were present, but no fish or other amphibians were noted. Sick larvae were lethargic and slow moving. They swam in circles, had buoyancy problems, and were unable to remain upright. Red spots or swollen areas were noted on the ventral surface near the gills and hind limbs.

Diagnostic findings

Most salamander larvae from all three mortality sites had small multifocal areas of subcutaneous hemorrhage on the ventral body surface and in particular on the plantar surface of the feet and in the inguinal areas and around the vent. Edema (Fig. 1) was present in the subcutaneous and intramuscular tissue of the gular and ventral thoracic areas. Livers were pale and swollen with multifocal hemorrhages (Fig. 1). The intestine was thin-walled, mildly distended with clear fluid or blood, and areas of hemorrhage were evident on the serosal surface.

Although tissues from all salamanders were examined microscopically, only four tiger salamander larvae from Lake Desolation, Utah were in suitable postmortem condition for subsequent analysis. Heart (Fig. 2), lung, trachea, glottis, gill, liver, pancreas, stomach, intestine, spleen, thymus, skeletal muscle, thyroid gland, gonad, brain (Fig. 3), skin, and adipose tissue from these tiger salamanders were examined microscopically. Microscopic pathology in these tissues was similar to that described and summarized for tiger salamanders in Saskatchewan by Bollinger et al. (1999). These changes included spherical amphophilic inclusions, consistent with viral inclusions, in the cell cytoplasm of all affected organs accompanied by varying degrees of necrosis. In addition to the changes described by Bollinger et al. (1999), intracytoplasmic inclusions also were present in cells of the meninges with mild multifocal meningitis and inclusions with necrosis were seen in brain, neuroepithelium of the nasal cavity, gill, adipose tissue, subcutaneous tissue, trachea and submucosa of the trachea, muscles of the neck and intermandibular space, and rarely involving multi-nucleated cells (possible osteoclasts) in the periosteum of long bones. Thyroid gland and thymus in these salamanders were not affected. Electron microscopy of ultra thin sections of spleen (Fig. 4), liver (Fig. 5) and intestine revealed intracellular icosahedral 130–150 nm diameter iridovirus-like particles.

Viruses were isolated, in all three cell culture types, from a variety of organs including spleen, gut, liver/kidney pool, testes, skin, and entire carcass, indicating a systemic infection (Table 1). Virus isolates produced a cytopathic effect in FHM which consisted of rounding up of infected cells followed by formation of focal plaque-like areas. Within 72–96 hr after initial inoculation these plaque-like areas coalesced resulting in 100% involvement

