

# Comparative Pathology of Ranaviruses and Diagnostic Techniques

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## 1 Ranaviral Disease

### 1.1 Introduction

Ranaviruses were detected and diagnosed as a disease agent in amphibians in the 1960s, and in reptiles and fish in the 1980s (Duffus et al. 2015). Since these initial cases, ranaviruses have been linked to numerous epizootic mortality events in these three classes of lower vertebrate animals (Duffus et al. 2015). Although long-term population data are generally lacking, there is evidence that ranaviruses can cause population declines in amphibians (Teacher et al. 2010; Beebee 2012; Earl and Gray 2014; Price et al. 2014). Further, the nearly complete loss of entire age classes of fish and amphibians due to ranavirus outbreaks have been reported (Petranka et al. 2003; Todd-Thompson 2010; Waltzek et al. 2014; Wheelwright et al. 2014), and some species of global conservation concern are highly susceptible (Geng et al. 2010; Sutton et al. 2014a). More recently, the potential economic impact of ranaviruses in farmed fish and amphibians (Mazzoni et al. 2009; Waltzek et al. 2014), and on recreational fisheries (Grizzle and Brunner 2003) has been recognized. Given

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these concerns, ranaviruses that infect amphibians and *Epizootic hematopoietic necrosis virus* (EHNV) are listed as notifiable agents by the World Organization for Animal Health (OIE, Schloegel et al. 2010).

Despite the global awareness of ranaviruses, the distribution of ranaviruses and their effects on host populations and international commerce remain poorly understood. Further, there is limited information on the mechanisms that affect host–ranavirus interactions and factors that lead to mortality events (Gray et al. 2009). Various field studies and controlled experiments are expanding our knowledge of ranaviruses (Duffus et al. 2015; Brunner et al. 2015; Jancovich et al. 2015b); however, more research is needed. Properly designed studies (Gray et al. 2015) are a first step to investigating hypotheses associated with ranavirus emergence. Use of appropriate diagnostic techniques is key to identifying ranavirus infections and determining the effects of infection on host species. Understanding the differential diagnoses for ranaviral disease is important, and combining infection data with pathological and environmental information is essential to confirm that ranavirus is the etiologic disease agent.

In this chapter, we begin with an overview of the gross and microscopic lesions associated with ranaviral disease followed by a discussion of the current diagnostic tests in use by research and veterinary diagnostic laboratories worldwide. We point out the limitations of certain diagnostic techniques, and identify needed areas of improvement. Finally, we briefly discuss research into treatment and vaccine development for ranaviruses.

## ***1.2 Ranaviral Disease Pathology***

### **1.2.1 Field and Clinical Findings**

In amphibians, outbreaks of ranaviral disease are most often observed in larvae and recently metamorphosed animals (Green et al. 2002; Docherty et al. 2003; Balseiro et al. 2009, 2010); however, outbreaks that include adult animals are increasingly recognized (Cunningham et al. 2007; Cheng et al. 2014). Moreover, Earl and Gray (2014) demonstrated that ranavirus-associated mortality of larvae or metamorphs was sufficient to cause population declines in highly susceptible species. Researchers have demonstrated a strong correlation between infection prevalence and mortality in laboratory experiments with *Frog Virus 3* (FV3)-like ranaviruses (see Haislip et al. 2011; Hoverman et al. 2011; Brenes et al. 2014a); thus, high infection prevalence during field surveillance may be an indicator of an impending die-off (Gray et al. 2015).

Mortality events often present as sudden and massive deaths across multiple species (Todd-Thompson 2010; Wheelwright et al. 2014). Deaths may continue for weeks, with later deaths due to individuals succumbing to secondary bacterial or fungal infections (Jancovich et al. 1997; Cunningham et al. 2007; Miller et al. 2008; Cheng et al. 2014). Field signs vary but lethargy is commonly reported in all classes

(Table 1). Chelonians can have additional signs of respiratory distress (Ruder et al. 2010; Farnsworth and Seigel 2013). Prior to death, diseased amphibians and fish may exhibit erratic swimming, loss of buoyancy, and loss of righting reflex (Mao et al. 1999; Bollinger et al. 1999; Zilberg et al. 2000; Geng et al. 2010; Miller et al. 2011). Recently, a vestibular syndrome has been observed in cultured adult bullfrogs (*Lithobates catesbeianus*) in Brazil (R. Mazzoni, CRMV-GO, Brazil, personal communication), and may explain these changes in coordination. Future investigation of the histologic changes within the brain of animals displaying a lack of coordination is needed.

### 1.2.2 Gross Pathology

The appearance of disease in individual animals reflects the systemic distribution of the virus and associated host response. Clinical disease is typically acute and can affect a high proportion of the population. In wild populations, the acute course of disease and rapid mortality might prevent detection of the disease event (Gray et al. 2015), or outbreaks might present as a large number of dead individuals. Clinically affected individuals are generally preferred to use for testing because they are most likely to yield a diagnosis. Affected individuals present with hemorrhages, edema, and necrosis as the most common gross lesions; however, the presentation of these

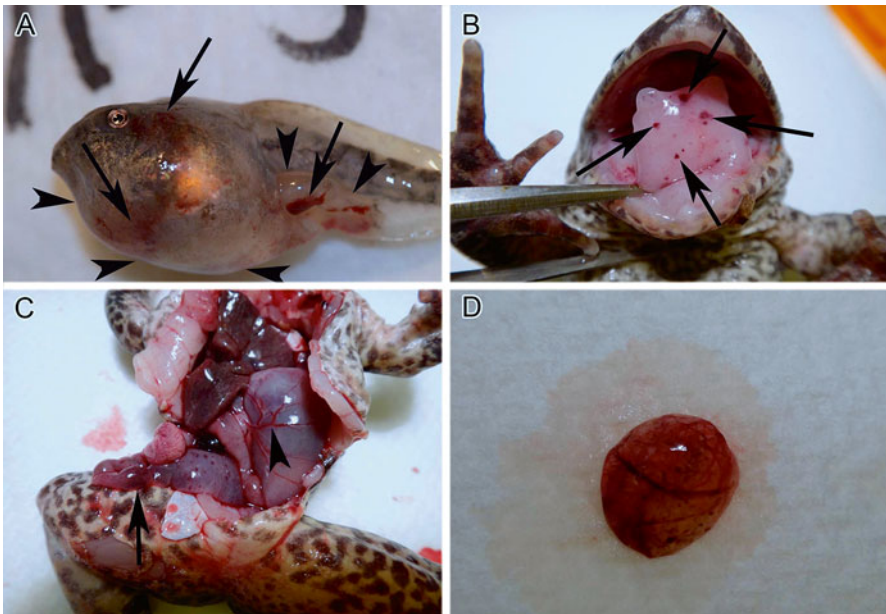
**Table 1** Examples of field (clinical) signs and gross changes that can be observed in individuals with ranaviral disease

Class	Lesion
Amphibian larvae	Loss of buoyancy; erratic swimming; anorexia; swelling (edema) of the body, head, legs, and internal soft tissues; external hemorrhages (especially around the vent, periocular, gular region, legs); occasional internal hemorrhages (especially pronephros, liver, spleen)
Anuran adults	Lethargy; anorexia; loss of buoyancy and erratic swimming (aquatic species); swelling (edema) of legs, feet, body, and internal soft tissues; skin ulcers; dermal, oral, and internal hemorrhages (ecchymotic, petechial); friable (necrotic) organs
Caudate adults	Lethargy; anorexia; loss of buoyancy and erratic swimming (aquatic species); hemorrhages (especially on tail and plantar surfaces of feet); swelling (edema); skin ulcers; internal hemorrhages (ecchymotic, petechial); friable (necrotic) organs; necrosis of extremities (Chinese Giant Salamanders)
Fish	Loss of buoyancy; erratic swimming; anorexia; red swollen gills; hemorrhages (especially periocular, fat bodies, swim bladder); overinflated swim bladder; friable (necrotic) organs; multiple pale foci in liver
Chelonians	Respiratory difficulty; anorexia; oral necrotic plaques; swelling (edema or rarely necrosis) of head, neck, legs, internal soft tissues, periocular; skin ulcers; friable (necrotic) organs; hemorrhages (especially internal)
Lizards	Lethargy; anorexia; oral necrotic plaques; skin ulcers; friable (necrotic) organs; occasional internal hemorrhages and edema
Snakes	Lethargy; anorexia; oral and nasal ulcers

changes can vary depending on the species affected and whether exposure to environmental stressors or other pathogens also occurs (Table 1).

