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Repeated Detection of an Invertebrate Iridovirus (IIV) in Amphibians

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Abstract

Invertebrate iridoviruses (IIVs) (family: *Iridoviridae*) are known pathogens for invertebrates, causing high mortality and reduced fertility in affected insects. Over the past 2 decades, IIVs have also been increasingly found in lizards in association with nonspecific clinical signs. It has been hypothesized that IIVs from insects can also infect reptiles. From 2010-2011, IIVs were repeatedly detected via polymerase chain reaction testing and virus isolation methods in routine diagnostic samples from different amphibians: 3 blue poison dart frogs (*Dendrobates tinctorius azureus*), 4 edible frogs (*Pelophylax* kl. *esculentus*), a giant ditch frog (*Leptodactylus fallax*), an Amazon milk frog (*Trachycephalus resinifictrix*), mixed organs from agile frogs (*Rana dalmatina*), a black-spined toad (*Bufo melanostictus*), and one Lake Urmia newt (*Neurergus crocatus*). IIVs were found in skin swabs from apparently healthy animals, as well as in multiple organs of frogs that died of unknown causes. Prey insects (crickets) from one owner also tested positive for the presence of IIV. The obtained partial sequences from the major capsid protein (MCP) gene (222nt) from each of these were 100% identical to each other and 98% identical to IIV-6, the type species of the genus *Iridovirus*. Although the pathogenicity of IIV in amphibians remains unclear, these findings provide further evidence that IIVs may be able to infect vertebrates under some conditions and underline the importance of the genus *Iridovirus* in vertebrates.

Keywords: anuran, frog, Invertebrate Iridovirus 6, newt, urodele, cricket

Introduction

Iridoviruses are large double-stranded, cytoplasmic DNA viruses. The family *Iridoviridae* consists of five recognized genera, which are important pathogens for ectothermic vertebrates (genera: *Lymphocystivirus, Megalocytivirus, Ranavirus)* and insects *(*genera: *Iridovirus, Chloriridovirus)* (Jancovich *et al*., 2012). Another group of closely related viruses with a predilection for red blood cells, Erythrocytic iridoviruses (EIVs), have been found in squamates, fish, and amphibians. Phylogenetic investigations on squamate EIVs indicate that these viruses may represent a novel iridovirus genus (Wellehan *et al*., 2008; Alves de Matos *et al*., 2011).

To date, the genus *Iridovirus* comprises two species: *Invertebrate iridescent virus 1* (IIV-1, syn. Tipula iridescent virus [TIV]) and *Invertebrate iridescent virus 6* (IIV-6, syn. Chilo iridescent virus [CIV]), as well as numerous unclassified isolates. Confirmed or putative infections with invertebrate iridoviruses (IIVs) have been reported in more than 100 species of invertebrates from various habitats on all continents except Antarctica. The infection causes lethal disease in susceptible insects manifested by hypertrophy and bluish iridescence of the affected fat body cells arising from the quasicrystalline arrangement of virus particles in the host cells. However, covert sublethal infections were reported in several host species and may have reduced the former interest in their potential use for controlling important agricultural pest and vector insect species (Williams, 2008).

Cricket iridovirus (CrIV), a variant of IIV-6, was identified as the causative agent for unusual mortalities and reduced fertility and lifespan in diseased animals (field crickets [*Gryllus campestris*], house crickets [*Acheta domesticus*]) from a commercial cricket producer in the Netherlands (Kleespies *et al*., 1999; Jakob *et al*., 2002). Another closely related iridovirus

(Gryllus bimaculatus iridovirus [GbIV]) has been detected in crickets from a breeder of field crickets (*Gryllus bimaculatus*) in Germany (Just and Essbauer, 2001). Investigation on the host range of CrIV demonstrated that the virus can be transmitted orally to other orthopteran species, reflecting a considerable problem for commercial insect breeders.