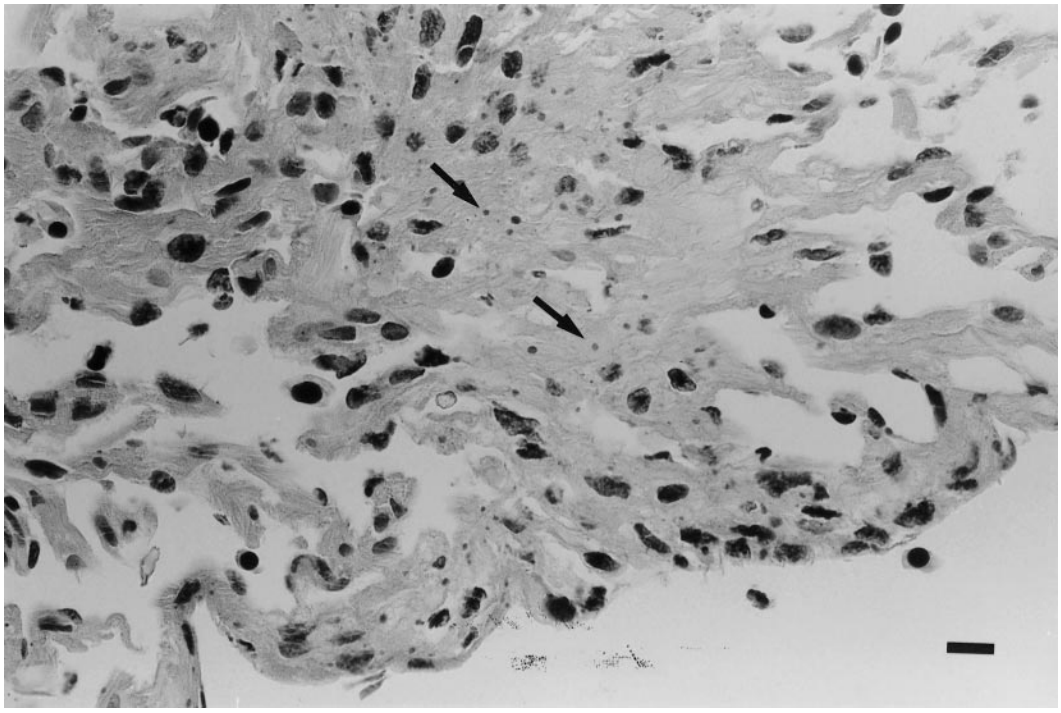

FIGURE 2. Intracytoplasmic inclusions (arrows) and necrosis at the ventricle of a tiger salamander heart. Heart failure may have contributed to the dependent edema pictured in Figure 1. HE. Bar=20 μm.

of the monolayer. When negatively stained preparations were viewed with an electron microscope, enveloped iridovirus-like virus particles (170–180 nm; Fig. 6) were seen.

Bacteriologic culture yielded a variety of organisms including *Acinetobacter baumannii, Aeromonas hydrophila, Plesiomonas shigelloides, Hafnia alvei,* and *Enterococcus* sp.

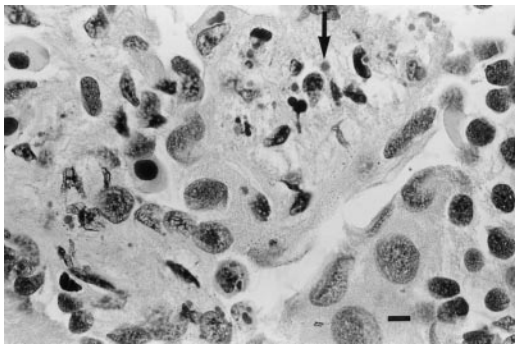

FIGURE 3. Salamander brain with focal necrosis associated with intracytoplasmic inclusions (arrow). HE. Bar=10 μm.

Molecular findings

Molecular characterization of virus isolates: Fathead minnow cell cultures were infected with each of the viral isolates and viral protein synthesis was analyzed at a time when CPE was marked. The right panel of Figure 7 compares profiles following infection with FV3 and SsME. The

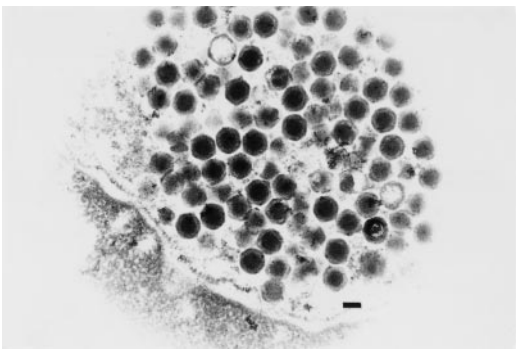

FIGURE 4. Electron micrograph of an intracytoplasmic inclusion containing non-enveloped icosahedral virus particles in the spleen cells of a tiger salamander. Bar=100 nm.

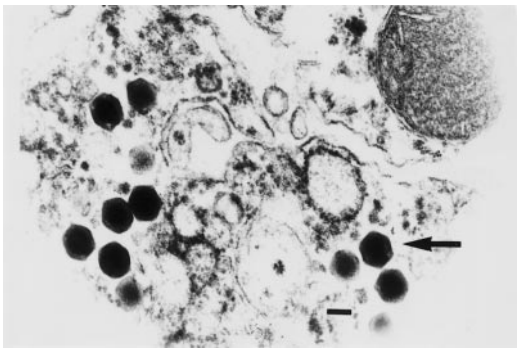

FIGURE 5. Electron micrograph of the icosahedral iridovirus-like particles (arrow) noted in the hepatocytes of a tiger salamander. Bar=100 nm.

