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Ranavirus detection by PCR in cultured tadpoles (*Rana catesbeiana* Shaw, 1802) from South America

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Abstract

Diseases in farmed tadpoles (*Rana catesbeiana*) are a common event, being an economically important threat for Uruguayan and Brazilian farms. Based on clinical signs and epizootiology, pathogens belonging to the Family *Iridoviridae* were suspected as the possible etiology. Although these viruses have already been widely incriminated affecting aquatic organisms including frogs, their presence in Brazil and Uruguay was never mentioned so far. The objective of this work was to detect the presence of ranaviral agents in affected tadpoles using Polymerase Chain Reaction (PCR) technique as a primary approach to the study of the disease. Primers were designed based on highly conserved iridoviral sequences. Major Capsid Protein (MCP) and Immediate Early Protein (IE) genes were the selected targets. A positive PCR result was obtained for both genes when sick tadpoles from Brazil and Uruguay were analyzed. To confirm the amplification of an *Iridoviridae*, PCR products were purified and sequenced. Amplified products showed high degree of homology with several members of the *Iridoviridae*, mostly with those belonging to the genus *Ranavirus*. Obtained sequences were registered in the GenBank with accession nos. AY585203, AY585204 and AY744387. This report indicates that *Ranavirus* should be considered into the aquatic organism disease etiologies throughout this geographical region.

Keywords: PCR; Iridovirus; Ranavirus; Frog

1. Introduction

Frog culture (*Rana catesbeiana*) is an expanding activity in several Latin American countries, mainly Brazil, Argentina, Ecuador and Uruguay (Mazzoni, 2000a; Mazzoni et al., 2003). Improvements obtained

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in culture techniques allowed frog farmers to increase densities and as a consequence disease susceptibility also has arisen. Nevertheless, identification of etiological agents and selection of an appropriate therapy were not easy to achieve due to unspecific symptomatology and lack of information.

Disease outbreaks in cultured tadpoles and frogs have been reported without a clear etiology. *Aeromonas hydrophyla* and multiple estreptococci were the main isolates obtained but without a clear definition about the importance and function as primary pathogens (Amborski et al., 1983; Hipólito et al., 1987, 1988a,b; Guimaraes et al., 1988; Hipólito, 1995, 1997; Fiorio et al., 1997; Mazzoni, 2000a,b; Mauel et al., 2002). With the increasing information on frog diseases related to amphibian populations declines, several viral agents members of the Family *Iridoviridae*, genus *Ranavirus* have been reported as responsible for disease outbreaks (Cunningham et al., 1996; Hyatt et al., 2000; Chinchar, 2002; Marsh et al., 2002). Ranaviruses were also reported in cultured frogs *Rana grylio* (Zhang et al., 2001) and *Rana tigrina rugulosa* (He et al., 2002; Weng et al., 2002) in Asia.

The analysis of mortality episodes in cultured tadpoles in South America based on epizootiological and clinical observations suggested that an iridoviral agent may have been the primary cause involved. Thus, the objective of this work was to detect the presence of an *Iridoviridae* agent in tadpoles with Polymerase Chain Reaction (PCR) technique, considering the lack of scientific trials directed to the identification of these pathogens in this geographical region.

2. Materials and methods

Tadpoles sampled for this study belong to one farm located in Brazil, 200 km south west from Brasilia, and a second farm in Uruguay, nearby Montevideo. Brazilian farm used surface water, distributed per gravity from a dam , and the Uruguayan farm used pumped water from a 40-m deep well.

For virus genes detection with polymerase chain reaction (PCR) DNA of skin and muscle from sick tadpoles was isolated by saline extraction according to Miller et al. (1988). Briefly, tissue samples were fixed in 95% ethanol and pieces of 50 mg were macerated and homogenized in 400 μL grinding buffer (NaCl 0.1 M, Sucrose 0.5 M, Tris 0.1 M, EDTA 50 mM, SDS 0.5%) into a 1.5-mL eppendorf tube and incubated for 30 min at 65 °C. Then 57 μL potassium acetate 8 M was added and incubated for 30 min on ice bath followed by centrifugation at 10000×*g* for 15 min. Supernatant was transferred to a new tube and 1 mL iced pure ethanol was added, gently mixed, incubated for 5 min at room temperature and finally centrifugated at 10000×*g* for 15 min. DNA was precipitated with ethanol and resuspended in 50 μL TNE buffer (Tris 6 mM, NaCl 6 mM, EDTA 0.2 mM) and stored at 4 °C.

2.1. Primers design

DNA oligonucleotides were designed looking for highly conserved genomic regions into the Family *Iridoviridae* with the aid of Blast search tool (http://www.ncbi.nlm.nih.gov/BLAST/). *Rana grylo* virus Major Capsid Protein (RGV-9807-MCP) gene (GenBank accession number AF192508) and Frog virus 3 Immediately Early protein (IE) gene (GenBank accession number U15575) were selected for primers design (Table 1). Estimated amplicon size was 357 bp for MCP and 479 bp for IE products. Selected primers were analyzed for melting temperature determination, GC percentage and complementarity.

2.2. PCR reaction and sequencing

PCR reaction was performed in a Biorad Thermo Cycler. For each reaction, mixture was 1× PCR buffer (Tris–HCl 20 mM, pH 8.4, KCl 50 mM), dNTP 200 μM, 2 mM $MgCl_2$, 2.5 μM of each primer, 1 U of Taq

Table 1
Primers used for PCR analysis

Primer	Location	Nucleotide sequence
MCP forward	61–81[a]	5′ TAC TTT GTC AAG GAG CAT TAC 3′
MCP reverse	398–418[a]	5′ TCA TGT TAT AGT AGC CTA TGC 3′
IE forward	104–22[b]	5′ ATG ATC CAA GCC TAC CTG TGC 3′
IE reverse	563–583[b]	5′ AAA TGT CCT AAT CTA TAC ACC 3′

[a] Referred to sequence AF192508.
[b] Referred to sequence U15575.