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Development of a multiplex real-time PCR surveillance assay for monitoring the health status of Ecuadorian amphibians at risk of extinction

G. Burgos[a], D. A. Narváez-Narváez[b], B. Freire-Paspuel[c], A. Merino-Viteri[d], C. Muslin[e], A. Genoy-Puerto[e,*]

[a] *Escuela de Medicina, Facultad de Ciencias de la Salud, Universidad de Las Américas (UDLA), Quito-Ecuador.*
[b] *Farmacia y Tecnología Farmacéutica y Fisicoquímica, Facultad de Farmacia y Ciencias de la Alimentación, Universitat de Barcelona, Barcelona-España.*
[c] *Laboratorios de Investigación, Universidad de Las Américas (UDLA), Quito, Ecuador*
[d] *Museo de Zoología (QCAZ), Escuela de Ciencias Biológicas, Pontificia Universidad Católica del Ecuador, Quito-Ecuador.*
[e] *Escuela de Medicina Veterinaria, Facultad de Ciencias de la Salud, Universidad de Las Américas (UDLA), Quito-Ecuador.*
**Tel +593 992624539; Corresponding author: alexandergenoy@gmail.com*

Abstract. Chytrid fungi and viruses within the genus *Ranavirus* have been associated with mass mortality events and declines in amphibian populations worldwide. The fungus *Batrachochytrium dendrobatidis* (*Bd*) was reported in Ecuador; however, other chytrid fungi like *Batrachochytrium salamandrivorans* (*Bsal*) or ranaviruses have not been described in the country so far. To prevent the introduction of pathogens into amphibian populations under conservation programs and to implement a successful disease surveillance program, the development of a sensitive and specific diagnostic assay was required. We describe here the optimization of one TaqMan probe-based multiplex quantitative polymerase chain reaction (qPCR) assay that enables the simultaneous detection of *Bsal* and ranavirus, and one monoplex TaqMan qPCR assay for the detection of *Bd*. Standard curves, with a high linear correlation ($r2 > 0.995$), were generated using a synthetic genome template (gBlocks®) containing the target sequences from all three pathogens. Different samples from skin, liver, kidney, spleen, and lung from six different amphibian species were tested, and both qPCR assays showed highly reproducible and reliable results. To our knowledge, this method is the first multiplex qPCR system developed in Ecuador for identifying amphibian pathogens and represents a valuable tool for the early detection of these pathogens and for infection and co-infection monitoring in future epidemiological surveillance of amphibian species at risk of extinction.

Keywords: *Chytridiomycosis; Ranavirus; Ecuadorian amphibians*

1. Introduction

Emerging infectious diseases threaten the survival of wildlife populations and species worldwide [1]. Amphibians have experienced population losses and extinctions due to mortal diseases, such as chytridiomycosis and ranavirus infections. However, the presence of these pathogens in some South American countries has not yet been established. Viruses within the genus *Ranavirus* are the causative agents of a disease with high mortality in frogs, salamanders and reptiles. Ranavirus has been found in several regions of North America, Asia, Europe, and Australia. Chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsa*l) affect anurans and caudate amphibians in all continents. They cause a skin infection associated with significant population decline. The aim of this study was to develop a TaqMan probe-based multiplex quantitative polymerase chain reaction (qPCR) assay that enables the simultaneous detection of *Bd*, *Bsal*, and ranavirus to support the sanitary surveillance in Ecuadorian conservation projects and in the natural areas of the country.

2. Methods

2.1 Samples

Full necropsy was made and samples from different organs and tissues such as skin, heart, lungs, gastrointestinal, kidney and liver were collected. Epithelial surface swabs were also taken. In total, 106 tissue samples from 23 animals of six different amphibian species were analyzed.

2.2 Positive control

The target sequences of all three pathogens were commercially synthesized in a single gene template (gBlocks®; Supplementary material) for use as positive control and quantitation standard (Fig. 1A). For *Bd* and *Bsal* detection, 146 and 180 base pair (bp) portions of the internal transcribed spacer (ITS-1 and ITS-2) and 5.8 ribosomal genes were targeted as previously described [2]. For ranavirus detection, a 435 bp segment of a portion of the major capsid protein (MCP) was integrated into the gBlock®.

2.3 Primers and probes design

Primers and probes were designed according to the gBlock target sequences, using Primer-BLAST and Primer3web V 4.1.0 (Table 1). Primers and probes were evaluated for dimers and hairpins with Autodimer V 1.0 and OligoAnalyzer V 3.1.