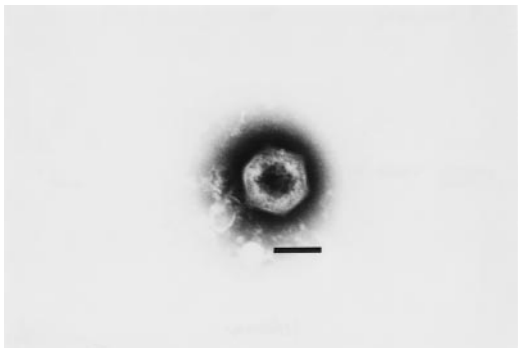

FIGURE 6. Electron micrograph of the tiger salamander iridovirus isolated in fathead minnow cell culture. Negative stained 2% phosphotungstic acid (pH 6.2). Bar=100 nm.

left panel of Figure 8 compares intracellular protein synthesis in cultures infected with FV3, RRV, TsUT, and TsND. Frog virus 3 and RRV were chosen for comparison because RRV is an iridovirus known to infect tiger salamanders whereas FV3 is the type species of the genus *Ranavirus* (Bollinger et al., 1999; Williams et al., 1999). In all cases, a prominent band at ~48 kDa was seen and is likely the major capsid protein. Profiles of protein synthesis in RRV, TsUT, and TsND-infected cells were remarkably similar with only minor differences in band intensity and electrophoretic mobility and are clearly different from that seen in FV3-infected cells. Likewise, there was marked similarity between FV3 and SsME. Despite the overall similarity, especially among proteins of mol weight <66 kDa, the three virus isolates differed in the mobility of several high molecular weight proteins. These results clearly indicate that the two isolates from tiger salamanders from Utah and North Dakota (TsUT and TsND) were similar to the earlier isolate from Canada (RRV), whereas the virus isolated from the Maine spotted salamander (SsME) is more similar to FV3 than to RRV, TsUT, and TsND.

To further characterize these isolates, viral DNA was subjected to RFLP analysis following digestion with *Hind* III and *Xba* I. These two enzymes were chosen since they were known to generate distinctive RFLP profiles for both FV3 and RRV (Mao et al., 1997; Bollinger et al., 1999). The two left panels of Figure 8 compare *Hind* III and *Xba* I digests of FV3 with RRV, TsUT, and TsND and show that although similar, these three isolates can be distinguished by their RFLP profiles. Likewise FV3 and SsME are similar (Fig. 8, right two panels), but distinguishable from each other and markedly different from the tiger salamander isolates. Thus the combination of SDS-PAGE and RFLP analysis allows us to place these six isolates into two groups, one composed of RRV, TsUT, and TsND and a second containing FV3 and SsME.

To more definitively determine the tax-

TABLE 1. Iridovirus isolations from the tissues of salamanders involved in the North Dakota, Utah, and Maine mortality events.

State	Tissues cultured	Iridovirus isolated
North Dakota	Liver and spleen	Spleen
Utah	Gut, liver/kidney pool, testes, brain swab, skin	Gut, liver/kidney pool, testes, skin
Maine	Liver, spleen, skin, entire carcass	Liver, spleen, skin, entire carcass