## Amphibians

Hemorrhages (ecchymotic and petechial) and erythema are common in the skin of anurans and caudates (Fig. 1a, b). Hemorrhages are most often present on the ventral surfaces near the vent, rear legs, and gular regions, but can also be observed around the eyes, ear drum, tongue, tail, and feet (Balseiro et al. 2009; Cheng et al. 2014; Cunningham et al. 2007; Docherty et al. 2003; Geng et al. 2010; Kik et al. 2011; Meng et al. 2014; Sutton et al. 2014a). Raised skin plaques or polyps have been described in tiger salamanders (*Ambystoma tigrinum*) and Chinese giant salamanders (Jancovich et al. 1997; Bollinger et al. 1999; Geng et al. 2010). Other



**Fig. 1** Gross lesions seen in amphibians with ranaviral disease. (a) Hemorrhages (arrows) and edema (arrowheads) in a wood frog (*Lithobates sylvaticus*) tadpole experimentally challenged with an FV3-like ranavirus. (b) Tongue hemorrhages (arrows) in a dusky gopher frog (*Lithobates sevosus*) experimentally challenged with an FV3-like ranavirus. (c) Intestinal hemorrhage (arrow) and congested blood vessels (arrowhead) in a dusky gopher frog experimentally challenged with an FV3-like ranavirus. (d) Tan friable and hemorrhagic spleen of a dusky gopher frog experimentally challenged with an FV3-like ranavirus

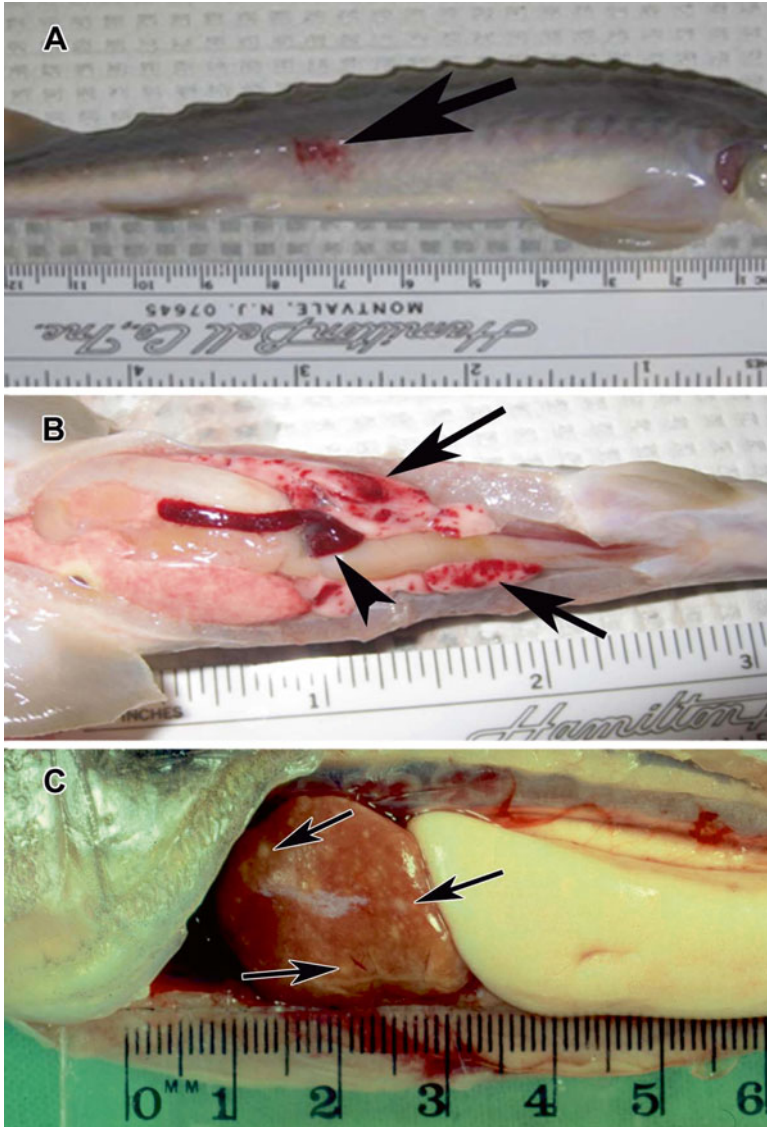
findings in the skin of anurans and caudates can include ulceration or rough discolored gray areas (e.g., Bollinger et al. 1999; Cunningham et al. 2007; Kik et al. 2011). Swelling of the legs, body, and head from accumulation of fluid (i.e., edema) within the tissues, lymph sacs, and body cavity is commonly seen in amphibians, and is especially evident in larvae (e.g., see Wolf et al. 1968; Miller et al. 2011; Meng et al. 2014).

Internally, hemorrhage and necrosis are common findings, especially in the spleen, pronephros and mesonephros (kidney), and liver (Fig. 1c, d). The cause of cell death may be associated with apoptosis or virus replication (Grayfer et al. 2015). Necrosis may present as generalized friable organs or as discrete pale foci scattered throughout an organ. Splenomegaly and hepatomegaly have also been reported (Kik et al. 2011) and may be related to congestion and hemorrhage. Intestinal hemorrhage has been seen in mortality events and in experimentally challenged anurans and caudates (Bollinger et al. 1999; Geng et al. 2010; Cheng et al. 2014; Meng et al. 2014). In Brazil, hemorrhage and necrosis are seen in the vestibular region of ranavirus-positive bullfrogs displaying vestibular syndrome (R. Mazzoni, CRMV-GO, personal communication).

In Europe, two syndromes have been described in adult common frogs (*Rana temporaria*), one which is systemic hemorrhages and the other which is extensive cutaneous ulcerations. Cunningham et al. (1996) first reported these syndromes from mortality events that occurred throughout Britain and were observed by members of the general public. Subsequently, both syndromes were experimentally reproduced in the common frog (Cunningham et al. 2007). Based on this result, the authors concluded that route of exposure and specific ranavirus isolate influenced the pathogenesis; however, both syndromes can develop within the same frog. The hemorrhagic syndrome is similar to pathological changes reported by Sutton et al. (2014a) in adult dusky gopher frogs (*Lithobates sevosus*).

## Fish

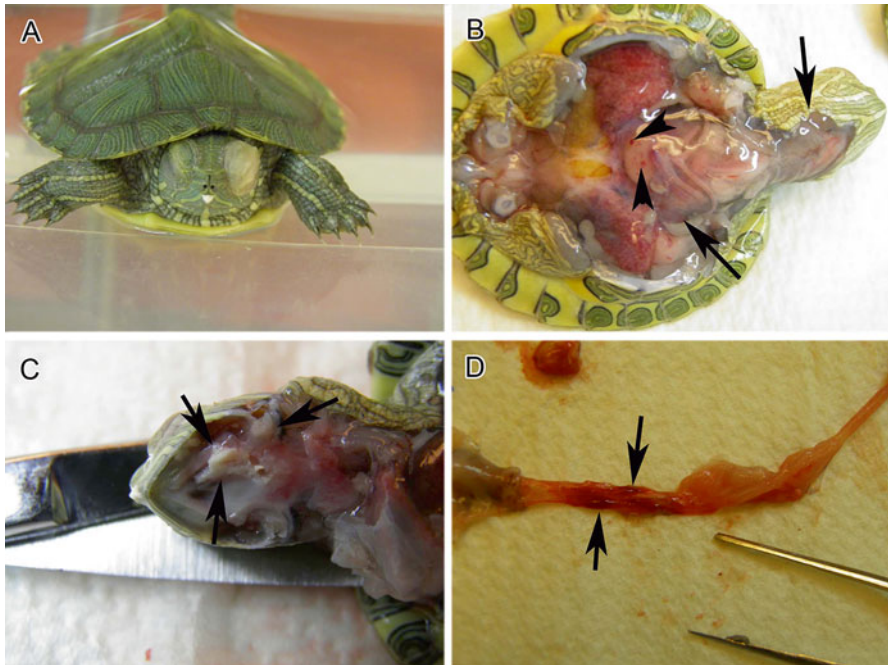
In fish, multifocal, random cutaneous hemorrhages are seen (Fig. 2a; Waltzek et al. 2014), and fish may have red swollen gills (Mao et al. 1999). Internally, hemorrhages may occur in any organ including the fat bodies and swim bladder, and organs may be friable (Fig. 2b, Zilberg et al. 2000; Waltzek et al. 2014). Over-inflation of the swim bladder has been reported (Grizzle and Brunner 2003). Reddacliff and Whittington (1996) provided detailed descriptions of lesions due to EHNV in redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*). Sick fish were dark, stopped eating, and sometimes were ataxic. Gross lesions included a swollen abdomen with swelling of the spleen and kidney; multiple pale foci were sometimes present in the liver. Zilberg et al. (2000) reported necrosis of the gastrointestinal (GI) mucosal epithelium, gills, and heart in largemouth bass (*Micropterus salmoides*) experimentally challenged with *Santee-Cooper ranavirus*.



**Fig. 2** Gross lesions seen in fish with ranaviral disease. (a) Pallid sturgeon (*Scaphirhynchus albus*) with cutaneous ecchymotic hemorrhage (arrow) due to an FV3-like ranavirus. Photo by Thomas B Waltzek, University of Florida. (b) Hemorrhage in the fat bodies (arrows) and spleen (arrowhead) of a pallid sturgeon with FV3-like ranavirus. Photo by Thomas B Waltzek, University of Florida. (c) Multifocal hepatic necrosis evidenced by areas of pale discoloration in the liver (arrows), and ecchymotic hemorrhage in the retroperitoneum of an adult redbfin perch (*Perca fluviatilis*) infected with EHNV

## Reptiles

Most lesions in reptiles have been described in chelonians and include periocular swelling, ulceration and necrosis of the oral cavity, swollen head and extremities, ocular and nasal discharges, and occasional skin ulcerations (Fig. 3, e.g., Johnson et al. 2008; Ruder et al. 2010). Similarly, Hyatt et al. (2002) reported ulceration of the oral mucosa in green pythons (*Chondropython viridis*). In lizards, skin lesions are common and include gray discoloration, ulcerative and necrotizing dermatitis, and hyperkeratosis (Stöhr et al. 2013). Multifocal to confluent tan friable areas (necrosis) may be seen internally (especially in the GI and respiratory tracts). Occasionally, hemorrhages of the GI tract may be the only change observed in water turtles (Fig. 3d, DLM, personal observation); however, it often is unclear if this is due to secondary infections.



