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Review

"Ranaviruses: An emerging threat to ectothermic vertebrates" Report of the First International Symposium on Ranaviruses, Minneapolis MN July 8, 2011

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ABSTRACT

This is a report of the First International Symposium on Ranaviruses held on July 8, 2011 in conjunction with the annual Joint Meeting of Ichthyologists and Herpetologists (JMIH) in Minneapolis, Minnesota, USA. The emerging threat of ranavirus infectious diseases to the global biodiversity of ectothermic vertebrates was addressed by 23 scientists from nine countries with expertise in ecology, pathology, virology, veterinary medicine and immunology.

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1. Introduction

Ranaviruses (RVs) are large (150–170 nm) icosahedral viruses with double-stranded DNA genomes of 105–140 kbp that belong the family *Iridoviridae*. Infections caused by RVs have become increasingly prevalent worldwide and involve a large number of wild and captive fish, amphibian and reptilian species. Thus, RVs are an emerging threat to ectothermic vertebrates, and RV infections of amphibians now require notification of the World Organization for Animal Health (http://www.oie.int/eng/en_index.htm). While emerging infectious diseases caused by RVs are of great concern for conservation biology and international trade, they also raise fundamental issues about the protective role of anti-viral immunity in cold blooded vertebrates.

To discuss fundamental and applied issues of emerging infectious disease caused by RVs, the First International Symposium on Ranaviruses was held on July 8, 2011 in conjunction with the annual Joint Meeting of Ichthyologists and Herpetologists (JMIH) in Minneapolis, Minnesota, USA. The symposium was organized by Matthew Gray (U. Tennessee-Knoxville, TN, USA) with help from Debra Miller (U. Tennessee-Knoxville, TN, USA), Jesse Brunner (Washington State U., WA, USA), Jason Hoverman (U. of Colorado, Boulder, CO, USA), and Andrew Storfer (Washington State U., Pullman, WA, USA), and included 23 speakers with expertise in ecology, pathology, virology and immunology from nine countries. Overall more than 60 scientists interested in ranaviral disease attended this symposium. This meeting provided comprehensive updates of ranaviral diseases, ranavirus biology, and host–pathogen interactions. In addition, roundtable discussions at the end of the symposium allowed participants to define future research directions as well as to identify and prioritize needs. Slides and video of most presentations are available on the symposium website (http://fwf.ag.utk.edu/mgray/ranavirus/2011Ranavirus.htm). In addition, a Global Ranavirus Consortium was created to stimulate interaction among ranavirus researchers, veterinarians, and biolo-

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gists, and to provide frequently updated information (http://fwf.ag.utk.edu/mgray/ranavirus/Ranavirus.htm). Below we provide a synopsis of the symposium highlights.

2. Ranavirus taxonomy, morphology and replication

The taxonomy, structure and replication of ranvrisues (RVs) were presented within an historical context by the keynote speaker, Greg Chinchar (U. Mississippi Medical Center, Jackson, MS, USA). RVs were first isolated from North American leopard frogs (*Rana pipiens)* in 1965 by Allan Granoff[1]. One of these isolates, Frog virus 3 (FV3), became type species of the genus and is the most extensively studied member of the family. Initially, FV3 and other RVs were not considered major pathogens of ectothermic animals. However, this benign view of RVs changed in the mid-1980s with realization that the rapid decline of amphibian populations worldwide was associated with infections caused by not only a chytrid fungus but also by RVs. Chinchar summarized current knowledge of the structure and life cycle of RVs. Notably, RVs contain 100 or more open reading frames of which only a few have been experimentally characterized. Moreover, two-thirds of these putative gene products share no sequence similarity with known viral or eukaryotic proteins, and therefore are of unknown function. Deciphering the role of these genes is crucial for understanding the success of this pathogen including its capacity to adapt and expand its host and geographic ranges. To reveal functions of putative FV3 genes, two approaches were discussed: knockdown mediated by antisense morpholino oligonucleotides and the generation of knock out mutants using an improved technique developed by J. Robert and colleagues (U. of Rochester, Rochester, NY, USA). Together these approaches will allow researchers to not only investigate the roles of specific viral replicative genes, but also those that play critical roles in virulence and immune evasion.

Following the keynote presentation, Matt Gray discussed whether RVs directly contribute to amphibian declines. Compelling evidence indicates that the chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), constitutes a major factor in amphibian species decline, whereas RVs have been regarded to date as an epiphenomenon, i.e., a minor player in amphibian declines. Gray reviewed three main conditions that would lead to local extirpation of a host by RV pathogens, and presented evidence from two long-term studies showing that these three conditions, namely frequency dependent transmission, a broad host range with asymptomatic carriers, and existence of an environmental reservoir, occurred with RV infections. Thus, based on epidemiological theory, RVs have the capability of contributing to amphibian population declines.

