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Conservation

Modified preservation and fungalysin description for *Batrachochytrium dendrobatidis*

Preservación modificada y descripción de la fungalisina para *Batrachochytrium dendrobatidis*

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Abstract

Batrachochytrium dendrobatidis is a pathogenic fungus causing chytridiomycosis, a cutaneous affection resulting in reduction on Anura populations around the world, because of fungalysins, the most important virulence factor. The *B. dendrobatidis* maps (http://www.bd-maps.net/) is the dataset of information about the *B. dendrobatidis* -related isolates. An alternative available for reconstitution of *B. dendrobatidis* strains from cryopreserved cells include methods relying on the World Organization for Animal Health (OIE) protocol. Most recently, much of the interest in the *B. dendrobatidis* research has focused on its DNA sequencing, especially *B. dendrobatidis* JAM81 and *B. dendrobatidis* JEL423 genomes. OBJECTIVE. To evaluate a modified form from OIE protocol for *B. dendrobatidis* strain criopreservation, and *in silico* analysis of *B. dendrobatidis* fungalysin. OIE protocol was modified using *B. dendrobatidis* EV001, focused on cryopreservant concentrations, antibiotics and recovering to −80°C, and microscopic viability evaluation on Tryptone Gelatin hydrolyzed agar. Besides, Bioinformatics was used for the determination of biochemical characteristics from a *B. dendrobatidis* JAM81 fungalysin. Modified OIE protocol was useful by viability, recovering *B. dendrobatidis* EV001 strain. The characterization of *B. dendrobatidis* JAM81 fungalysin showed a molecular weight of 85 kDa, isoelectric point of 8.33, and tertiary structure among others. This indicated that the protein is a metalloproteinase, it has a PepSY domain for protease inhibition, and a catalytic domain that destroy protein barriers.

Keywords: *Batrachochytrium dendrobatidis*, anura, pathogen, zoospore, cryopreservation, fungalysin.

Resumen

Batrachochytrium dendrobatidis es un hongo patógeno que causa quitridiomicosis, una afección cutánea que resulta en la reducción de las poblaciones de anuros en todo el mundo, debido a las fungalisinas, su factor de virulencia más importante. Los mapas de *B. dendrobatidis* (http://www.bd-maps.net/) contienen la información sobre los aislamientos relacionados con *B. dendrobatidis*. Una alternativa disponible para la reconstitución de cepas de *B. dendrobatidis* es a partir de células criopreservadas, los métodos para esto se basan en el protocolo de la Organización Mundial de Sanidad Animal (OIE). Recientemente, gran parte del interés en la investigación de *B. dendrobatidis* se ha centrado en la secuenciación de su ADN, se encuentran disponibles los genomas de *B. dendrobatidis* JAM81 y *B. dendrobatidis* JEL423. OBJETIVO. Evaluar una forma modificada del protocolo de la OIE para la criopreservación de cepas de *B. dendrobatidis* y el análisis *in silico* de su fungalisina. Se modificó el protocolo de la OIE utilizando la cepa de *B. dendrobatidis* EV001, enfocado en los ítems de concentraciones de criopreservantes, antibióticos y recuperación, desde −80°C, y evaluación de viabilidad microscópica en agar hidrolizado con triptona y gelatina. Además se utilizó Bioinformática para la determinación de características bioquímicas de la fungalisina de la cepa de *B. dendrobatidis* JAM81. El protocolo modificado de la OIE resultó útil para la recuperación de la viabilidad de la cepa *B. dendrobatidis* EV001. La caracterización de la fungalisina de *B. dendrobatidis* JAM81 mostró un peso molecular de 85 kDa, punto isoeléctrico de 8,33 y estructura terciaria característica que indicó que la proteína es una metaloproteinasa, tiene un dominio PepSY para la inhibición de proteasas, y un dominio catalítico asociado a destrucción de barreras proteicas.

Palabras clave: *Batrachochytrium dendrobatidis*, anura, patógeno, zoospora, criopreservación, fungalisina.