Since 1998, IIVs have also been repeatedly detected in reptilian hosts including bearded dragons (*Pogona vitticeps*), a four-horned chameleon (*Chamaeleo quadricornis*), a high-casqued chameleon (*Chamaeleo hoehnelii*), a frilled lizard (*Chlamydosaurus kingii*), green striped tree dragons (*Japalura splendida*), an Asian glass lizard (*Dopasia gracilis*), and a green anole (*Anolis carolinensis*) (Just *et al*., 2001; Weinmann *et al*., 2007; Behncke *et al*., 2013; Stöhr *et al*., 2013a), as well as in numerous other insectivorous lizards (authors unpublished observations; Papp *et al*., 2014).The IIV detected in the high-casqued chameleon was 100% identical to GbIV on partial sequences of the major capsid protein (MCP) gene. It has therefore been hypothesized that IIV from prey insects might be transmitted to reptiles. Over a period of two years (2010-2011), IIVs were repeatedly detected in diagnostic samples from amphibians. This paper describes these cases from different amphibian species and the partial characterization of the isolated viruses.

Materials and Methods

Samples

Samples from apparently healthy, quarantined animals, as well as from dead amphibians were submitted for virological testing. The different samples (skin swabs / organs) were obtained from blue poison dart frogs (*Dendrobates tinctorius azureus*) (n=4), edible frogs (*Pelophylax* kl. *esculentus*) (n=4), a giant ditch frog (*Leptodactylus fallax*), an Amazon milk frog (*Trachycephalus resinifictrix*), agile frogs (*Rana dalmatina*) (n=6), a black-spined toad (*Bufo*

melanostictus), and Lake Urmia newts (*Neurergus crocatus*) (n=3). Prey insects (crickets) from one owner were also submitted for virological examination. Short case histories and a list of tested samples are given in Table 1. No samples were available for histopathological examination.

Virological testing

Samples were taken from each animal separately and submitted in cell culture medium (Dulbecco's modified Eagle medium (DMEM), Biochrom AG, Berlin, Germany) supplemented with antibiotics. The samples were sonified, centrifuged at low speed, and inoculated onto iguana heart cells (IgH-2, ATCC: CCL-108) for virus isolation as described previously (Stöhr *et al*., 2013b). DNA was extracted from the original sample or from the cell culture supernatant using a commercial DNA extraction kit (DNeasy Kit®, Qiagen GmbH, Hilden, Germany), and routine diagnostic polymerase chain reactions (PCRs) for the detection of IIV targeting a part of the MCP gene were done as described previously (Weinmann *et al*., 2007). All samples were also tested for the presence of ranaviruses (Mao *et al*., 1997; Marschang *et al*., 1999). The obtained PCR products were separated by agarose gel electrophoresis (1.5% agarose gel; Biozym Scientific GmbH, Hessisch Oldendorf, Germany) in TAE buffer containing 0.5 µg/mL ethidium-bromide and visualized under 320 nm UV light. Afterwards, the PCR amplicons were cut and gel purified using a gel extraction kit (peqGOLD Gel Extraction Kit®, Peqlab Biotechnologie GmbH, Erlangen, Germany) and sent for sequencing from both directions to a commercial company (MWG Biotech AG, Ebersberg, Germany). Obtained sequences were edited, assembled, and compared using STADEN Package version 2003.0 Pregap4 and Gap4 programmes (Bonfield *et al*., 1995). Finally, the sequences were compared to those in GenBank (National Center for Biotechnology Information, Bethesda, MD) online (http://www.ncbi.nih.gov/blast/) using

BLASTN option and to the local iridovirus database of the Fachgebiet für Umwelt- und Tierhygiene at Hohenheim University.