3. Genetics, pathology and immunology

As with *Bd*, the reasons for the relatively recent emergence and rapid expansion of RV infections appear to be complex and likely include both host and viral factors. The large RV genome with over 100 putative genes represents a mine for fundamental and comparative studies of host-pathogen interactions, but also poses serious challenges. The genomes of six RVs have been sequenced and annotated. James Jancovich (Arizona State U., Tempe, AZ, USA) presented an evolutionary view of the relationship among RV isolates by comparing full genome sequences. His analysis revealed unique rearrangements of RV genomes, and suggested that the ancestral RV was a fish pathogen and that several recent host shifts have taken place with subsequent adaption of the viruses to new reptilian and amphibian hosts. The capacity of RVs to cross numerous poikilothermic species barriers further increases concern of the potential threat of RVs to the biodiversity. The study presented by Andrew Storfer (Washington State U., WA, USA) is consistent with rapid adaptation of RV to their hosts. Comparative phylogenetic data indicate that tiger salamanders and viruses are coevolved, but human introduction of infected salamanders as fishing bait has disrupted coevolutionary patterns. Due to increased densities of captive populations, increased virulence is observed in a virus strain isolated from a bait salamander population. Even more concerning is the case presented by Thomas Waltzek (U. California, Davis, CA, USA) of an interclass host shift of an FV3-like virus found to pallid sturgeon (*Scaphirhynchus albus*), an endangered species. The sequence of the major capsid protein of the virus infecting the pallid sturgeon is identical to FV3. Although the source of infection has not yet been identified, it is quite likely FV3 has cross-infected the sturgeon either passively through contaminated water or actively from a sympatric amphibian population with recurring FV3 infections. Similarly, R. Marschang (U. Hohenheim, Detmold, Germany) who reviewed several cases of RV outbreaks among tortoises, noted that, although MCP sequence analysis indicates a close phylogenetic relationship between the reptilian and the amphibian RVs, genomic restriction endonuclease profiles showed considerable variation among RVs suggesting that RVs rapidly adapt to their new hosts.

Identifying the pathological features associated with RV infections is critical for accurate diagnosis and understanding host and tissue range, host-pathogen interactions, and the basis of cytopathology. Notably cells and tissues targeted by RVs may differ among viral isolates and species, hosts, and developmental stage. As noted by Debra Miller (U. Tennessee, Knoxville, TN, USA) comparative pathology is just beginning to be studied. While gross lesions such as swelling, erythema, cutaneous ulceration and hemorrhage are useful for diagnosing RV infections, Debra Miller, David Green (National Wildlife Health Center, Madison, WI, USA) and Ana Balseiro (Centro de Biotecnología Animal, SERIDA, Spain) argued that examination of microlesions in various tissues by histology is more revealing. Notably, necrosis of kidneys, liver, and spleen tissue, and particularly the presence of intracytoplasmic inclusion bodies, is a more reliable indicator of RV infection. Comparison of pathology in fish, amphibian and reptile species underscore the overall similarity in the host responses to RV infection, although there are differences in the relative occurrence of signs and in the time of appearance among species. Interestingly, occurrence of RV infections in apparently healthy animals has been found by histology examinations. Besides current *post mortem* diagnostic methods based on conventional PCR and histopathology, M. Allender (U. Illinois, IL, USA) described ELISA assays using sera and blood smears to diagnosis infections in live gopher tortoises. Such assays permit longitudinal studies and can readily be applied to amphibians.

Host anti-viral immune defenses are a chief bulwark against the establishment of productive, life-threatening infections. However, a major challenge in studying antiviral immunity in cold blooded vertebrates, especially adaptive immune responses, is the absence of species-specific tools (i.e., antibodies and primers specific for immunologically-relevant gene products) and MHC-matched host systems. The use of appropriate animal models is, therefore, critically important in examining viral–host interactions. With regard to RVs, the FV3-*Xenopus laevis* model developed by J. Robert (U. Rochester, Rochester, NY, USA) provides a powerful model for investigating host immune responses. *Xenopus* adults resist and clear FV3 infection by developing rapid innate immune responses followed by an efficient CD8 T cell response and the generation of potent anti-FV3 antibodies. In contrast, *Xenopus* tadpoles cannot clear FV3 and die within a few weeks after infection. Although sus-

[1] Granoff, A., Came, P.E., Rafferty, K.A. 1965. The isolation and properties of viruses from *Rana pipiens:* their possible relationship to the renal adenocarcinoma of the leopard frog. Ann. NY Acad. Sci. 126(1), 237–255.

