

## Status of *Ranavirus* in Anurans of Two National Parks in Gabon

Amphibian populations are declining worldwide at an accelerated rate compared to other taxa, such as birds and mammals (Stuart et al. 2004). Known causes of population declines include habitat destruction, pollution, and the spread of amphibian pathogens (Beebee and Griffiths 2005). Infection with viruses of the genus *Ranavirus* results in high mortality rates in many amphibian species and has been identified as a contributing factor to declines in several populations around the world (Teacher et al. 2010; Miller et al. 2011; Price et al. 2014). At present, *Ranavirus* is known to infect amphibians on all continents, except Antarctica, where amphibians appear to be absent (Duffus et al. 2015). However, knowledge of the status of *Ranavirus* in Africa, a continent with high amphibian diversity, is extremely limited. *Ranavirus* has only been detected on the mainland of Africa as a single positive case in *Xenopus longipes* at Lake Oku, Cameroon (Doherty-Bone et al. 2013). Wild-captured animals that tested positive for *Ranavirus* have also been detected in Madagascar (Kolby et al. 2015). Prevalence of this pathogen and the potential associated threat to amphibians throughout Africa remain largely unknown.

### METHODS AND MATERIALS

This study tested for presence of *Ranavirus* DNA in anurans of Gabon, a central African country that shares a border with Cameroon. Field sampling of anurans was performed during October 2009 in pristine forest, natural forest clearings, and disturbed forests at Monts de Cristal National Park, northwestern Gabon, and Ivindo National Park, northeastern Gabon. Field sampling methods, habitat, elevation ranges, and status of *Bd* infection are fully described in Bell et al. (2011). Swabbed individuals were euthanized, prepared as voucher specimens, and deposited at Cornell University Museum of Vertebrates (CUMV), Museum of Comparative Zoology at Harvard University (MCZ), and North Carolina Museum of Natural Sciences (NCSM). Skin swabs were obtained following methods of Hyatt et al. (2007) and were initially used (Bell et al. 2011) to detect the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). The same extracted DNA used to assess *Bd* was used here to detect *Ranavirus*. Our analyzed dataset included swabs from 150

individual anurans of at least 40 different species representing nine families (Table 1).

DNA that had been previously isolated from sample swabs as in Boyle et al. (2004) was stored at -20°C for ~6 months and then at -80°C for long-term (> 1 year) preservation. These samples were then evaluated for the presence of *Ranavirus* DNA using a quantitative polymerase chain reaction (qPCR) with forward (5'-AACGCCGACCGAAAAGT-3') and reverse (5'-GCTGCCAAGATGTCGGGTAA-3') primers developed by Allender et al. (2013). These primers amplify a region of the MCP gene within the sequence targeted in the Cameroon study, however they do not overlap with the sequence targeted in the Madagascar study (Pearman et al. 2004; Kolby et al. 2015). Extracted DNA samples were diluted 1:2 in DNase/RNase free DEPC-treated water and assayed by qPCR in duplicate. Analysis was performed with both SYBR Green and TaqMan assays in 96-well plate format using a CFX Connect Real-Time PCR Detection System (BioRad). SYBR Green reagents were used initially on 107 samples, however the limit of detection determined for this assay (~110 genome equivalents) and maximum signal observed in negative controls (~342 genome equivalents) left the possibility that virus might be present in samples at low copy number. Subsequent analysis, including the same 107 samples as well as 43 additional samples, was performed with TaqMan reagents to achieve greater sensitivity. The limit of detection determined for this assay was ~5 genome equivalents and the maximum signal observed in a negative control was ~3 genome equivalents. SYBR Green reactions were prepared in a 25- $\mu$ l volume (2.5- $\mu$ l DNA sample, 12.5- $\mu$ l iTaq™ Universal SYBR Green Supermix [BioRad], 0.75- $\mu$ l forward primer, 0.75- $\mu$ l reverse primer, and 8.5- $\mu$ l DEPC-treated H<sub>2</sub>O). Cycling parameters were denaturation at 95°C for 5 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 15 seconds, 72°C for 30 seconds. TaqMan reactions were prepared according to Allender et al. (2013) in a 25- $\mu$ l volume (2.5- $\mu$ l DNA sample, 12.5- $\mu$ l Platinum qPCR SuperMix-UDG (Invitrogen), 0.5- $\mu$ l forward primer, 0.5- $\mu$ l reverse primer, 0.25- $\mu$ l FV3-MCP probe [5' 6FAM-CCGGCTTTCGGGC-MGBNFQ 3']). Cycling parameters were inactivation of UDG at 50°C for 2 minutes, denaturation at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, followed by a final extension of 72°C for 10 minutes. Each qPCR plate included duplicate wells of a 10-fold dilution curve of pCRII-TOPO plasmid carrying 531 bp of FV3 Major Capsid Protein gene restricted with *EcoRI* (kindly provided by Matt Allender). Positive and negative controls were included in every experiment and consisted of total DNA from FV3-infected FHM cells (positive), total DNA from naive FHM cells (negative), AE buffer used to elute DNA samples (negative), and water (negative). Linear regression analysis of the standard curve was used to determine limit of quantitative detection in genome copy number.