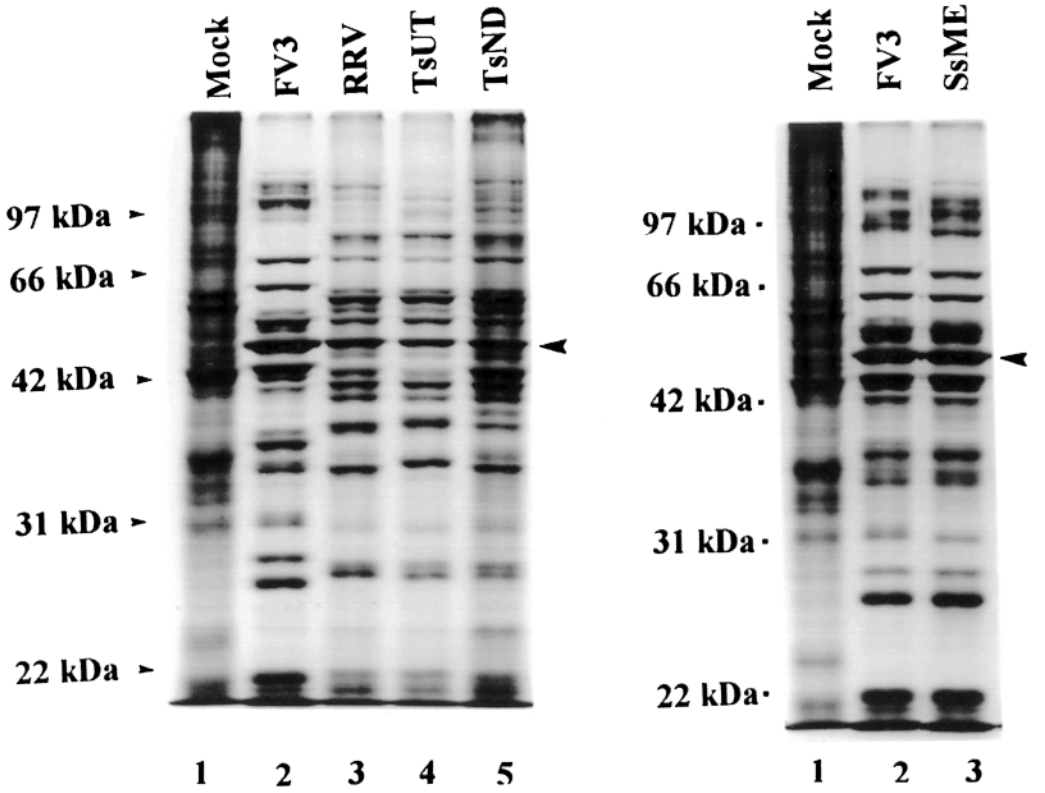

FIGURE 7. Protein synthesis in ranavirus-infected FHM cells. (Left Panel) FHM cells were mock-infected or infected with FV3, RRV, TsUT, or TsND and, when CPE was marked, labeled with [^{35}S]methionine. Cell extracts were analyzed by electrophoresis on denaturing 10% SDS-polyacrylamide gels and radio-labeled proteins were visualized by autoradiography. (Right Panel) FHM cells were mock-infected, or infected with FV3 and SsME and analyzed as above. Molecular weight markers are shown to the left of each panel and the position of the major capsid protein is marked with an arrowhead.

onomic relatedness of these six isolates, DNA corresponding to the 5′ end of the major capsid protein gene was amplified, cloned, and sequenced. A multiple alignment of the deduced amino acid sequence of the MCP from SsME, TsUT, TsND, FV3, and RRV is shown in Figure 9. Inspection of this 177 amino acid fragment indicates that SsME and FV3 are essentially identical. Likewise, TsUT, TsND, and RRV are similar to each other, but clearly distinguishable from FV3 and SsME. Although slight sequence differences among each group were noted, it is not known whether these differences represent PCR or sequencing artifacts, or micro-heterogeneity within the virus population. Taken together, these results indicate that mortality among western tiger salamanders was due to a RRV-like virus, whereas a die-off among Eastern spotted salamanders was attributed to a FV3-like virus.

DISCUSSION

Isolation of viruses from tissue samples taken from individuals involved in amphibian mortality events contributes critical information when determining the etiology of the event and the relationship of secondary infections. In earlier reports of salamander mortality there is no mention of virus isolation attempts. Worthylake and Hovingh (1989) described recurrent annual mortality of breeding adults and mass mortality of larval tiger salamanders at Desolation Lake, Utah, during July and