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ceptibility to FV3 is likely to be mainly due to the known weakness of larval antibody and T cell responses, recent study by J. Robert and colleagues revealed that FV3 infection also results in low and delayed induction of innate immune responses. Another important area of research that has been opened by using the *Xenopus* model concerns viral persistence and the potential role of resistant species including *Xenopus* in the dissemination of RV infections. Several lines of evidence suggest that a subset of *Xenopus* macrophages are permissive for FV3 infection and harbor quiescent virus. It is currently unknown how common and relevant is the ability of FV3 or other RVs to establish transient quiescent infections in their hosts and what are the mechanisms involved. However, subclinical infections of several species have been documented, which are consistent with a quiescent phase of RV infection. Finally, the implementation of an improved method to generate FV3 knock out mutants provides a powerful way to identify viral genes involved in virulence and immune evasion and to develop an attenuated viral vaccine.

4. Pathology, ecology and conservation

The comprehensive and compelling documentation of the worldwide distribution, diversification, and ongoing expansion of RV infections was a major achievement of the symposium. Detailed accounts of RV infections were reported from Australia (Ellen Ariel, James Cook U., Australia), Thailand (Somkiat Kanchanakhan, Aquatic Animal Health Research Institute, Bangkok, Thailand), Japan (Yumi Une, Azabu U., Kanagawa, Japan), Europe including the United Kingdom, Croatia, Spain, Denmark and The Netherlands (Ana Balseiro, Centro de Biotecnología Animal, Spain; Amanda Duffus, Gordon College, MA, USA; R. Marschang, U. Hohenheim, Germany), South America (Rolando Mazzoni, U. Federal de Goiás, Brazil) and North America (Danna Schock, Keyano College, Alberta, Canada). The remarkable spatial distribution of these viruses and the extent of their host range highlight the worldwide reach of these pathogens. RVs infect more than 10 fish species, 40 amphibian species encompassing 12 different families (including the giant salamander from China!), and several reptile species (e.g., box turtles and tortoises).

Several presentations addressed viral transmission within and among species, as well the possible influence of environmental factors. J. Brunner (Washington State University, WA, USA) explained that although RVs can persist and remain infectious for several days in water, their long term persistence is likely dependent on an animal reservoir. RV transmission can occur via several routes, including contaminated water, fomites, casual contact, and ingestion via cannibalism and necrophagy. However, Brunner stressed that most transmissions seem to require close contact, which suggests that transmission can be density-dependent. David Lesbarrères (Laurentian University, Sudbury, ON, Canada) presented further evidence of density-dependent transmission, and showed that RV infection is relatively more severe in animals held at low density. Consistent with data from others (e.g., Jason Hoverman, U. Colorado, Boulder, CO, USA), his laboratory and field studies indicate that amphibian species differ in their susceptibility to RVs, and isolates within different strains (ATV, FV3) are numerous. Furthermore, J. Kerby (U. South Dakota, Vermillion, SD, USA) showed that a synergistic effect between some insecticides (chlorpyrifos, atrazine, carbaryl) and RV infections led to increased mortality.

Lastly, several presentations addressed the risk of introducing exotic RVs into naïve populations via the trade in infected pet animals. Brit Jensen (Norwegian Veterinary Institute, Oslo, Norway) discussed the RANA project that has been developed to increase knowledge of susceptible hosts and improve diagnostic tools, as well as to assess the risks of introducing exotic RVs into Europe. Risk assessment was based on World Animal Health Organisation (OIE) guidelines. Angela Picco (United States Fish and Wildlife Service, USA) focused on the global trade of amphibians as a potential means to spread disease into new areas and contribute to amphibian die-offs and declines – a phenomenon known as pathogen pollution. She presented two case studies implicating pathogen pollution in North America, the translocation of larval tiger salamanders (*Ambystoma tigrinum*) as fishing bait and the sale of American bullfrogs (*Lithobates catesbeianus*) for human consumption. Jason Hoverman, (U. Colorado, Boulder, CO, USA) also discussed the potential role of amphibian culture facilities as the sources of novel highly infectious RVs, and highlighted the potential threat of pathogen pollution associated with the international and interstate commerce of American bullfrogs. Likewise, the study reported by Rolondo Mazzoni suggests that the importation of American bullfrogs from the USA was at the origin of recent RV outbreaks in Brazil. These alarming reports increase concern about the threat of RVs to biodiversity as well as captive and farmed cold-blooded vertebrates world-wide.

5. Future directions

The conference ended with two concurrent roundtable discussions on priorities and future directions. These discussions reinforced the general consensus that what is needed is more integrated interactions among the different investigators, which is the *raison d'être* for the Global Ranavirus Consortium. A list research of research priorities and directions have been posted (http://fwf.ag.utk.edu/mgray/ranavirus/2011Symposium/FutureDirections.pdf), along with a list of recent publications (http://fwf.ag.utk.edu/mgray/ranavirus/2011Publications.htm) and a list of the sponsors who have contributed to the success of this symposium (http://fwf.ag.utk.edu/mgray/ranavirus/2011Ranavirus.htm). Among the important issues identified, it was felt most urgent to develop a consensus for defining, identifying, and naming RV isolates.

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