To be defined as “positive” for *Ranavirus*, a sample had to produce a signal within the linear range of detection for the assay in both of the duplicate reactions. This result also had to be repeated in a second verification qPCR assay. Using these criteria, 0 out of 107 samples analyzed by SYBR Green and 0 out of

#### ETHAN J. FRITCH

Biotechnology Program, North Carolina State University,  
Raleigh, North Carolina 27695, USA

#### RAYNA C. BELL

Department of Vertebrate Zoology, Smithsonian Institution,  
National Museum of Natural History,  
Washington, DC 20560, USA

#### BRYAN L. STUART

North Carolina Museum of Natural Sciences,  
Raleigh, North Carolina 27601, USA

#### THOMAS B. LENTZ\*

Biotechnology Program, North Carolina State University,  
Raleigh, North Carolina 27695, USA

\*Corresponding author; e-mail: tlentz@uwsp.edu

TABLE 1. Results of qPCR test for *Ranavirus* in skin swab samples of anurans from Gabon.

Species	Preliminary experiment (# Positive specimens/ Total # specimens tested)		Verification experiment (# Positive/Total # tested)
	SYBR Green <sup>1</sup>	TaqMan	TaqMan
<b>Arthroleptidae</b>			
<i>Arthroleptis adelphus</i>	NE	0/1	
<i>Arthroleptis poecilonotus</i>	0/4	0/5	
<i>Arthroleptis</i> sp.	NE	0/1	
<i>Arthroleptis sylvaticus</i>	0/2	0/3	
<i>Astylosternus batesi</i>	NE	0/2	
<i>Cardioglossa elegans</i>	NE	0/1	
<i>Cardioglossa leucomystax</i>	NE	0/2	
<i>Leptopelis aubryi</i>	NE	0/1	
<i>Leptopelis aubryioides</i>	0/2	0/2	
<i>Leptopelis boulengeri</i>	NE	0/1	
<i>Leptopelis brevirostris</i>	NE	0/2	
<i>Leptopelis calcaratus</i>	0/4	0/4	
<i>Leptopelis crystallinoron</i>	0/3	1/5	0/1
<i>Leptopelis notatus</i>	NE	0/1	
<i>Leptopelis ocellatus</i>	0/8	0/11	
<i>Leptopelis rufus</i>	0/3	0/5	
<i>Scotobleps gabonicus</i>	0/4	0/8	
<i>Trichobatrachus robustus</i>	NE	0/1	
<b>Bufonidae</b>			
<i>Amietophrynus camerunensis</i>	NE	0/2	
<i>Amietophrynus gracilipes</i>	NE	0/1	
<i>Nectophryne batesii</i>	NE	0/3	
<b>Conrauidae</b>			
<i>Conraua crassipes</i>	0/7	0/7	
<b>Hyperoliidae</b>			
<i>Afrixalus fulvovittatus</i>	0/6	0/6	
<i>Afrixalus paradorsalis</i>	0/3	0/3	
<i>Hyperolius bolifambae</i>	0/3	0/3	
<i>Hyperolius cinnamomeoventris</i>	0/7	0/7	
<i>Hyperolius kuligae</i>	NE	0/1	
<i>Hyperolius ocellatus</i>	0/10	0/10	
<i>Hyperolius phantasticus</i>	NE	0/1	
<i>Hyperolius tuberculatus</i>	0/4	0/4	
<i>Opisthothylax immaculatus</i>	0/2	0/2	
<i>Phlyctimantis leonardi</i>	0/4	0/4	
<b>Petropedetidae</b>			
<i>Petropedetes newtoni</i>	NE	0/2	
<i>Petropedetes palmipes</i>	0/5	0/5	
<b>Phrynobatrachidae</b>			
<i>Phrynobatrachus auritus</i>	NE	0/3	
<i>Phrynobatrachus africanus</i>	NE	0/1	
<b>Ptychadenidae</b>			
<i>Ptychadena</i> sp.	0/6	1/7	0/1
<b>Ranidae</b>			
<i>Amnirana (Hylarana)</i> sp. A	0/12	0/12	
<i>Amnirana (Hylarana)</i> sp. B	NE	0/2	
<b>Rhacophoridae</b>			
<i>Chiromantis rufescens</i>	0/8	0/8	
<b>Total</b>	<b>107</b>	<b>150</b>	