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1 Introduction

There are several possible reduction causes of the amphibian population, including the following: commercial consumption; introduced species introduction, land usage, pollutants, climate change, and pathogens. There are more than 4,000 species concerning the anurans, and America tropical areas have high diversity of anurans (32% are in South America), e.g., Cali region (Colombia) had a high index of anuran diversity (35 species), Pichincha area (Ecuador) has a richness of anuran species (20 species), and Bolivar region (Venezuela) reported 26 species (Duellman, 1988). Colombia has a richness of anuran species (Flechas et al., 2017; Lynch et al., 1997), but there are more than 50 threatened species (Fisher and Garner, 2020; Spitzen-van der Sluijs et al., 2017; Rueda-Almonacid et al., 2004). Infectious diseases are one of the factors affecting the status of anurans, and among these is the chytridiomycosis, a disease caused by the fungus *Batrachochytrium dendrobatidis*. The fungus has harmful effects on frogs and toads across nations. *B. dendrobatidis* has an affinity with keratinized dermis (Lindauer et al., 2019; Green et al., 2010; Vredenburg et al., 2010; Berger et al., 1998). *B. dendrobatidis* seems to affect anurans according to species, alkaloid synthesis, or relationship between skin and environmental temperature. *B. dendrobatidis* proliferates during the rainy season, and water ecosystems stimulate the pathogen presence in the host (Richmond et al., 2009). The disease has been reported in a total of six areas in Colombia: Amazonas, Boyacá, Cauca, Cundinamarca, Nariño and Valle del Cauca, these regions have a tropical climate with little variation in temperature and high humidity (Flechas et al., 2017; Vásquez-Ochoa et al., 2012; Velásquez et al., 2008).

B. dendrobatidis has two stages: a zoospore stage which is motile and flagellated, where zoospores appeared to initiate a colonization of skin frog. Once inside a host, zoospore can develop a thallus which develops into a zoosporangium (or sporangia), containing the next generation of spores, renewing the cycle. Early zoosporangium is present in viable cells causing hyperkeratosis and hydric-electrolite balance disruption, but matured zoosporangium is predominant in the outer keratinized stratums. The discharge tubes are turned towards the skin frog surface, releasing zoospores into the environment (Rosenblum et al., 2010). *B. dendrobatidis* genome shows a high change rate in lineages, which has promoted theories of their pathogenic evolution: NPH (the novel pathogen hypothesis) which is based on an invasive spread model into new ecosystems or hosts, and EPH (the endemic pathogen hypothesis) which is focused on *B. dendrobatidis* disease as a consequence of an altered association among pathogen-anura-environment (Scheele et al., 2019; Rosenblum et al., 2009).

The method of *B. dendrobatidis* cryopreservation was described by the World Organization for Animal Health (OIE) (OIE-World Organization for Animal Health, 2012). This method is based solely on the microorganism culture for an increase in zoospore and zoosporangium concentration; hence, Dimethyl sulfoxide (DMSO) 10% and fetal bovine serum (FBS) 10% at -80 °C must be used. During thawing, cultures are to be subjected to 43 °C, lactose and antibiotics.

The evidence suggests that enzymatic activity of *B. dendrobatidis* directly influences its degree of pathogenicity. The *B. dendrobatidis* penetration into frog epidermal cells probably requires digestive enzymes. A reference strain of *B. dendrobatidis* is cultured and used to produce proteases, which degrade casein and gelatin. Knowledge on genetic bases, and interactions at molecular level lead to gene expression for serin proteases and fungalysin metallopeptidase, two pathogenesis-related genes identified in other fungus (Rosenblum et al., 2010). *B. dendrobatidis* synthesizes fungalysin metallopeptidases, a class of proteins that are essential for microorganims such as *Trichophyton sp.* and *Microsporum sp.* Fungalysin metallopeptidase gene of *B. dendrobatidis* has high expression levels in zoosporangium (McDonald et al., 2020; Rosenblum et al., 2009). This work evaluates changes to OIE protocol for *B. dendrobatidis* cryopreservation and subsequent recovery of this microorganism and *in silico* description of its fungalysin.

2 Materials and Methods

2.1 Strain

The *B. dendrobatidis* strain EV001 was used, which was donated by Universidad de los Andes (Bogotá-Colombia). *B. dendrobatidis* EV001 represents the

first strain isolated in Colombia (from Ubaqué, Cundinamarca), which was recovered from *Rheobates palmatus* (Flechas et al., 2013). The *B. dendrobatidis* culture medium used is a mixture of tryptone and hydrolysed gelatin (TGh) (tryptone 1%, hydrolysed gelatin 0.4%, agar 1%). Tryptone 1% broth has been used for zoospore production. *B. dendrobatidis* EV001 cells were examined under a microscope using lactophenol cotton blue and Congo red stains.

2.2 *Batrachochytrium dendrobatidis* cryopreservation

A modified OIE preservation method was applied to *B. dendrobatidis* EV001. A 2.0 mL portion of 1-week-old tryptone medium inoculated with *B. dendrobatidis* EV001 was diluted with 13 mL of a fresh tryptone medium in a 25 cm^2 cell culture flask and incubated at room temperature for 4 days by gentle agitation. A scraping of the flask walls was done, then the product was centrifuged at 1700 g for 10 min. The supernatant was eliminated, and the pellet was suspended in 0.6 mL of tryptone broth containing DMSO 10% and freezed at -80 °C. A month later, the frozen pellet were placed in water bath at 43 °C for 30 s and transferred to TGh medium without lactose or antibiotics. A randomized design with three replications was used.