**Fig. 3** Gross lesions in a red-eared slider (*Trachemys scripta elegans*) experimentally challenged with an FV3-like ranavirus. (a) Periocular swelling. The swelling is bilateral but more prominent around the left eye of this turtle. (b) Internal soft tissue edema (arrows) and hemorrhages (arrowheads). (c) Necrotic plaques (arrows) on the oral mucosa. (d) Hemorrhage (arrows) of the intestinal mucosa

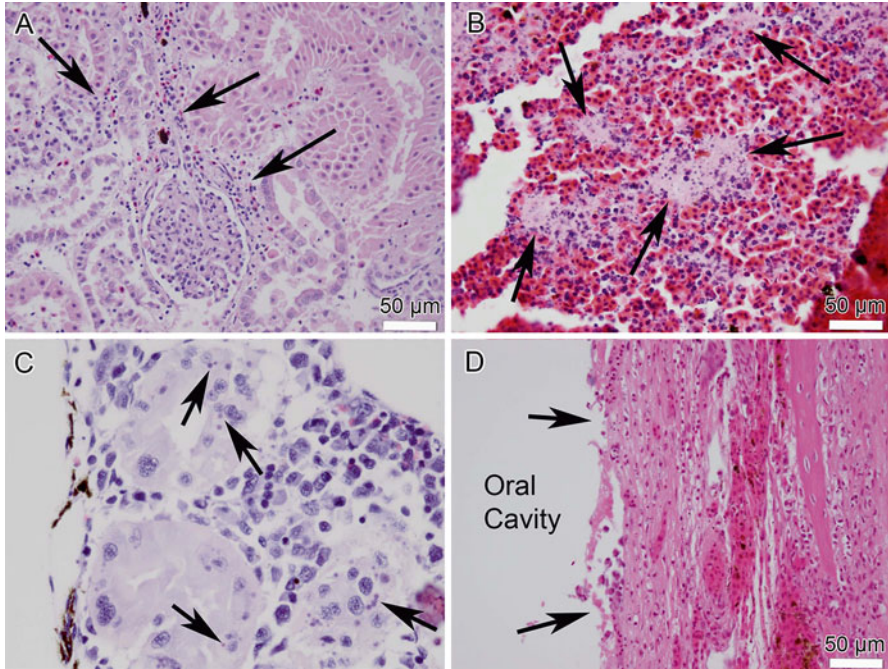
### 1.2.3 Histopathology

Necrosis of the hematopoietic tissues, vascular endothelium, and epithelial cells, hemorrhage, and intracytoplasmic basophilic inclusion bodies are common microscopic lesions in all hosts (Table 2, Fig. 4; Reddacliff and Whittington 1996; Cunningham et al. 2007; Allender et al. 2013b; Bayley et al. 2013; Cheng et al. 2014; Waltzek et al. 2014). The liver, spleen, and kidney (including pronephros and mesonephros) are most commonly affected in fatal cases, and can involve both the hematopoietic and non-hematopoietic components of these tissues (Fig. 4a–c). In amphibians, a wide tissue tropism has been observed and additional changes that have been reported include degeneration and ulceration of the epidermis (Cunningham et al. 2007; Geng et al. 2010; Cheng et al. 2014; Meng et al. 2014), necrosis of the GI mucosa (Bollinger et al. 1999), necrosis of lymphoid tissue (Bollinger et al. 1999; Balseiro et al. 2009; Meng et al. 2014), necrosis of neuroepithelial tissue (Docherty et al. 2003), skeletal muscle degeneration (Miller et al. 2008), necrosis of the pancreas (Balseiro et al. 2010; Kik et al. 2011), multicentric hemorrhage, vestibular hemorrhage and necrosis (R. Mazzoni, CRMV-GO, personal communication), and an ocular malformation (Burton et al. 2008). In chelonians, common findings include fibrinoid vasculitis, myositis, and necrotizing pharyngitis, esophagitis, and stomatitis (Fig. 4d; Johnson et al. 2007, 2008; Ruder et al. 2010; Allender et al. 2013b). Similarly, Hyatt et al. (2002) reported necrosis of the pharyngeal submucosa, ulceration of the nasal mucosa, and hepatic degeneration and necrosis in snakes (green pythons, *Chondropython viridis*). In lizards, ulcerative-necrotizing glossitis, hepatic necrosis, ulcerative dermatitis, and

**Table 2** Examples of histopathological changes that can be observed in individuals with ranaviral disease

Organ	Histopathological change
Kidney (including pronephros of larvae and mesonephros of adult amphibians and fish)	Degeneration or necrosis (tubular epithelial cells and glomeruli), intracytoplasmic inclusions, necrosis of hematopoietic tissue
Liver	Degeneration or necrosis (sinusoids, melanomacrophage centers, hepatocytes), intracytoplasmic inclusions, necrosis of hematopoietic tissue
Spleen	Necrosis, intracytoplasmic inclusions
Pancreas	Necrosis, intracytoplasmic inclusions
Muscle	Degeneration of muscle fibers, hemorrhage
Skin (especially lizards, fish, and adult amphibians)	Erosion, ulceration, hemorrhage, intracytoplasmic inclusions
Thymus, lymphoid tissue	Depletion, apoptosis, necrosis
Gastrointestinal tissue	Apoptosis, necrosis of epithelial cells, intracytoplasmic inclusions
Vessels	Necrosis (endothelial cells)
Upper respiratory tract (especially cheolians and snakes)	Necrosis of epithelial cells, intracytoplasmic inclusion



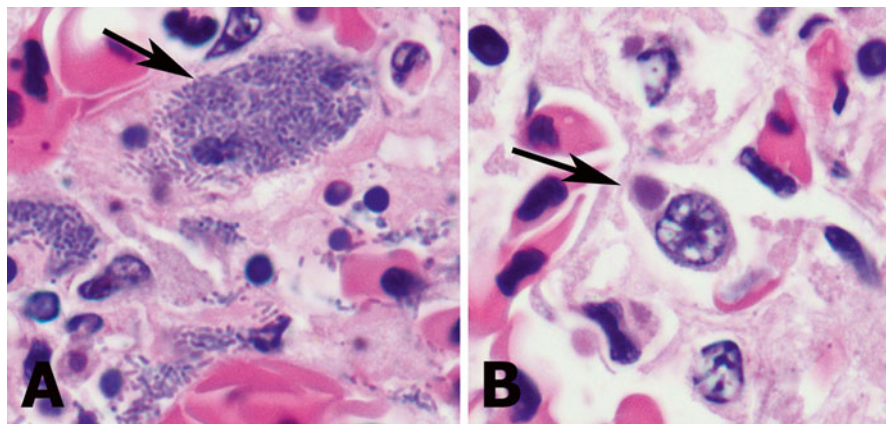


**Fig. 4** Histopathological changes seen in individuals with ranaviral disease from experimental challenges with FV3-like ranaviruses. **(a)** Necrosis (*arrows*) of the hematopoietic tissue within the kidney of a pallid sturgeon (*Scaphirhynchus albus*). **(b)** Necrosis (*arrows*) of the spleen of a dusky gopher frog (*Lithobates sevosus*). **(c)** Intracytoplasmic inclusion bodies (*arrows*) within the renal tubular epithelial cells of a Southern leopard frog (*Lithobates sphenoccephalus*). **(d)** Necrosis (*arrows*) of the mucosa of the oral cavity of a red-eared slider (*Trachemys scripta elegans*)

secondary infections have been reported (Marschang et al. 2005; Behncke et al. 2013; Stöhr et al. 2013, Fig. 5). In chelonians, lesions of necrotizing stomatitis and pharyngitis overlap significantly with those seen with herpesvirus and adenovirus infections (Johnson et al. 2005; Rivera et al. 2009).

#### 1.2.4 Subclinical Infection

Subclinical infections may play an important role in the epidemiology of rana viruses (Brunner et al. 2015). It is instructive to note that the original amphibian ranavirus isolates (Frog virus-1, -2, and -3) were from animals that were, with the exception of one with a renal tumor, ostensibly normal (Granoff et al. 1966). Subclinical infections have been detected in wild amphibians (Gray et al. 2007, 2009; Rothermel et al. 2013), chelonians (Allender et al. 2013b; Goodman et al. 2013), and fish (Goldberg 2002; Whittington et al. 2010). Additionally, subclinical infections have been experimentally produced in amphibians (Brunner et al. 2004; Harp and Petranka 2006; Robert et al. 2007, 2011), chelonians (Johnson et al. 2007;



**Fig. 5** Histopathology of ranaviral disease with superimposed secondary bacterial infection in an American bullfrog (*Lithobates catesbeianus*). (a) Bacterial colonies and necrosis in the spleen (arrows) can obscure subtle evidence of ranavirus infection. (b) Careful examination of the histologic sections from this individual revealed rare intracytoplasmic inclusion bodies highly suggestive of ranavirus infection

Brenes et al. 2014a, b), and fish (Bang Jensen et al. 2011; Becker et al. 2013; Brenes et al. 2014a, b). Individuals that are subclinically infected sometimes have nonspecific histologic changes such as vacuolation of renal tubular epithelium and hepatocytes (Miller et al. 2011; Allender et al. 2013b). Ana Balseiro (SERIDA, personal communication) has observed positive immunohistochemical staining in renal glomeruli of adult common midwife toads (*Alytes obstetricans*) with no clinical signs of disease. In most of the studies above, it is unknown whether the subclinical infections would have developed into clinical disease, because individuals were euthanized or released into the environment. It is likely that subclinical infections represent early stages of ranaviral disease in some cases, while in other cases, infections are resolved or persistent infections are maintained.

## 2 Diagnostic Testing

### 2.1 World Organization for Animal Health (OIE) Standards

The OIE provides recommendations and protocols on diagnostic testing for various animal pathogens (<http://www.oie.int>). One goal of the OIE is to provide procedures to declare an international shipment of animals or a site, region, or nation as pathogen free for a particular agent. Agents of concern to the OIE are called notifiable and deemed to be a risk to international commerce or human health. Ranaviruses that infect amphibians and EHNV are listed as notifiable, hence sampling and

diagnostic procedures are provided in the Diagnostic Manual for Aquatic Animal Diseases published by the OIE (OIE 2012c, d). The OIE reference laboratory for ranaviruses can provide reagents and procedures for diagnostic testing as well as perform diagnostic testing (Table 3). Gray et al. (2015) discuss how to perform a risk analysis for introduction of ranavirus into an area following OIE procedures.