<sup>1</sup>NE = Not Evaluated

150 samples analyzed by TaqMan were determined to be positive (Table 1). Two samples produced a signal meeting the criteria of a positive in the initial TaqMan analysis, but were near the limit of detection. Neither of these samples produced a positive signal when re-tested in the verification assay. Looking at these results in aggregate, which assumed all species analyzed share similar susceptibility to the virus, we found no evidence of *Ranavirus* infections in anurans from Gabon (95% confidence interval of 0–2.5%). Susceptibility of the tested species to ranaviruses is not known at present. However, *Ranavirus* spp. are known to have broad host ranges in anurans and our survey data remain useful for evaluating general prevalence of these pathogens in this region of Africa (Duffus et al. 2015).

#### RESULTS AND DISCUSSION

We found no evidence for the occurrence of *Ranavirus* spp. in anurans at the two sampled national parks in Gabon. The only other survey for *Ranavirus* conducted on the African continent detected virus in 1 out of 11 diseased *Xenopus longipes* at Lake Oku, Cameroon (Doherty-Bone et al. 2013). Although the virus was determined to not be the cause of disease in that animal, the finding indicates virus is present in tropical Africa, although small sample size precluded a precise estimate of prevalence. The primer set used here targets a 54 bp region of the major capsid protein (MCP) gene (base coordinates 97,501–97,554; Genbank accession #AY548484) and will detect FV3, common midwife toad virus, and other closely related *Ranavirus* species or strains, but is unlikely (based on sequence homology) to detect others (e.g., tiger frog virus, European shellfish virus, epizootic haematopoietic necrosis virus, *Ambystoma tigrinum* virus), which could have been detected by the primers used by Doherty-Bone et al. (2013). Therefore, it is possible that certain ranaviruses went undetected in the analyses. Future research should use a more inclusive primer set to detect all possible amphibian ranaviruses. Additionally, though swabbing has the advantage of being a non-lethal means of detection, this method detects infections in only ~66% of animals determined to be positive by liver tissue sampling (Gray et al. 2012). Sensitivity may also be reduced in our study by the storage of the samples for an extended period (> 1 yr) prior to analysis. In any case, it appears that *Ranavirus* are rare at best, which might reflect the regional absence of these viruses or perhaps resistance in these amphibian species. Experimental challenges would be necessary to determine whether these amphibian communities are naive or largely resistant to *Ranavirus* infections. Continued surveillance with swabbing at regular intervals, serological surveys to detect prior exposure, or active surveillance during mortality events would provide a more accurate assessment of presence or absence of ranaviruses in these regions.

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