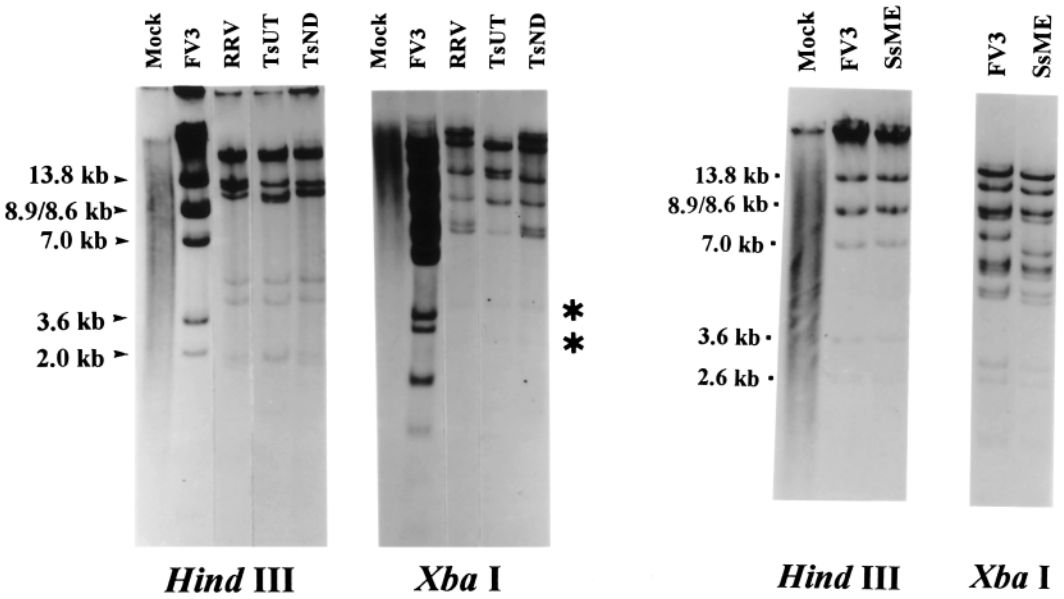

FIGURE 8. Restriction fragment length polymorphism analysis of viral DNA. (Left two panels) FHM cells were mock-infected, or infected with FV3, RRV, TsUT, or TsND and, when CPE was marked, labeled with [^{3}H]methyl-thymidine. DNA was isolated, digested with *Hind* III or *Xba* I, separated by electrophoresis on 0.7% agarose gels, and visualized by fluorography. (Right two panels) Analysis of FHM cells infected with FV3 and SsME. Molecular weight markers are shown to left of each panel. Asterisks (*) mark two faint bands detected in digests of RRV, TsUT, or TsND DNA.

August of 1982–86. In each year, maximum adult mortality was never greater than 37 individuals, however larval mortality in 1983, 1984, and 1985 was 4,949, 12,703, and 26,780 respectively. The hind limbs and vent of affected larvae were described as red and swollen and on internal examination diffuse hemorrhage was noted in the kidney, spleen, and stomach. Although the etiologic agent in the 1983–85 mortality events was identified as an *Acinetobacter* sp., virus cultures were not attempted. The necropsy findings, histopathology, and virology in the tiger salamanders we examined from Desolation Lake in 1998 clearly indicated that an iridovirus was the cause of death. *Acinetobacter baumannii* and other bacteria were isolated from organs of the larvae submitted to the NWHC in 1998. The larvae were in good body condition with no significant inflammation or bacteria noted microscopically in any tissue. This suggests that these bacteria were likely incidental or secondary to the iridovirus infection. Cunningham et al. (1996) hypothesize that primary iridovirus infections, with or without secondary infections by opportunistic pathogens, may cause outbreaks of redleg, which was previously considered to be of bacterial etiology only. Because virus isolation was not attempted on the samples from the previous Desolation Lake tiger salamander mortality events, the possibility of viral etiology remains an open question.

The gross and microscopic changes in the salamanders described in this paper were consistent with those reported in tiger salamanders from Canada (Bollinger et al., 1999). In addition, there was severe myocarditis, focal encephalitis, mild meningitis, and necrosis of neuroepithelial tissue associated with viral inclusions. Severity of the virus-associated necrosis in the heart may have contributed to the gular edema, similar to bottle jaw in cattle with congestive heart failure. The lesions described in tiger salamanders dying in Can-