2.3 *Batrachochytrium dendrobatidis* fungalysin

A bioinformatic analysis of fungalysin-like virulence factor was done using the corresponding amino acid sequence from *B. dendrobatidis* JAM81 genome deposited in National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) (NCBI ID: XP_006675712.1). A comparison between the *B. dendrobatidis* JAM81 fungalysin and a fungalysin from *Aspergillus* (Protein Data Bank ID: 4k90) was made with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). ProtParam tool from ExPASy (http://www.expasy.org/) was applied for theoretical description of the *B. dendrobatidis* fungalysin (isoelectric point, molecular weight, hydropathicity, positive residues and aliphatic index). Functional domains were determined using the Conserved Domain Database (CDD) from NCBI. A three dimensional (3D) model of the *B. dendrobatidis* JAM81 fungalysin was calculated by homology modelling using Swiss-model (https://swissmodel.expasy.org/), the model was submitted for refinement to 3Drefine (http://sysbio.rnet.missouri.edu/3Drefine/index.html) and validated, applying QMEAN (https://swissmodel.expasy.org/qmean/). The figure was visualized using Jmol (https://www.bioinformatics.org/firstglance/fgij/).

3 Results

3.1 *B. dendrobatidis* EV001 morphology

B. dendrobatidis EV001 viability was evidenced by means of growth in TGh medium and its morphological structures. *B. dendrobatidis* EV001 shows granular and cream colonies (Figure 1A), and a morphology-like pan can be seen when we examine a Lactophenol Cotton Blue preparation under the microscope, a characteristic of chytridiomycetes (Figure 1B).

B. dendrobatidis EV001 was grown on TGh medium for morphology determination involving zoospores, zoosporangium, discharge tube, thalli or rhizoids (Figure 2 and 3) to establish *B. dendrobatidis* development stages (Figure 4).

3.2 *B. dendrobatidis* cryopreservation method

The *B. dendrobatidis* cryopreservation method based on OIE protocol (OIE-World Organization for Animal Health, 2012) was modified as shown in Table 1. Viability of *B. dendrobatidis* EV001 was confirmed into TGh medium (Figure 5).

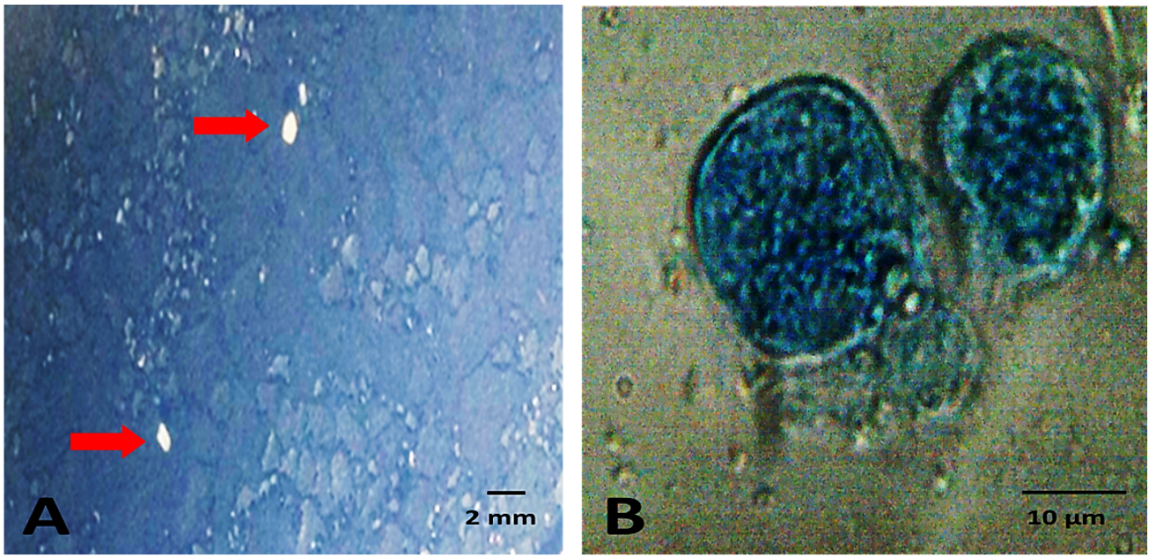

Figure 1. Macroscopic and microscopic morphology of *B. dendrobatidis*. (A) *B. dendrobatidis* EV001 granular breadcrumb colonies on TGh medium (arrows). (B) *B. dendrobatidis* EV001 pan morphology of the fungus (light microscopy at a 40× lens); they typically have 10-40 μm in diameter (Berger et al., 2005).