2.4 DNA extraction and Multiplex PCR reaction

The collected samples were laid and mixed in a microtube with 300 µL of Phosphate-buffered solution (PBS) 0.1 M and subjected to nucleic acid extraction with phenol-chloroform. Two qPCR reactions were performed, a duplex (*Ranavirus and Bsal*) and a monoplex (*Bd*) qPCR. Each 10 µl duplex qPCR reaction contained 10 ng DNA, 5 µl of 2X GoTaq® Probe qPCR Master Mix (Promega, WI, USA), 400 nM of each primer, and 250 nM of each probe. Each 10 µl monoplex qPCR reaction contained 10

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ng DNA, 5 µl of 2X GoTaq® Probe qPCR Master Mix (Promega), 800 nM of forward primer, 400 nM of reverse primer and 150 nM of probe. Amplifications were performed using CFX96 Touch System® (Bio-Rad©), as follows: 2 min at 95 ºC and 45 cycles of 15 sec at 95 ºC and 1 min at 63 ºC.

Table 1: Sequences of primers and probes used in multiplex quantitative polymerase chain reaction (qPCR) for detection of *Batrachochytrium dendrobatidis* (*Bd*), *B. salamandrivorans* (*Bsal*) and Ranavirus (frog virus 3, FV3).

Pathogen	Name	Sequence (5'–3')	Concentration (nM)	Reference
Ranavirus-Frog Virus 3	FV3-MCP-F	TGCGGATAATGTTGTGGTTGATG	400	This study
	FV3-MCP-R	GTGGAGCCCTGGTACTATGC	400	This study
	FV3-MCP-Probe	Cy5-GTCTACCGTAATTGGTGGATCCGGATGGG-BHQ	250	This study
Bsal	Bsal-ITS2-F	ATCTCCCCCTCTTCATCCCTAA	400	This study
	Bsal-5.8S-R	CATCGAATCTTTGAACGCACATTG	400	This study
	Bs-5.8S-Probe	HEX-TGATTCTCAAACAGGCATACTCTAC-BHQ-1	250	This study
Bd	ITS-1Chytr3	CCTTGATATAATACAGTGTGCCATATGTC	800	[3]
	Bd-5.8S-R	GCATTTCGCTGCGTTCTTCA	400	This study
	Bd-ITS1-Probe	FAM-TTGACAACGGATCTCTTGGCTCTCGCAA-BHQ	150	This study

3. Results and discussion

Monoplex Bd: Eleven samples from skin tissues and swabs were found positive and corresponded to seven animals of three species. Eight from *Gatrotheca riobambae*, two from *Gastrotheca* sp. *(pseustes* complex*)* and one from *Ambystoma mexicanum* (exotic species) (Fig. 1B). *Duplex Ranavirus – Bsal*: Five samples were found positive for ranavirus: four from *Gastrotheca riobambae*, and one from *Boana geographica*. Viral DNA was amplified in samples of heart, gastrointestinal, and kidney tissues (Fig. 1C). None of the samples was positive for *Bsal*.

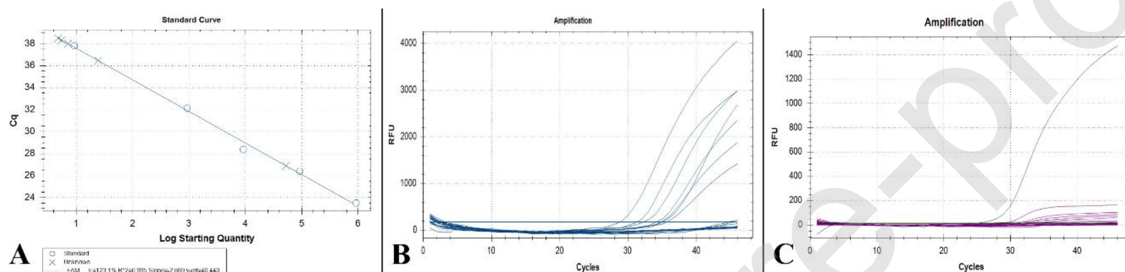

Fig. 1. A. Standard curve for the monoplex *Bd* qPCR obtained with 10-fold serial dilutions showing a high linear correlation (RSq = 0.995). **B.** Monoplex *Bd* amplification. Several positive samples for *Bd* are shown. **C.** Duplex Ranavirus – *Bsal* amplification. Several positive samples for Ranavirus are shown. None for *Bsal* except the positive control.

The presence of *Bd* is not surprising as it is still reported routinely in Ecuador, but its detection in exotic amphibians is of high concern. A larger sample size of animals from different bioregions would allow us to establish the presence or absence of the *Bsal* fungus in Ecuadorian territory. *Ranavirus* results would indicate the first report of the virus in Ecuador. This fact highlights the importance of epidemiological surveillance by molecular methods because one of the positive species, *Gastrotheca riobambae,* is at high risk of extinction and, although the other *Boana geographica* is not, the presence of the virus would constitute a threat to its conservation.

4. Conclusion

The duplex and monoplex real-time PCR assays showed highly reproducible and reliable results. To our knowledge, this method is the first multiplex qPCR system developed in Ecuador for identifying amphibian pathogens and represents a valuable tool for the early detection of these pathogens and for infection monitoring in future epidemiological surveillance of amphibian species at risk of extinction. Moreover, it can be used to estimate the prevalence of infections and co-infections of these pathogens.

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7. Conflict of interest statement: None.

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