Considering the scope of OIE is to detect ranaviruses for the purpose of international trade, the sampling procedures and diagnostic techniques they outline may not be applicable for all investigations. In general, we recommend that the procedures in Gray et al. (2015) be followed for determining required sample size and designing studies for ranaviruses. The diagnostic procedures recommended by OIE also may not be applicable for all regions of the world or may be cost prohibitive. Below, we review the majority of diagnostic techniques used to detect ranavirus infection and determine if individuals are in a diseased state. Table 4 provides guidance as to what techniques can be used given general study directions. In general, we recommend that investigators consult with experts that routinely perform ranavirus diagnostics so that appropriate techniques are chosen to address the particular goals of a study. Importantly, the appropriate sample collecting procedures can depend on the diagnostic technique that is used. The Global Ranavirus Consortium (GRC) maintains a list of laboratories on their website that routinely perform ranavirus diagnostics (<http://www.ranavirus.org/>).

**Table 3** Contact persons for the OIE Reference Laboratory for ranavirus reagents, protocols, and testing

Contact person	Contact information
Dr. Nick Moody	CSIRO Livestock Industries Australian Animal Health Laboratory Private Bag 24 (Ryrie Street) Geelong Victoria 3220 Australia Tel: +61-3 52 27 00 00 Fax: +61-3 52 27 55 55 Email: <a href="mailto:nick.moody@csiro.au">nick.moody@csiro.au</a> Web: <a href="http://www.csiro.au">www.csiro.au</a>
Dr. Richard Whittington	University of Sydney Faculty of Veterinary Science 425 Werombi Road Private Bag 3 Camden NSW 2570 Australia Tel: +61-2 93 51 16 19 Fax: +61-2 93 51 16 18 Email: <a href="mailto:richardw@camden.usyd.edu.au">richardw@camden.usyd.edu.au</a>

**Table 4** Examples of diagnostic tests that might be used based upon the study goal

Tests <sup>a</sup>	Study goal							
	Detection/surveillance	Investigating mortality events	Phylogenetic studies	Ecological studies	Viral morphometric studies	Host immune studies		
Conventional PCR	X	X		X	X	X		
qPCR	X	X		X	X	X		
DNA sequencing	X	X	X	X	X	X		
Ag-capture ELISA	X	X		X	X	X		
Virus isolation		X	X		X	X		
Serology	X	X				X		
Bioassay		X			X	X		
Histology, cytology		X		X	X	X		
IHC, ISH, EM		X			X	X		

<sup>a</sup>PCR polymerase chain reaction, qPCR quantitative real-time PCR, IHC immunohistochemistry, Ag-capture ELISA antigen capture enzyme linked immunosorbent assay, ISH in situ hybridization, EM electron microscopy

## 2.2 *Matching Diagnostic Tests with Study Goal*

Investigations into ranaviruses have various purposes, and depending on the goal, some diagnostic techniques may be more appropriate than others. Moreover, the diagnostic needs for field vs. controlled studies can be different. In general, most investigations can be classified as detection of ranavirus for facilitating trade according to OIE guidelines (Gray et al. 2015), detection for mapping distribution and estimating prevalence or incidence (Gray et al. 2015), isolation for phylogenetic classification (Jancovich et al. 2015a), viral morphometrics and host immune response (Jancovich et al. 2015b; Grayfer et al. 2015), ecological factors related to emergence (Brunner et al. 2015), and diagnosis of mortality events (Duffus et al. 2015). Although techniques are constantly being developed and improved, common diagnostic tools used for ranavirus investigations include those that can detect ranavirus and those that can detect the host response to infection. For example, the polymerase chain reaction (PCR) simply detects the presence of nucleic acid for a virus-specific sequence, but does not provide evidence as to whether the virus is active (i.e., able to replicate and cause disease). However, virus isolation demonstrates the presence of infectious virus. Similarly, other tests (e.g., histology, cytology, gene expression, antibody production) detect the cellular response to the infection. Techniques that demonstrate the presence of virus within a lesion such as electron microscopy (EM), immunohistochemistry (IHC), and in situ hybridization (ISH) are particularly useful for demonstrating an association between the presence of the virus and expression of disease. In general, investigations with the primary goal of ranavirus detection (e.g., surveillance studies) will use PCR or an antigen detection technique, phylogenetic studies will use virus isolation and genomic sequencing, ecological studies will use molecular modalities and histology, and viral morphometric studies, host immune studies, and mortality investigations may use all techniques (Table 4). Additionally, confirmation of ranavirus as the etiologic agent of a die-off requires use of multiple techniques, and information on pathological changes within cells is needed. Importantly, diagnosis of ranaviral disease cannot be inferred solely with infection data or the observation of gross signs.

Along with study goals, the type of sample may dictate the type of test that can be performed (Table 5). Nonlethal samples generally include swabs, tail or toe clips, and blood because these are generally easily collected (Greer and Collins 2007; Gray et al. 2012). Swabs should have plastic or wire (not wood) shafts to avoid PCR inhibitors (Pessier and Mendelson 2010). Swabbing should be done by firmly but not forcefully swiping (one to multiple times) the swab along the surface to be tested. Surfaces that are swabbed for ranaviruses typically include the oral cavity, cloaca, or skin lesions (Pessier and Mendelson 2010). Swabbing the vent might provide evidence of intestinal shedding. Tail or toe clips also can be effective at detecting ranavirus infection, and might result in fewer false negative test results compared to swabs (Gray et al. 2012). For salamanders, there generally are natural breakpoints near the tip of the tail where light pressure can be applied to autotomize the tail and collect tissue without cutting (Sutton et al. 2014b). Swabs or tissues can be stored frozen or in ethanol.

**Table 5** Various specimens used for *Ranavirus* testing, the type of test that can be performed, and the limitations of the test result

Specimen	Test	Limitations
Swab	PCR, virus isolation	False positives (environmental contamination); total DNA may be minimal; no histology
Tail or toe clip	PCR, virus isolation	False positives (environmental contamination); no histology
Whole body or internal organs	PCR, virus isolation, histology, IHC	Dead animals
Fixed tissue	PCR, histology, IHC	No virus isolation, electron microscopy is possible
Blood	PCR, virus isolation	Best obtained from live animals; can be difficult to obtain; often cannot obtain large enough quantity from small individuals
	ELISA if serum separated	
	Differential cell count if blood smear is prepared	

Disposable gloves should be worn and changed between animals to minimize cross-contamination of samples, and to prevent unintentionally transmitting the virus among animals. Additionally, individual animals should not be co-housed, and processing in the field should occur on a sterile surface (Fig. 6). For tail or toe clips, sterile instruments must be used to avoid sample contamination. We recommend using a different sterile (e.g., autoclaved) set of instruments for each animal. Although ranavirus can be quickly inactivated by various disinfectants (Byran et al. 2009), cross-contamination (and thus false positive results with molecular testing) can occur if ranavirus DNA is not degraded. While degradation studies have not been performed for ranaviruses, autoclaving, flaming, and long duration soaking (>12 h) in full strength bleach (6 % NaOCl) have been used to degrade DNA for other pathogens (Cashin et al. 2008). Some researchers are investigating the use of liver aspirates (Forzan and Wood 2013); however, the feasibility in field collection and the need for expertise in collection may limit their use. Blood can be collected from multiple locations, including the caudal vein (fish, caudates, snakes), subcarapacial sinus or occipital venus sinus (turtles, Martínez-Silvestre et al. 2002; Allender et al. 2011), abdominal and facial veins (anurans, Forzan and Wood 2013), and heart (anurans, snakes).

Detailed reviews of sample collection protocols for amphibian necropsy and mortality events are available (Green et al. 2009; Pessier and Mendelson 2010). In brief, submission of whole moribund animals sent overnight to a diagnostic laboratory are preferred. However, if this is not possible, tissue samples may be collected and submitted. It is important to realize that if a sample is submitted for only one test, the result will only be positive or negative for that one pathogen by that one test. If positive, the pathogen may or may not have played a role in morbidity and mortality. If negative, the cause of the morbidity and mortality will remain undetermined. Thus, ideally multiple samples should be submitted and multiple diagnostic tests performed. At minimum, tissue collection should include the major organs (liver, kidney, spleen, lungs/gills, heart, skin, digestive tract) and any lesions



**Fig. 6** Field sampling station demonstrating sterile collection techniques. Animals should be placed in separate containers, such as glass jars containing sterile water for aquatic stages (a) or plastic sealable bags for terrestrial stages (b). A portable table can serve as a processing station that can be easily disinfected after each field site (c). A sterile set of instruments should be used for each animal. For some species (e.g., Hellbenders, *Cryptobranchus alleganiensis*) a small slice can be collected from the dorsal tail using a sterile disposable scalpel and forceps (d). For many salamander species, there are natural break points in the tail that can easily yield a tail sample by simply applying gentle pressure near a break point, and can be done without removing the animal from the bag (e). When testing for concurrent *Batrachochytrium dendrobatidis* infection, swabbing can be accomplished within the containers as well (e inset). All equipment, waders, and boots should be disinfected (e.g., with 1 % Nolvasan<sup>®</sup>) before leaving a field site (f)

noted. A set of these tissues should be submitted fresh or frozen for pathogen testing, and a set should be submitted fixed (e.g., 10 % buffered formalin) for histological evaluation. Samples should be triple packaged in leak-proof containers and shipped following the guidelines of the carrier (e.g., [http://www.fedex.com/downloads/hk\\_english/packagingtips/pointers.pdf](http://www.fedex.com/downloads/hk_english/packagingtips/pointers.pdf)). Autolyzed samples are of little diagnostic value but, if they are all that remain, it may be possible to glean some information. It is best to contact the diagnostic laboratory before submission of any samples, but especially before submitting autolyzed samples. As mentioned, a list of diagnostic laboratories can be found on the GRC website. Importantly, researchers should disinfect footwear and sampling gear that comes in contact with potentially infected animals or water that contains ranavirus. Nolvasan® (1 %, Fort Dodge Animal Health, Fort Dodge, Iowa, USA) and bleach (4 %) are effective at inactivating ranavirus, as well as other pathogens such as the amphibian chytrid fungus (Bryan et al. 2009, Gold et al. 2014).