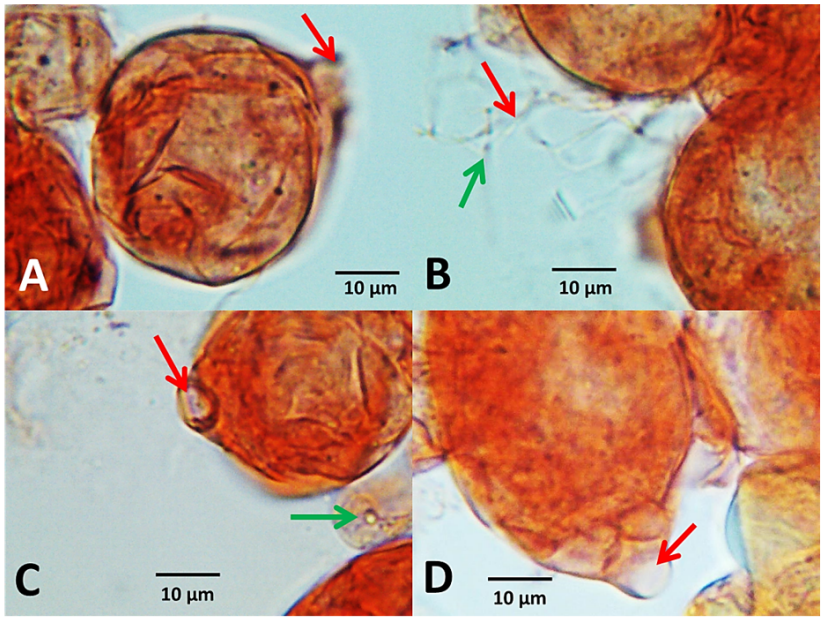

Figure 2. *B. dendrobatidis* EV001 morphology on TGh using Congo red stain. (A). *B. dendrobatidis* EV001 zoosporangium and open discharge tube (red arrow). (B). *B. dendrobatidis* EV001 zoosporangium with thalli (red arrow) and rhizoids (green arrow). (C) *B. dendrobatidis* EV001 zoosporangium with open discharge tube (red arrow), which contains cellulose and chitin, and a zoospore (green arrow). (D) Inoperculate *B. dendrobatidis* EV001 zoosporangium (red arrow). Light microscopy at a 100× lens. Zoosporangium typically has 10-40 μm in diameter (Berger et al., 2005).

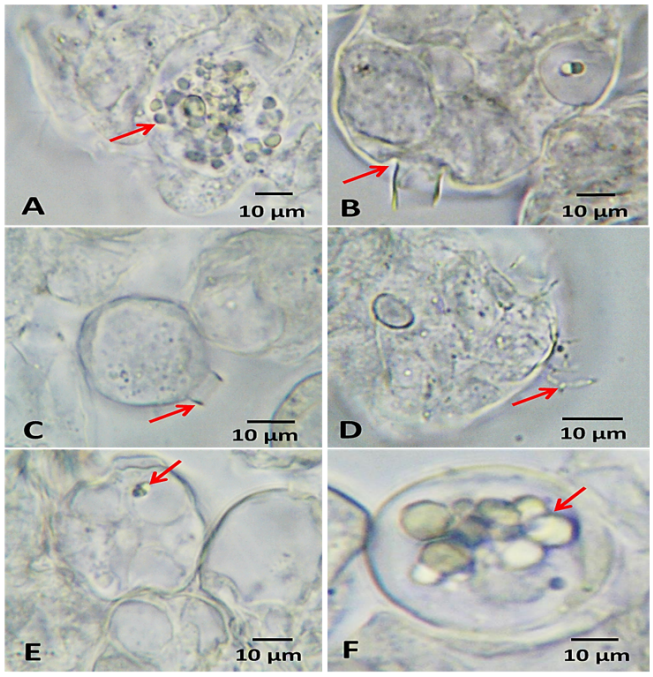

Figure 3. *B. dendrobatidis* EV001 wet mount microscopic examination. (A) *B. dendrobatidis* EV001 zoosporangium and inner zoospores (red arrow). (B) *B. dendrobatidis* EV001 zoosporangium with dictyosomes (red arrow). (C) *B. dendrobatidis* EV001 zoosporangium and discharge tube (red arrow). (D) *B. dendrobatidis* EV001 rhizoid (red arrow) (E) *B. dendrobatidis* EV001 zoospore (red arrow) (F) *B. dendrobatidis* EV001 plasmalemma and inner saccules (red arrow). Light microscopy at a 40× lens. Zoospores typically have 0.7 to 6.0 μm of diameter (Berger et al., 2005).