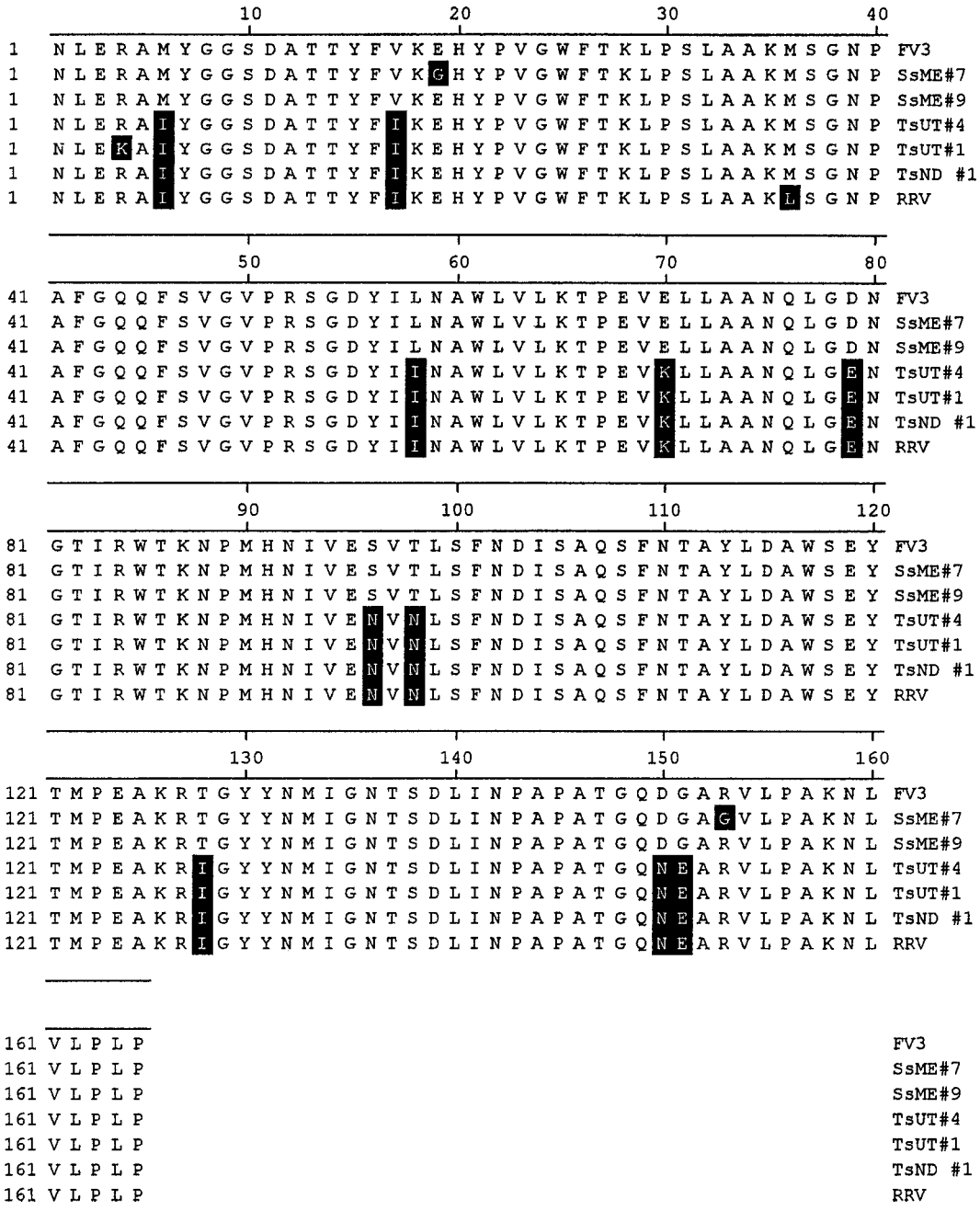

FIGURE 9. Sequence analysis of the major capsid protein. The 5′ end of the MCP gene was amplified using primers targeted to conserved regions within the coding region. Amplified DNA was cloned, sequenced, and the deduced amino acid sequences aligned using the program MegaAlign (DNASTAR). The sequences of two clones each of SsME (#7 and #9) and TsUT (#1 and #4) and a single clone of TsND (#1) were compared to RRV and FV3. Differences between FV3 and a specific sequence are indicated by shading in black. The six amino acids encoded by the primers (DLATYD and FFFSRD) are not shown.

ada (Bollinger et al., 1999) and those we examined from Utah, were consistent with a lethal, sudden onset viremia with viral inclusions in almost all organs. These changes differ from the chronic proliferative skin disease described in the Sonora tiger salamanders from Arizona (Jancovich et al., 1997).