### **2.3 Diagnostic Tests**

Confirmation of the presence of ranavirus in host tissues can be achieved using various methods, including virus isolation, EM, antigen-capture enzyme-linked immunosorbent assay (Ag-capture ELISA), IHC, and PCR. Of these tests, all may be performed on specimens collected from dead organisms with minimal postmortem change, and most can be performed on specimens collected from live organisms (Table 5). Some tests (e.g., PCR) may yield results even with advanced autolysis, but this is not ideal. For all tests, it is necessary to prepare tissues in order to release ranavirus from the host cells. Several validated methods that incorporate automated, semi-automated, and manual homogenization of tissues have been described and compare the extraction efficiencies of ranavirus from tissues (Whittington and Steiner 1993; Rimmer et al. 2012). Extraction efficiency is especially important if viral loads are low in tissues, such as in subclinical infections. Additionally, all tests should include positive and negative controls. For example, positive PCR controls typically include extracted DNA from a virus isolate and known infected animal; whereas, negative controls typically include extracted DNA from a known negative animal and DNA grade water. In this scenario, the controls verify that the test was effective and had low contamination likelihood.

#### **2.3.1 Polymerase Chain Reaction**

Both conventional and quantitative real-time PCR (qPCR) are used to detect ranavirus DNA. The PCR assays can be performed on a variety of specimens, including fresh tissue, formalin-fixed paraffin-embedded tissues, and swabs. In the most



commonly used assays, the major capsid protein (MCP) gene is targeted (Mao et al. 1997; Hyatt et al. 2000; Kattenbelt et al. 2000; Marsh et al. 2002; Pallister et al. 2007). The MCP gene is highly conserved, which makes it a desirable region to target for identifying the presence of ranavirus DNA; however, it has limitations if the goal is to explore subtle genomic or phylogenetic differences among isolates within a particular *Ranavirus* species (Jancovich et al. 2015a). Other targets for PCR have included the neurofilament triplet H1 protein (Holopainen et al. 2009), DNA polymerase (Holopainen et al. 2009), and an intergenic variable region (Jancovich et al. 2005).

The sensitivity of PCR may vary depending on the type of specimen tested. In general, it is considered that tissue samples from internal organs represent infection status better than nonlethal sampling techniques such as tail clips, toe clips, and swabs (Greer and Collins 2007; Gray et al. 2012). Given that ranavirus tropism differs among tissue types and host postexposure duration (Robert et al. 2011; Ma et al. 2014), it is expected that PCR test results will depend on the tissue used. In frogs infected with FV3, ranavirus is first detected in the kidney and intestines (Robert et al. 2011), then the liver, spleen, and other major organs. Thus, testing different tissues can provide evidence of infection severity. Commonly, researchers test for infection using a homogenate of different tissues (e.g., Hoverman et al. 2011) to increase detection probability.

Testing for ranavirus in nonlethal samples can lead to false-negative and -positive results when compared to whole animal or liver samples (Greer and Collins 2007; Gray et al. 2012). False negative results may be caused by insufficient virus in swabs or tail- and toe clips; whereas, false positive results could be caused by virus on the outside of the animal being detected (Gray et al. 2012). Regardless, nonlethal sampling techniques can be useful for ranavirus surveillance if animal collections are not allowed or population abundance is low.

It is important to note that PCR assays are not perfect, implying that some rate of false positive and false negative results are expected even when a laboratory procedure with excellent analytical characteristics is performed without error. Accurate interpretation of PCR results requires estimation of the diagnostic sensitivity and diagnostic specificity of the assay so that the positive and negative predictive value of the result can be calculated (Greiner and Gardner 2000). These characteristics are measured when a test protocol is validated for a specific sample type and host species. Occasionally, when the quantity of virus present is low, different results from the same sample can occur. At minimum, we recommend that all samples tested by qPCR are run in duplicate (although triplicate is preferred) on the same qPCR machine, and only samples with consecutive positive results are declared positive. If one sample appears positive and the other negative, a third sample can be run and the declaration of infection made based on the majority of the results. Ongoing research (E. Grant, U.S. Geological Survey, and DLM) is estimating detection probabilities of qPCR using the protocol of Picco et al. (2007) and double sampling procedures.

## Conventional PCR

Conventional PCR has been used to detect ranaviral infection in fresh or fixed tissue specimens from surveillance studies and mortality events, as well as the identification of cultured virus (Miller et al. 2007; Gray et al. 2009; Meng et al. 2014). Phylogenetic mapping of banked DNA sequences (GenBank) representing isolates from throughout the world reveals significant sequence identity, which has been exploited in designing primers for detection of ranaviruses by PCR. In addition, sequence polymorphisms within the MCP can be used to distinguish some isolates to genus level (e.g., FV3, EHNV) by restriction digestion of amplicons from conventional PCR (Marsh et al. 2002; Holopainen et al. 2009). Conventional PCRs for detection of ranaviruses and restriction endonuclease analysis (REA) for subtyping are described in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2012c, d).

DNA sequencing of conventional PCR products to confirm positive results may not always be necessary, especially in endemic regions. However, amplification of non-ranaviral DNA can occur with the commonly used MCP primer sets (A. Pessier, unpublished data). Thus, sequencing of at least a subset of positive results is suggested when new hosts are affected or ranavirus is detected in regions previously thought to be free of these viruses. Sequencing of PCR products can also be informative for preliminary genus-level virus identification (e.g., FV3-like or *Ambystoma tigrinum virus* [ATV]-like).

## Quantitative Real-Time PCR

The advent of qPCR has provided significant advances in the study of various pathogens and their virulence. Studies have found that qPCR is more sensitive than conventional PCR as it can detect lower viral loads and be more sensitive than virus isolation (e.g., Pallister et al. 2007; Jaramillo et al. 2012). Pallister et al. (2007) found that qPCR could differentiate between a number of ranaviruses that infect fish, amphibians, and reptiles, and was especially effective at distinguishing European from Australian ranaviruses. A useful protocol for amphibians was reported by Picco et al. (2007), which amplifies a 70-bp region of the *Ranavirus* MCP gene; Allender et al. (2013a) developed a similar assay for chelonians. Viral load can be estimated when the genomic DNA in samples is quantified, and equal amounts of DNA used from each specimen tested. Viral load is predicted by entering the cycle threshold (Ct) value for a sample into a regression equation (called the standard curve) that relates Ct values and known virus quantities for the PCR, system that is used for testing (Yuan et al. 2006). Importantly, Ct values on different PCR systems are not equivalent; thus, conversion of Ct values to a standard unit of virus quantity using a standard curve is necessary for interpretation among studies. Typically, the standard unit of measurement is a log<sub>10</sub>-transformed value of virus concentration per unit of genomic DNA or tissue. For example, Brenes et al. (2014a) reported virus levels as plaque-forming units (PFU) per 0.25 µg of genomic

DNA. Results of PCR systems can vary among runs; thus, it is ideal to estimate a standard curve for each run (i.e., plate). Guidelines are available for reporting results for quantitative PCR assays (Bustin et al. 2010). Slope and intercept parameters for the standard curve can be averaged among multiple independent runs for a more robust estimate of viral load, similar to model averaging. Standard curves should be published with qPCR results.

Given that pathogen load often is positively related to morbidity in animals, qPCR might provide insight into the disease state (clinical vs. subclinical), but this remains to be shown for ranaviruses. Caution must be exercised when interpreting high Ct values (e.g., Ct values >35), because they can represent amplification or fluorescence artifacts, or cross-contamination (Caraguel et al. 2011). Conventional PCR, followed by DNA sequencing or virus isolation, can be used to verify viral DNA presence within samples having high Ct values. Additionally, a standard curve for the PCR system can be used to identify a Ct threshold where the sample is declared PCR-positive. For example, Brenes et al. (2014a, b) conservatively declared a positive result if the Ct value was less than the lower bound of a 95 % confidence interval at a predicted virus quantity of zero. In general, data on the diagnostic sensitivity and specificity of published qPCR tests for ranavirus are lacking. However, the test described by Jaramillo et al. (2012) has been validated for EHNV and will be described in the forthcoming new edition of the OIE Manual. Similarly, Allender et al. (2013a) validated the qPCR for detection of FV3-like virus in eastern box turtles (*Terrapene carolina carolina*).

### Differentiation of Ranavirus Species and Strains

In most routine diagnostic and research situations, existing PCR assays based on the MCP gene work well for determining the presence or absence of a ranavirus or to categorize a virus into a major species group (e.g., an “FV3-like” virus, Jancovich et al. 2015a). However, in other instances such as epidemiologic investigations that need to determine the distribution of different ranavirus strains or translocation programs for wildlife species where it may be important to determine if the same ranavirus is present in both source and destination populations, these assays are not as useful because the MCP gene is so highly conserved (Jancovich et al. 2015a). As an example, it is now well documented that different strains of FV3 as determined by genomic REA frequently have identical DNA sequences within regions of the MCP gene commonly used in diagnostic conventional PCR (Schock et al. 2008; Duffus and Andrews 2013).