Results

IIVs were found in at least 12 animals belonging to 7 amphibian species from 5 collections (Table 1). In some cases, IIVs were found in skin swabs from apparently healthy animals. Other animals died and IIVs were detected in different organs from these animals; one of these animals, a Lake Urmia newt, was coinfected with a ranavirus. Crickets collected from one owner also tested positive for the presence of IIV. The partial sequences from the MCP genes (222nt) of all detected IIVs were 100% identical to each other and 98% identical to IIV-6, the type species of the genus *Iridovirus* (AF303741), as well as 100% identical to CIV and GbIV, which have been previously found in crickets and lizards (Kleespies *et al*., 1999; Just and Essbauer, 2001; Just *et al*., 2001).

Discussion

This is the first description of IIVs in amphibians. Unfortunately, little is known about the impact of these pathogens in vertebrates. Clinical signs observed in lizards infected with IIVs have been relatively non-specific and included poor body condition, skin lesions, pneumonia, hyperemic liver, and enlarged spleen (Just *et al*., 2001; Weinmann *et al*., 2007; Papp *et al*., 2014). Recently, coinfections of IIVs with other viruses (ranavirus and/or adenovirus) have been described in a number of severely diseased lizards with various clinical signs, as well as skin alterations (Behncke *et al*., 2013; Stöhr *et al*., 2013a). In these animals, IIVs have been found in the skin of Asian glass lizards, green anoles, and in the skin and internal organs of dead green striped tree dragons. Ranaviruses – which are known pathogens for ectothermic vertebrates – were also found in these samples and were considered to be one of the causative

agents for the disease. Interestingly, IIVs were also detected in the skin (swabs/tissue samples) from 8 amphibians in this study. However, the severe course of disease observed in the Lake Urmia newts was most likely caused by a ranaviral infection. IIVs are relatively commonly found in oral or cloacal swabs from insectivorous lizards (authors unpublished observations); however, it is unclear whether the virus replicates in these animals or if the viruses detected only passed through the animals via ingestion of infected prey insects. The interpretation of the presence of IIV in samples from the skin or the gastrointestinal tract in amphibians poses the same problem of possible environmental contamination from infected prey sources. However, the fact that IIV was found in samples from internal nondigestive organs during this study clearly indicates that these animals were infected with the virus: IIV was detected in pooled samples (liver and kidney) from agile frogs and in the kidney from an edible frog which died of unknown causes during hibernation. The edible frog was the only animal in which macroscopic pathological changes were found in the tested tissue sample (reddening of the kidneys) and no other virus was detected. Nevertheless, these results have to be interpreted with caution, as no histopathological examination was carried out; this could have helped confirm virus-related tissue alterations in these organs. Virus detection in tissue extracts by PCR or cell culture could also reflect the presence of virus in the blood (either in the plasma or in the cell component or both) and not necessarily in the tissue parenchyma itself. No clinical signs were observed in the prey insects submitted by one of the owners, which tested positive for the presence of IIV, and sequencing results indicated that the same virus was found in all tested animals.

A number of viruses affecting lower vertebrates are known to have a wide host range, but the diversity of host specificity patterns is still poorly understood (Bandin and Dopazo, 2011). However, the ability of large DNA viruses to replicate completely in the cytoplasm seems to

be strongly connected with an increased ability to jump hosts, compared to those with intranuclear replication (Pulliam and Dushoff, 2009). Field data, experimental trials, and genomic studies have demonstrated that members of the family *Iridoviridae* (genus: *Ranavirus*) are capable of infecting hosts from different poikilothermic classes (Duffus *et al*., 2015). Ranaviruses have also been found in invertebrates, and mosquitoes may be a possible vector for ranavirus transmission to terrestrial turtles (Kimble *et al*., 2014). Previous studies provided evidence that highly infected but clinically healthy insects might infect insectivorous reptiles with IIV (Weinmann *et al*., 2007). In per os infection trials with bearded dragons, IIVs were also detected in nondigestive organs (Papp, 2014). It is therefore possible that the amphibians in our study were infected by the crickets fed to them.