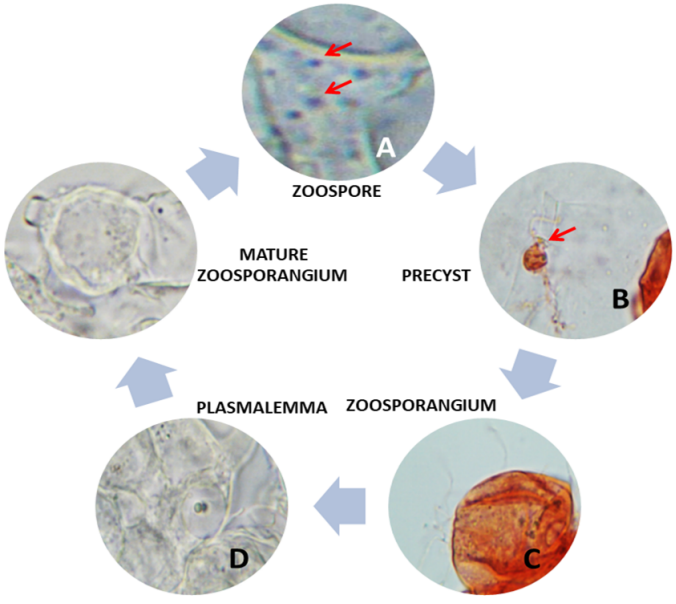

Figure 4. *B. dendrobatidis* EV001 life cycle. (A) *B. dendrobatidis* EV001 zoospores (red arrows). (B) *B. dendrobatidis* EV001 thalli-forming zoospore (precyst) (red arrow). (C) *B. dendrobatidis* EV001 zoosporangium with thalli and rhizoids. (D) *B. dendrobatidis* EV001 plasmalemma, saccules and zoospores. (E) *B. dendrobatidis* EV001 zoosporangium before release of zoospores. Light microscopy at a 100× (A, B, C) and a 40× (D, E) lens.

Table 1. Proposal for modification of OIE method (OIE, 2012) dedicated to the *B. dendrobatidis* cryopreservation using *B. dendrobatidis* EV001.

CRYOPRESERVATION	
OIE protocol	**Changes**
Take 2 ml of culture broth with active growth (one week). Add 13 mL of new broth in a 25 cm^2 flask. Incubate for 3-4 days. Cultures must have a lot of active zoospores, zoosporangium on the broth. Scrape the flask walls and centrifuge to 1700 g per 10 min.	None
Pour off the supernatant	Supernatant was inactivated with hypochlorite 2.5 % (Supernatant contains active zoospores)
Suspend the sediment in 1 ml 10 % DMSO and 10 % FBS in broth and freeze.	0.6 ml of 5 % and 10 % DMSO were used, and FBS was not used. Freezing process was applied to -80 °C for a month
THAWING	
Place the vials directly from liquid nitrogen into water (43 °C) place the vials. Agitate for 30 seconds.	None
Pour the content onto the TGhL agar supplemented with lactose (TGhL) and antibiotics.	The contents of the vial were poured directly into TGh medium. No antibiotics or lactose were used.
Pipette liquid cryoprotectant onto the sample (in the Petri dish).	It has not been applied in this work.
Put on 1 ml of weak saline solution (0.001 M KH_2PO_4, 0.0001 M $MgCl_2$, 0.00002 M $CaCl_2$) into the transferred sample and incubate. Examine the cultures over 7-10 days for movement of zoospores.	To avoid contamination risks, all of the process is designed to be as non-intrusive as possible and reduce the amount of manipulation needed until the colonial growth was observed.

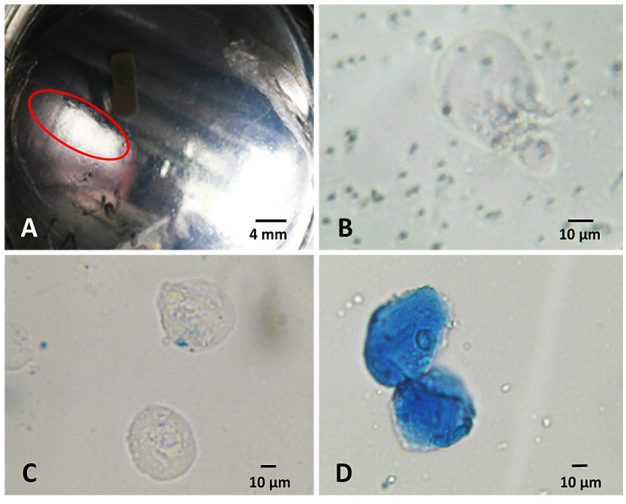

Figure 5. *B. dendrobatidis* EV001 after cryopreservation. A. TGh medium with translucent colonies of *B. dendrobatidis* EV001 are observed in the medium 20 days after thawing. B. *B. dendrobatidis* EV001 zoosporangium (red arrow) and zoospores. C. *B. dendrobatidis* EV001 sporangium with discharge tube. D. Lactophenol blue, *B. dendrobatidis* EV001 zoosporangium, zoospore (lactophenol cotton blue). Light microscopy at a 40× lens