The laboratory techniques used to differentiate specific ranavirus strains such as virus isolation and purification followed by genomic REA are not available in all laboratories and are not practical for some studies; therefore, there is a need to find and validate methods for rapid strain identification. One possible approach is genotyping using conventional PCR and DNA sequencing for genes such as the intergenic variable region (Jancovich et al. 2005; Weir et al. 2012) or neurofilament triplet H1-like protein (Holopainen et al. 2009; Cheng et al. 2014) that contain variable

repeating regions. By using consistent approaches for selection of sequencing targets, researchers ensure that advances in the understanding of ranavirus phylogeny and epidemiology can be more rapidly obtained.

### 2.3.2 Antigen-Capture ELISA

The analytical sensitivity of Ag-capture ELISA applied to fish tissue homogenates for detection of EHNV was  $10^3$ – $10^4$  TCID<sub>50</sub>/ml. Antigen-capture ELISA is useful for diagnosis and surveillance because it can be applied quickly (compared to virus isolation) and inexpensively (compared to molecular assays) to test large numbers of samples. Relative to virus isolation, the diagnostic specificity and sensitivity of Ag-capture ELISA was 100 % and 60 %, respectively (Whittington and Steiner 1993).

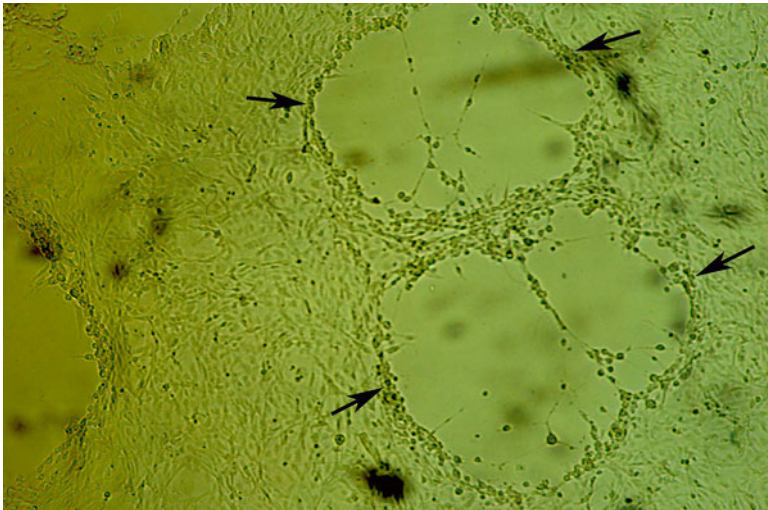
### 2.3.3 Virus Isolation

Virus isolation using well-characterized cell lines that are commercially available from cell repositories is another approach for determining the presence of ranavirus. Additional advantages of cell culture techniques include demonstration of viable virus, and amplification of it for further characterization. Culture specimens also may yield better PCR results and ultimately better products for sequencing than tissue specimens. EHNV replicates in many fish cell lines including fathead minnow (FHM), rainbow trout gonad (RTG), bluegill fry (BF-2), and Chinook salmon embryo (CHSE-214) at a range of temperatures from 15 to 22 °C (Langdon et al. 1986; Crane et al. 2005; Ariel et al. 2009; OIE 2012c). European catfish virus can be isolated using *Epithelioma papulosum cyprinid* (EPC) cells, FHM, and channel catfish ovary (CCO) cells at 15–25 °C, but BF-2 cells were tenfold more sensitive than EPC and CCO cells (Ahne et al. 1989; Pozet et al. 1992). *Santee-Cooper ranavirus* was originally isolated from largemouth bass in FHM cells (Plumb et al. 1996); and subsequently in BF-2, EPC, and CCO cells at 25–32 °C (McClenahan et al. 2005). Grouper iridovirus can be propagated on a range of cells including FHM and BF-2, but more rapid cytopathic effect (CPE) and higher titres were observed in Grouper (GP) embryo cells (Qin et al. 2003).

Ranaviruses commonly found in amphibians can be isolated on some fish cells as well as amphibian cells. Incubation temperature can vary and is a critical consideration for optimum results. For example, ATV reproduces on FHM, RTG, and bullfrog tongue cells at 25 °C (Docherty et al. 2003), FV3 on FHM cells or frog embryo fibroblasts at 27 °C (Cunningham et al. 1996, 2007) and EPC at 24 °C (Ariel et al. 2009), and CMTV can be propagated on EPC at 15 °C (Balseiro et al. 2009). Ma et al. (2014) and Geng et al. (2010) found that Chinese Giant Salamander Virus (CGSV; also designated as Chinese Giant Salamander Iridovirus, GSIV) could be propagated on EPC at 25 °C and 20 °C, respectively.

All ranaviruses produce a similar CPE with focal lysis of cells, which typically is followed within a few days by destruction of the entire monolayer (Fig. 7). Ranavirus CPE presents as a net-like appearance on monolayers of FHM cells (G. Chinchar, personal communication); however, the appearance of CPE is not sufficient to indicate that a ranavirus is the cause. When CPE is observed in cell monolayers after passage, several techniques can be used to confirm the presence of a ranavirus. Suitable techniques include indirect fluorescent antibody stain, Ag-capture ELISA, and PCR (OIE 2012c). Alternatively, viral nucleic acid sequencing or restriction enzyme digestion can demonstrate the presence of a ranavirus and allow for differentiation between species and strains (Hyatt et al. 2000; Marsh et al. 2002). The virus isolation technique can be adapted to determine the quantity of infectious virus (i.e., the 50 % tissue culture infective dose, TCID<sub>50</sub>; Rojas et al. 2005).

Virus isolation requires that viability of the virus is maintained from the field to the laboratory. Thus, this technique requires an appropriate cold chain throughout transport and transfer, or false-negative results could occur (OIE 2012d). Autolyzed tissues also should be avoided. Virus isolation using cell culture has a very high analytical sensitivity, but is not as sensitive as real-time PCR. For example, detection of EHNV using BF-2 cells was 100-fold less sensitive than qPCR (Jaramillo et al. 2012). When used for the purpose of surveillance and to detect subclinical infection, the sensitivity of virus isolation requires preparation techniques that ensure maximum release of cell-associated virus from the tissues (Whittington and Steiner 1993). Recently, modification of these methods using high-throughput laboratory technologies such as bead-beating were shown to be compatible with virus



**Fig. 7** A typical cytopathic effect (CPE) of EHNV in bluefill fry (BF-2) cells showing multiple foci of lysis of the cell monolayer. Note the rounding of cells on the margins of the lytic areas (arrows). After a few days, destruction of the entire monolayer can be expected

isolation and increased the sensitivity of qPCR for detection of EHNV (Rimmer et al. 2012). Ranaviruses are abundant in the liver, kidney, and spleen of clinically affected fish, amphibians, and reptiles. For *Santee-Cooper ranavirus*, it was suggested that gills, swim bladder, and posterior kidney should be used as a minimum sample (Beck et al. 2006), while a pool of kidney, liver, and spleen is preferred for EHNV.

Virus isolation provides an important tool for basic studies of the replication and morphogenesis of ranaviruses. For example, after initial descriptions of GCSV based on diagnostic tests including isolation on EPC cells (Geng et al. 2010), additional important information about the virus was acquired using EM and proteomic and RNA knockdown studies in EPC cells (Li et al. 2014b; Ma et al. 2014). These types of studies can provide valuable information on virus replication, which may in turn prove useful for developing methods of treatment and control of ranavirus infection.

### 2.3.4 Detecting Antibodies: Serology

An ELISA for detection of antibodies was developed using a combination of anti-ranavirus antibodies and species-specific anti-immunoglobulin reagents, and has been used to detect a specific adaptive immune responses to ranavirus infection in the serum of redbfin perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiss*), and the cane toad (*Bufo marinus*; Whittington et al. 1994, 1997; Whittington and Reddacliff 1995; Whittington and Speare, 1996). Seroconversion occurred when redbfin perch and rainbow trout were injected with inactivated EHNV (Whittington et al. 1994; Whittington and Reddacliff 1995). Serological approaches that target a long-term adaptive immune response to ranavirus infection potentially offer a sensitive method for differentiating host populations with endemic ranavirus infections from areas that are ranavirus free. For example, Whittington et al. (1999) reported a small proportion of seropositive adult rainbow trout in EHNV-infected farmed fish populations. Similarly, anti-ranavirus antibodies have been detected in populations of free-living anurans (Whittington et al. 1997; Zupanovic et al. 1998); and more recently, humoral immune responses have been measured in comprehensive investigations of the pathogenesis of ranavirus infection in *Xenopus laevis* (Gantress et al. 2003).

Given the success in fish, ELISA testing for an antibody response is being applied to reptiles. Ariel (1997) utilized methods developed by Hengstberger et al. (1993) to detect anti-*Bohle iridovirus* (BIV) antibodies in wild reptiles in Australia. More recently, Johnson et al. (2007, 2010) and Allender (2012a) used ELISA for surveillance and laboratory investigations to test for the presence of anti-ranavirus antibodies in plasma of various chelonians in the USA.

Serological testing is an easy and cost-effective technique, although it also presents limits. The application of serological tests is limited by the cross-reactivity of antibodies to all ranaviruses and the absence of a secondary test method to evaluate sensitivity and specificity. Virus neutralization tests cannot be used

because antibodies with neutralizing activity to EHNV were not evoked following immunization of rabbits (Hedrick et al. 1992) or from mouse monoclonal antibodies (Monini and Ruggeri 2002). Current knowledge of the amphibian, reptile, and piscine immune systems indicates limitations to serological techniques that are imposed by a lack of affinity maturation, poor immune memory, and temperature dependence of the response (McLoughlin and Graham 2007). For this reason, the OIE does not currently support serology as a useful strategy.