Only recently have large mortality events associated with ranaviruses been noted among amphibians (Chinchar, 2002). Ranaviruses have been linked to focal die-offs of frogs and salamanders in England, North America, China, and Thailand (Cunningham et al., 1996; Jancovich et al., 1997; Kanchanakhan, 1998; Bollinger et al., 1999; Zhang et al., 2001; and NWHC, unpubl. data). Sequence analysis of virus isolates from die-offs among frog species in China, Thailand, and the US support the view that illness is attributable to a FV3-like agent (Zhang et al., 2001). It is not known whether this reflects the appearance of new, highly virulent FV3 strains, severe illness secondary to immune suppression, or to enhanced awareness due to increased surveillance and virus isolation attempts.

Increased attempts to detect genomic differences may be the only way to determine whether there is strain or species variation among iridovirus isolates from amphibians. When characterizing the specific agent involved in an outbreak of iridoviral disease, molecular analysis is essential because electron microscopy and serologic analysis cannot distinguish among different species within the genus *Ranavirus.* It should be appreciated that of the three molecular techniques used (SDS-PAGE analysis; RFLP profiles, and sequence analysis), SDS-PAGE analysis is the least discriminatory technique. Moreover, while sequence analysis can provide useful phylogenetic and quantitative information, its sensitivity is limited by the gene analyzed. Restriction fragment length polymorphism analysis is potentially the most sensitive approach for detecting differences among isolates because it involves the complete viral genome and not just the sequenced gene, but the data developed are not useful for the construction of phylogenetic trees or for quantitation. Considering the results of the molecular techniques described here, the iridovirus isolates from TsND and TsUT are similar to each other and RRV, but dissimilar to FV3 or SsME. Coupled with sequence data from the Arizona tiger salamander isolate (Jancovich et al., 1997), the same viral species is capable of infecting tiger salamanders in a region extending from southern Arizona to central Saskatchewan. In contrast, the SsME isolate is similar to FV3 but not similar to the TsND, TsUT, or RRV.

Challenge assays are needed to determine the host range of the iridoviruses isolated from salamanders and to determine whether tiger salamanders and spotted salamanders are susceptible to the heterologous virus isolates. The 1998 mortality events in North Dakota, Utah, and Maine involved only salamanders and it has not been determined whether the isolates are capable of infecting other species of amphibians, fish, or reptiles. Earlier work has shown that some iridoviruses may not be species-specific. For example, experimental studies have shown that Bohle iridovirus, isolated from the ornate burrowing frog (*Limnodynastes ornatus),* is able to infect and cause death in other species of amphibians (Cullen, 1995) as well as in some species of fish (Moody and Owens, 1994). In addition, one FV3 strain, designated Redwood Creek virus, was recently identified among sympatric stickleback fish (*Gasterostelus aculeatus*) and red-legged frogs (*Rana aurora*) in California (Mao et al., 1999). Although sequence analysis readily distinguishes TsUT and TsND from SsME, it is not known whether TsUT and TsND are pathogenic for spotted salamanders or whether SsME is pathogenic for tiger salamanders.

The NWHC has more than 60 iridovirus isolates from 12 amphibian species involved in mortality events in 18 states from

1998 through 2001. Currently the NWHC is preparing RFLP analysis profiles to determine whether these isolates are similar to FV3 or each other. Any isolates that are dissimilar to FV3, will be analyzed further by SDS-gel analysis and sequence analysis.

Although molecular analysis can reveal genomic differences between iridovirus isolates, amphibian mortality events are seldom followed up with systematic epizootiologic studies. The method of virus transmission, virus maintenance in the environment, environmental conditions accompanying mortality events, species at risk, and effect of the event on the amphibian population are all important to understanding these local mortality events and their contribution to more widespread amphibian decline.

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