3.3 Bioinformatic analysis of fungalysin

According to the bioinformatic analysis, the predictions show that the *B. dendrobatidis* JAM81 fungalysin has an average life greater than 20 hours, a molecular weight of 85 kDa, an isoelectric point of 8.33, an aliphatic index of 59.68 (it has a high temperature tolerance similar to other proteins), a hydropathicity of -0,625 (hydrophilic protein). *B. dendrobatidis* fungalysin was modeled using a standard fungalysin of *Aspergillus* as template (PDB: 4k90) (Figure 6) (identity: 40.75%, query cover: 45%, E value: 1e-82). 3D theoretical model corresponds to 368 residues (46.8%) from F237 to G604 (Figure 7). The alpha-helices in the model are formed by 126 residues (34.23%), 46 residues have a beta-sheet conformation (12.5%) and 196 residues form loops (53.26%). A HEXXH signal is between residues 407 and 411, and the EXXXD signal is located between residues 437 and 441. Based on the results from CDD, *B. dendrobatidis* JAM81 fungalysin has a PepSY domain (propeptide peptidase) which goes from I164 to the V230, and the catalytic region is between residues Y239 and A590 (Fernández et al., 2013).

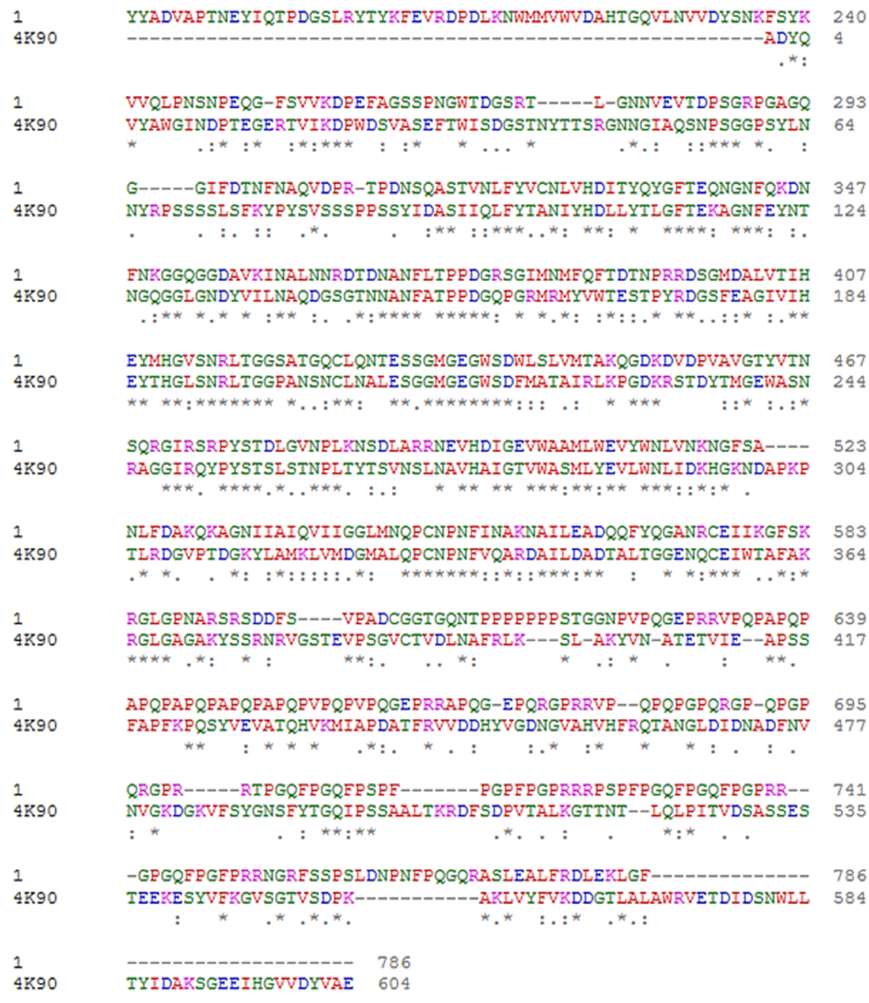

Figure 6. Sequence alignment of *B. dendrobatidis* JAM81 fungalysin and *A. fumigatus* fungalysin. Number 1 represents *B. dendrobatidis* JAM81 fungalysin and 4k90 is the sequence of *A. fumigatus* fungalysin. Asterisks indicate identity, two points are conserved substitutions and a point is a semiconserved substitution. Colors represent amino acid features according to Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