### 2.3.5 Testing in Animals: Bioassay

A bioassay or experimental transmission trial is an important part of the diagnostic process that is used in cases where a novel ranavirus is detected or disease is detected in a new host or novel ecological setting. An example of this can be found in Waltzek et al. (2014), which describes a mortality event in Pallid sturgeon caused by an FV3-like ranavirus. In this case, experimental transmission of the virus isolated from the die-off event was used to infect pallid sturgeon in the laboratory, resulting in a mortality event mimicking the one from which the virus was originally isolated. Subsequent sequencing of the virus revealed it to be most closely related to FV3 (i.e., FV3-like ranavirus).

### 2.3.6 Examining the Tissues

#### Histopathology and Cytology

Histopathology is an essential tool for investigating cases of disease with unknown etiology. Characteristic lesions can provide preliminary evidence of certain pathogens and guidance in selecting subsequent diagnostic tests. Histopathology is also important for determining the relevance of ranavirus infection detected during a surveillance study or disease event. Once death of the organism occurs, tissues breakdown (autolyze) quickly, especially in warm conditions, which can obscure microscopic detail. Thus, histology is performed best when using morbid specimens that were collected alive, humanely euthanized (AVMA 2013), and preserved in a fixative immediately after death. A 9:1 ratio of neutral buffered formalin (10 %) to tissue is generally used for fixation (Pessier and Mendelson 2010). Ethanol or other fixatives (e.g., Davidson's or Bouin's) may be preferred for fish tissues. Small animals (e.g., 10 cm or less) may be placed whole in a fixative if shipping to a diagnostic laboratory. A small (5 mm) incision into the coelomic cavity will aid in preserving internal tissues. In small fish, this is accomplished by removing the tail and operculum. Larger animals may be opened (necropsied) and representative samples collected from all tissues (see Sect. 2.2) so lesions can be observed grossly and differential diagnoses considered. At the time of necropsy, it is important to collect a second set of samples to be stored fresh (for immediate testing) or frozen

(for future testing) if histopathology results indicate that lesions consistent with ranavirus infection are present and confirmatory testing is necessary, or to rule out the presence of concurrent pathogens. To prevent cross-contamination, a different set of sterile instruments should be used for each individual.

Often cases of ranaviral disease are not straightforward and require the use of additional diagnostic methods such as IHC, EM, or molecular methods for confirmation. The observation of characteristic intracytoplasmic inclusion bodies is an inconsistent finding, especially in chelonians (DeVoe et al. 2004; Johnson et al. 2007, 2008). In other cases, subtle lesions of ranaviral infection can be obscured by secondary bacterial or fungal infection (Fig. 5).

Although not a definitive test of disease, cytology can be used to document changes in the innate immune system through blood cell counts, and for detection of viral inclusions. Blood may be collected as described above (see Sect. 2.2), and air-dried blood smears stained and subjected to cytological examination with oil-immersion light microscopy. Inclusion bodies may be observed within the cytoplasm of leukocytes (Allender et al. 2006). Although not definitive, inclusion bodies are suggestive of ranavirus infection, when corresponding clinical, gross, and histopathological changes are present. It should be noted that viral inclusions in erythrocytes may represent erythrocytic iridoviruses, which may be a different genus within the family *Iridoviridae*, especially when seen in reptiles (Wellehan et al. 2008; Grosset et al. 2014).

### Tests for Visualizing the Virus

There are several persistent questions regarding the pathogenicity of ranaviruses: Is ranavirus causing the lesion, where is the virus in subclinical infections, and what cells are infected by the virus and how does this vary by host species and developmental stage? A key factor in pathogenesis is identifying the cell types targeted by the virus. This can be done with histology; however, cellular changes are not always visible, such as with subclinical infections. Additionally, attributing cellular changes due to ranavirus can be challenging with histology, especially in cases of concurrent infection with other pathogens. Visualizing the virus within cells can be useful for attributing cellular changes to ranavirus (Miller and Gray 2010), and can be accomplished using IHC, in situ hybridization (ISH), and EM.

### Immunohistochemistry

For IHC, enzyme-conjugated, virus-specific antisera are used to demonstrate the intracellular location of viral antigens via an enzyme (e.g., horseradish peroxidase or alkaline phosphatase) catalyzed reaction (Fig. 8). Additional approaches have been developed to detect EHNV and European catfish virus (ECV) antigen using polyclonal antisera in Ag-capture ELISA and immunoelectronmicroscopy (Steiner et al. 1991; Hengstberger et al. 1993; Hyatt et al. 1991; Whittington and Steiner



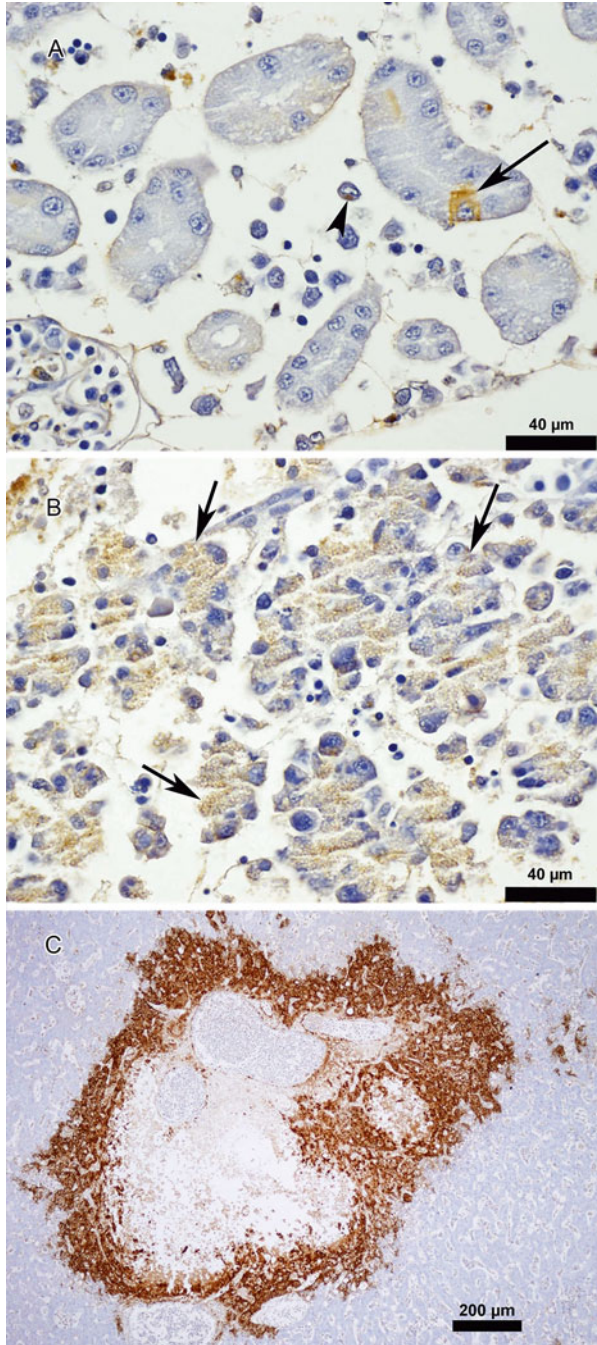
1993; Ahne et al. 1998). Secondary antibodies suitable for immunofluorescence or enzymatic detection enable the detection of ranavirus antigens in tissue sections and cell cultures (Reddacliff and Whittington 1996). Some antisera are directed against the MCP and are cross-reactive; thus, all ranaviruses can be detected, but it is not possible to distinguish between ranaviruses (Hedrick et al. 1992; Ahne et al. 1998). Other antisera have been developed against purified virions (Balseiro et al. 2009), but again, it might not be possible to distinguish between ranaviruses. Effective use of immunohistochemical staining has been reported for amphibians (see, Hyatt et al. 2002; Cunningham et al. 2008; Balseiro et al. 2009, 2010; Bayley et al. 2013). Balseiro et al. (2009, 2010) used IHC to detect the presence of ranavirus in various cells and found that glomeruli appear to have the most positive staining in larval toads but ganglia (particularly in the skeletal muscle) displayed the most positive staining in juvenile newts.

Despite these published reports, IHC is not widely used. Perceived difficulty in obtaining antibodies is often mentioned as the reason for not incorporating it as part of the basic laboratory testing. Primarily, IHC has been used in research studies and often the investigators develop their own antibodies or obtain them from other researchers (e.g., Hyatt et al. 2002; Cunningham et al. 2008; Balseiro et al. 2009, 2010; Bayley et al. 2013; Whitley et al. 2010; Chinchar et al., 1984). This technique may be especially useful to identify inclusion bodies within areas of necrosis, as this can be challenging when abundant cellular debris is present (Fig. 8). IHC may also be positive prior to development of necrosis and inclusions; thus, it may be useful in detecting the presence of virus in animals with subclinical infection. The antibodies recommended by the OIE reference laboratory were developed against a verified strain of highly purified ranavirus, have been pretested, and are of known titer and shelf life (Whittington and Deece 2004). It remains unknown if this is the case for other antibodies developed for IHC. Initial work by A. Balseiro and D. Miller (unpublished data) suggests that there may be differences in IHC results among ranavirus isolates and among hosts.

Another use for IHC is in cases where only fixed tissues are available, such as in diagnostic investigation of mass die-off events where shipping fresh or frozen tissues was not possible. Although PCR can be run on preserved samples and provide information on the presence or absence of ranavirus, the technique cannot verify the virus' involvement in a particular lesion (and thus possibly the cause of death). IHC provides supportive evidence of lesion etiology by microscopically visualizing the association of a pathogen with a lesion.