Investigations on ranaviruses have demonstrated that the MCP gene may not be a suitable target to distinguish different virus strains and that comparison of partial sequences may show viruses to be more closely related than they actually are (Duffus and Andrews, 2013). Since the MCP gene in IIVs is also highly conserved, and only a small portion has been sequenced, it is possible that the isolates detected in this study may also differ from each other. Sequencing of the complete MCP gene, or other more variable regions, would be useful to learn more about the isolated viruses.

Interspecies transmission has been demonstrated for different IIVs (Williams *et al*., 2005), and a GbIV isolated from a high-casqued chameleon has been shown to be pathogenic for crickets (*Gryllus bimaculatus*) (Weinmann *et al*., 2007). Experimental infection trials with amphibians supported by virological methods (e.g., electron microscopy of internal organs for the detection of iridoviral virions in infected tissue, in situ hybridization to determine invertebrate iridovirus infection in vertebrate cells, or the use of reverse transcriptase [RT]

PCR to prove virus replication) should be considered in future studies to determine if IIV is able to cause disease in amphibians. Furthermore, histopathological investigations of affected tissues (skin and internal organs) from animals with and without apparent clinical signs would help to elucidate ongoing changes at the cellular level consistent with viral infections. Although the pathogenicity of IIV in amphibians remains unclear, the detection of IIV in amphibians provides further evidence that these viruses may be able to infect vertebrate hosts under some circumstances and underlines the importance of the genus *Iridovirus* in vertebrates.

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279 Table 1: Samples from the different amphibian species included in this report with short case
280 histories, the results of virus isolation on cell culture (IgH-2), and the PCR for the presence of
281 invertebrate iridovirus (IIV) and ranavirus.

Date of testing	Species	Case history	Samples	IIV isolation in cell culture	IIV PCR from original sample	Detection of ranavius
09/2010	Blue poison dart frogs (*Dendrobates tinctorius azureus*)	Apparently healthy animals from a zoo in Switzerland. Newly obtained from a private breeder, in quarantine.	Skin swabs from 4 animals	3 positive	3 postive	negative
03/2011	Edible frogs (*Pelophylax* kl. *esculentus*)	Group of animals from various European ponds infected with ranaviruses. Dead + apparently healthy animals tested for virus shedding over a period of 3 years (Stöhr *et al*., 2013b). Animal died during hibernation, reddening of the kidneys.	Kidney, liver, skin	positive (kidney)	negative	negative
04/2011		See above – clinically healthy animals.	Skin swabs from 30 animals	3 positive	2 positive	negative
07/2011	Lake Urmia newts (*Neurergus crocatus*)	Group of animals imported from Iraq in April 2011. 10/11 animals died due to ranaviral infection. Clinical signs: anorexia, apathy, ulcerative dermatitis, systemic haemorrhages, granulomatous hepatitis (Stöhr *et al*., 2013c).	Skin + mixed organs (liver, kidney) from 3 animals	1 positive (skin)	negative	via PCR in this and one other animal (skin + organs)
07/2011	Giant Ditch Frog *(Leptodactylus fallax)*	Deaths of several amphibians in a zoological institution in the United Kingdom	Frozen large intestine wall lesion	positive	positive	negative
			Frozen large intestine mass	positive	positive	negative
07/2011	Amazon milk frog *(Trachycephalus resinifictrix)*		Frozen pyloric nodule	negative	positive	negative
07/2011	Agile frogs *(Rana dalmatina)*		Frozen mixed organs (liver, kidneys) from six animals	negative	positive	negative

			Frozen skin from six animals	negative	negative	negative
09/2011	Crickets	Prey animals from the zoological institution in the United Kingdom	Animals (fat bodies)	positive	not done	not done
08/2011	Black-spined toad (*Bufo melanostictus*)	Temporary housing in a reptile rescue center in Germany, no clinical signs.	Skin swab	positive	positive	negative

282