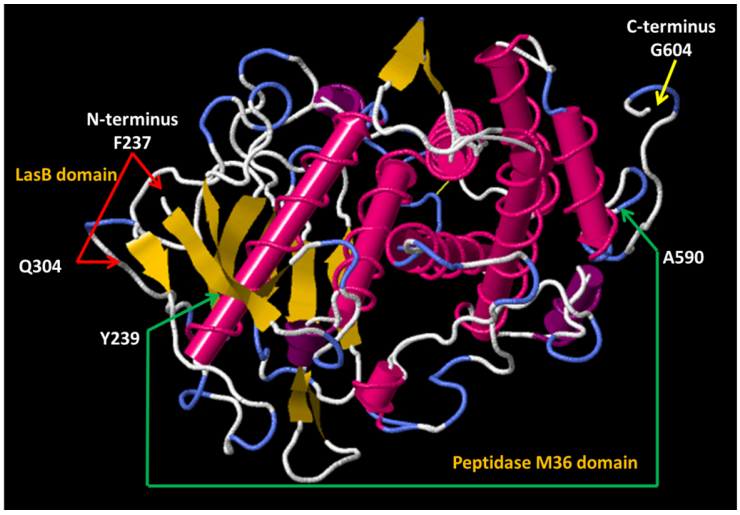

Figure 7. 3D model of *B. dendrobatidis* JAM81 fungalysin. The N-terminus end (F237) and the C-terminus residue G604 (yellow arrow) are indicated. Yellow arrows are beta sheets and pink tubes are helices. Two domains are determined, the LasB domain (F237-Q304, red arrows) is related to Zinc-dependent metalloproteases, and the peptidase M36 domain (Y239-A590, green arrows) is typical of fungalysins.

4 Discussion

4.1 *B. dendrobatidis* EV001 morphology

B. dendrobatidis has a first stage, consisting of a flagellate zoospore that colonizes anura skin. Subsequently, *B. dendrobatidis* zoospores change to spherical thalli that produce new asexually reproduced zoospores inside skin epidermal cells of frog, and then infective zoospores are released into skin surface (Greenberg et al., 2017; Kilpatrick et al., 2010). It is important to analyze this microorganism insofar as it is relevant to understand emergent pathogens that are rapidly disseminated (cosmopolitan), in addition to their aggressive nature toward anurans (Rosenblum et al., 2009). *B. dendrobatidis*-anura interactions are not fully understood (De León et al., 2019).

It should be noted that *B. dendrobatidis* zoosporangium has a refractive structure and abundant presence of zoospores in wet mount analyses (Robinson et al., 2020; Berger et al., 1998). The lactophenol cotton blue slides were similar to those raised in wet slides. Lactophenol cotton blue is a staining agent based on methyl blue and lactophenol; the first component serves as dye of fungal chitin, and lactophenol (phenol, lactic acid, glycerol, water) is a mountant (Leck, 1999). Congo red stain has proven to be a valuable tool for *B. dendrobatidis* identification, which acts similarly to lactophenol cotton blue by staining fungal chitin (Slifkin and Cumbie, 1988). *B. dendrobatidis* EV001 features correspond to the observations made by experts: zoospore presence, refractive zoosporangium or discharge tube among others (Briggs and Burgin, 2004; Berger et al., 1998).

4.2 *B. dendrobatidis* cryopreservation method

At the same time, analyses for the cryopreservation such as the testing of aseptic practices using techniques like storage at cryogenic temperature with 5% DMSO were applied. One month later, the thawing procedure was developed using TGh medium. It was determined that agitate by inverting the container (lid down) was positive for *B. dendrobatidis* EV001 growth, together with culture sealing flasks and their maintenance under aseptic laboratory conditions. Other cryoprotectants were evaluated: 10% skin milk, 10% DMSO with TGhL broth, and 10% DMSO with TGhL broth and 10% FBS. These assays were done using freezer at -80 °C and liquid nitrogen. An evaluation highlighted a better recovery with DMSO and FBS at 4-5 days using freezing at -80 °C, the outcome was similar for liquid nitrogen at 7-14 days (Boyle et al., 2003).