### In Situ Hybridization

Another approach to visualizing the virus is by ISH, which uses molecular probes to localize specific nucleic acid sequences within fixed tissue sections. For example, specific staining was observed in the kidney and spleen of Malabar grouper, (*Epinephelus malabaricus*) infected with Singapore grouper iridovirus (SGIV), an iridovirus which affects marine fish and has approximately 83 % MCP gene



**Fig. 8** (a) Immunohistochemical staining for ranavirus in renal tubular epithelial cells (*arrow*) of a Southern leopard frog (*Lithobates sphenoccephala*). An intracytoplasmic inclusion body within a hematopoietic cell (*arrowhead*) also stains positive. (b) Positive staining (*arrows*) is present throughout the spleen of the Southern leopard frog. (c) Positive staining in the liver of a redbfin perch (*Perca fluviatilis*)

sequence homology with FV3 (Huang et al. 2004). Although reports of the use of ISH for ranaviruses are few, future studies might consider using this technique as the need to document pathogenesis of ranaviral disease increases.

## Electron Microscopy

Electron microscopy (EM) is used for visual confirmation of the identity of cultured virus and for visualizing the virus within tissue sections. Verification of the cultured product involves negative staining and assessment via scanning EM (SEM); whereas, preserved tissues are used to visualize the virus within the tissues by transmission EM (TEM). For example, Burton et al. (2008) used TEM to examine the ultrastructure of tissues from a malformed eye of a preserved specimen. Electron microscopy revealed that the sample contained viral particles consistent with the family *Iridoviridae*, which allowed further characterization of ranavirus using PCR and DNA sequencing. Similarly, histological examination may reveal structures suggestive of intracellular viral inclusions, and EM can be used to verify the particles are viral origin (Cunningham et al. 1996, 2007; Balseiro et al. 2010; Cheng et al. 2014; Meng et al. 2014). In general, identification is only reliably made at the family (*Iridoviridae*) level; however, Hyatt et al. (2000, 2002) reported variations in size among members of the family, suggesting that more specific identification might be possible.

As early as the 1980s, cryo-electron microscopy (cryo-EM) has been used to examine the ultrastructure of the surface of viruses (see Adrian et al. 1984). This technique uses rapid freezing of purified virus, thus avoiding alterations from chemical preservatives. During the procedure, multiple photos are taken and reconstructed to yield a 3D image of the virus. Researchers have been using cryo-EM to evaluate the role of various surface proteins in viral assembly and replication (see Yan et al. 2009; Whitley et al. 2010; Tran et al. 2011). It is possible that these findings may provide insight into treatment protocols or vaccine development.

## 2.4 Test Validation and Efficiency

### 2.4.1 Gold Standards and Limitations of Diagnostic Tests

There is no single Gold Standard test for ranavirus, rather the tests vary based upon the question asked. In the case of disease diagnosis, laboratory results should be interpreted in conjunction with knowledge of the clinical status of the animal, pathological findings, and observed population effects. For example, isolation of ranavirus from tissues along with corresponding necropsy findings (gross and histopathological findings) and IHC provides strong evidence supportive of ranavirus as an etiology of death. Similarly, the use of diagnostic tests for surveillance or certification of infection-free individuals or zones requires a statistically valid

sampling protocol (Gray et al. 2015). Calculation of the sample size required to assess freedom from infection requires knowledge of the minimum expected prevalence and a diagnostic test with known sensitivity and specificity. For example, the prevalence of EHNV infection in rainbow trout can be as low as 4 % (Whittington et al. 1994, 1999). Gray et al. (2015) provide recommendations on sample sizes required to detect ranavirus given a range of assumed infection prevalence.

Given that diagnostic tests (e.g., qPCR) are imperfect, estimating the sensitivity and specificity of tests is important to accurately interpret results. Methods used by laboratories to demonstrate the validity of laboratory test results are outlined in an introductory chapter in *The Diagnostic Manual for Aquatic Animal Diseases* (OIE 2012a). The OIE guidelines outline the principles of using a laboratory quality management system to ensure that the results of tests are reliable by documenting the complete suite of measures required to minimize and identify false-positive and -negative results. In general, several laboratories working in collaboration can estimate false-positive and -negative results by testing the same samples, which can be used to estimate test sensitivity and specificity. This is accomplished by distributing control samples (i.e., known positive and negative) and standard reagents among laboratories in a blind experimental design, and estimating error rates among laboratories.

### 3 Treatment and Vaccine Development

Treatment and vaccination for ranavirus infection is probably most applicable in captive populations; thus, may be most useful in zoological collections or for conservation programs targeting rare species. Current treatments for ranavirus infection are limited. Allender et al. (2012b) reported the possibility of using guanine analogue antiviral drugs (acyclovir and valacyclovir) to treat chelonians for infection with iridoviruses and herpesviruses. Recently, Li et al. (2014a) reported antiviral activity of DNA aptamers when treating SGIV. Heat treatment is effective at inactivating many pathogens of ectothermic vertebrates such as *Batrachochytrium dendrobatidis* (Woodhams et al. 2003). However, the effectiveness of heat as a treatment for ranavirus likely varies among host species and viral strains. Rojas et al. (2005) reported that salamanders housed at elevated temperatures (26 °C) were more likely to survive exposure to ATV than those at lower temperatures. Similarly, Allender et al. (2013b) found greater pathogenicity of an FV3-like ranavirus at 22 °C compared to 28 °C in red-eared sliders (*Trachemys scripta elegans*). However, several other studies report faster replication and greater pathogenicity of ranaviruses at warmer temperatures (Whittington and Reddacliff 1995; Grant et al. 2003; Ariel and Jensen 2009; Ariel et al. 2009; Bayley et al. 2013). Given that most ranaviruses do not replicate above 32 °C (Chinchar 2002; Ariel et al. 2009), elevating body temperature above this threshold may be useful for some host species. Research is needed to determine the effectiveness of heat treatment, duration required for inactivation, and if there is variability among host species and viral strains.

To date, development of vaccines against iridoviruses has primarily been focused on fish species within the aquaculture industry. Some vaccines are commercially available, yet not applicable to all species or cross-reactive to other iridoviruses (Oh et al. 2014). Although live vaccines are often used, DNA vaccines are showing promise. For example, Zhang et al. (2012) found that turbot (*Scophthalmus maximus*) vaccinated with DNA vaccines were more likely to survive infection with rock bream iridovirus than unvaccinated fish. Similarly, Caipang et al. (2006) reported evidence of immunity against infection with red seabream iridovirus following administration of a DNA vaccine in red sea bream (*Pagrus major*). One aspect of vaccination that often limits its use is that it is delivered by intramuscular injection, which is labor intensive. Other researchers have been exploring more feasible delivery methods, such as oral formulations (Tamaru et al. 2006).

## 4 Summary Section with Final Recommendations

Ranaviral disease is devastating to susceptible hosts and causes hemorrhage, ulceration, edema, and organ necrosis. Although lesions vary among classes of ectothermic vertebrates, endothelial cell necrosis with subsequent hemorrhage is one of the changes that occurs across all classes. New technologies have enabled application of diagnostic techniques, such as IHC, that allows us to visualize viral antigens within tissues. Advances in techniques might include laser dissection of lesions followed by IHC and PCR, as well as 3D sequential tomography.

There are limitations to all diagnostic tests; thus, it is important that investigators and researchers use multiple tests for accurate diagnosis, and make the distinction between subclinical infection and disease. The most appropriate test to use is dependent upon the question that needs to be answered. For example, if one wants to determine whether an animal harbors a ranavirus, then PCR, qPCR, IHC, and virus isolation all might provide an answer. Importantly, there is a difference between pathogen detection and determining that a pathogen is associated with the observed clinical disease. There also are different tests recommended for determining prevalence of current or past infection (e.g., qPCR and antibody ELISA, respectively). A typical approach to determine infection status is to apply tests with known sensitivity and specificity to a population using a statistically valid sampling strategy to demonstrate freedom from infection or disease at a minimum expected prevalence (Cameron and Baldock 1998). High throughput qPCR laboratory methodologies are well suited to handle the large sample sizes required for these surveys. However, we still do not have a test that will verify that an individual animal is free from ranavirus infection, especially using nonlethal sampling. For example, if an animal tests positive by PCR but then negative when retested 2 weeks later, it is unknown if the animal has cleared the virus or if the virus remains hidden in the animal's system. Latent infections by ranavirus may be possible (Chapter 6).

We also need a cost-efficient, validated method for detecting and identifying different strains of *Ranavirus*. Current methods for doing so are not widely available

and can be cost prohibited; however, future success of reintroduction or translocation programs may hinge on our ability to differentiate between strains within source and destination populations. Likewise, strain identification will be important to include in future ranavirus reporting and mapping programs, as these data can be used in epidemiological studies.

There are many different protocols available for various tests. The OIE has guidelines for standardizing tests among laboratories. With the wide range of host species and sample types encountered during ranavirus studies, it is important to develop and share validation data for diagnostic techniques. We recommend that laboratories studying ranaviruses work together to standardize detection protocols. This task could be accomplished using a ring trial, where blinded samples are tested at many different laboratories internationally. The GRC is an entity that could lead initial discussions on how techniques could be standardized and help facilitate organization of ring trials and coordination of validation data.

There remains much to learn regarding the pathology of ranaviral disease and diagnostic testing for ranavirus infection and disease. Given our current understanding of pathogenesis and recent advances in the genetics of ranavirus and immune response of the host, we should be able to develop effective management and treatment modalities for use in conservation programs, commercial and zoological facilities and aquaria. Further, our understanding of the disease process coupled with our growing knowledge of the ecology and epidemiology of ranaviruses provides a basis for development of management plans for aquatic ecosystems.

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