It should be emphasized that antibiotic usage was not employed to avoid microbial contamination, which will reduce artificial selection (Brem et al., 2013); these results can often be achieved with strict use of aseptic conditions involving a biological safety set and previous material sterilization among other procedures. Growth medium can also be contaminated due to tryptone, which is the best nutritional source for microorganisms. Therefore, *Penicillium sp.* and *Bacillus sp.* contamination was observed in our work. Other authors have previously described that *B. dendrobatidis* handling needs a regular examination of flasks and vials for minimizing the impact of contamination variables, besides it was necessary to develop an appropriate set of constant passages (at least every 14-21 days in agar, at least every 4-5 months in broth, storage at 4-6 °C) (Boyle et al., 2003).

The use of lactose is suggested (Flechas et al., 2012), but was not necessary in our study. TGh medium is ideal because *B. dendrobatidis* grow best in tryptone or peptonized milk, and does not require additional sugars; *B. dendrobatidis* synthesizes proteases, which can degrade casein and gelatin (Piotrowski et al., 2004).

On the other hand, weak saline solution replaces pond water as natural environment of *B. dendrobatidis* (Boyle et al., 2003). In this work, weak saline solution was omitted with no effects on *B. dendrobatidis* recovery. Thus, it is said that when environmental and physico-chemical conditions are modified for this microorganism, as has occurred in culture media, changes in zoosporangium size may occur (Garcés et al., 2003). Concerning the observations of stages regarding *B. dendrobatidis* cycle, young zoosporangium formation was observed (germination process), besides discharge tube in 1% tryptone broth related to humidity for zoospore release can be determined, and longer tubes can be viewed in liquid media; however, zoospore release should be in solid media. Zoosporangium requirements on frog skin can be variable and unpredictable in occurrence; however, *B. dendrobatidis* can be viable using 1% tryptone broth, and their rhizoids can be observed with congo red (Berger et al., 2005). This approach can be particularly effective for smaller laboratories, especially those with limited-resource settings, but which can be highly effective in studying *B. dendrobatidis* if good microbiology practices are applied.

4.3 Bioinformatic analysis of fungalysin

B. dendrobatidis fungalysyn-related genes (approximately 25) are expressed primarily in zoosporangium (76%) and only a small percentage is expressed in zoospore (Rosenblum et al., 2008). 3D experimental structure of an *A. fumigatus* fungalysin (AfuMep) has been described, showing relevant parameters for this virulence factors, such as propeptides presence that supress the host proteases (residues 19-245), and C-terminal catalytic domain (residues 245-634) that degrade proteins from extracellular matrix (elastin and collagen). It was found that fungalysin belongs to zinc metalloproteases M36. This paper describes fungalysin as a non-glycosilated protein of 68 kDa, containing a catalytic signal HEXXH for zinc atom, involving 12 alpha-helices (35%) and 22 beta-sheets (12%).

B. dendrobatidis JAM81 fungalysin complies with the M36 family requirements regarding molecular weight (85 kDa), disposal of helices and sheets, HEXXH metalloprotease-like signal, a R470 residue, which is regarded as the important element in folding, and catalytic activity of this enzyme, because elastases and thermolisins have a calcium ion near to zinc ion (16 Å) from catalytic site, with ability to protect the enzyme from autolysis. AfuMep and *B. dendrobatidis* JAM81 fungalysin have a R470 that would protect these virulence factors (Fernández et al., 2013).

The precise mechanisms underlying the chytridiomycosis process are not fully understood, for that reason, several hypotheses have been put forward (Berger et al., 2005). Frog susceptibility to *B. dendrobatidis* is related to how amphibian take their water directly through skin while preserving animal homeostasis, reflecting the fact that *B. dendrobatidis* inhibits sodium channels which are related to chytridiomycosis. It was determined that frog transports sodium from pod to plasma, and epithelial sodium channels and their influence in amphibian kidneys are important in this process, and there is deterioration of the cardiac function before death (Campbell et al., 2012). *B. dendrobatidis* is a nonspecific pathogen, which does not affect all amphibian species equally (Strauss and Smith, 2013) due presumably to skin microbiota, or antimicrobial peptides, and could play an important role in the defense against fungi (Flechas et al., 2013; Berger et al., 1999). *B. dendrobatidis* has alternative hosts and re-

servoirs such as nematodes and river crabs, but infections in other vertebrates are not lethal (Rosenblum et al., 2010; Strauss and Smith, 2013).

5 Conclusion

This work shows a *B. dendrobatidis* EV001 description as an introduction to a modified cryopreservation method, which would be a significant contribution to the laboratories focused on this area. Additionally, a theoretical model of fungalysin is added as a contribution towards understanding the virulence factors of this anura pathogen.

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