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Combining ethidium monoazide treatment with real-time PCR selectively quantifies viable *Batrachochytrium dendrobatidis* cells

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ABSTRACT

Detection of the lethal amphibian fungus *Batrachochytrium dendrobatidis* relies on PCR-based techniques. Although highly accurate and sensitive, these methods fail to distinguish between viable and dead cells. In this study a novel approach combining the DNA intercalating dye ethidium monoazide (EMA) and real-time PCR is presented that allows quantification of viable *B. dendrobatidis* cells without the need for culturing. The developed method is able to suppress real-time PCR signals of heat-killed *B. dendrobatidis* zoospores by 99.9 % and is able to discriminate viable from heat-killed *B. dendrobatidis* zoospores in mixed samples. Furthermore, the novel approach was applied to assess the antifungal activity of the veterinary antiseptic F10® Antiseptic Solution. This disinfectant killed *B. dendrobatidis* zoospores effectively within 1 min at concentrations as low as 1:6400.

Introduction

Amphibian populations are currently facing declines on a global scale. One of the main causes of these declines is the amphibian disease chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Berger *et al.* 1998; Daszak *et al.* 1999; Skerratt *et al.* 2007; James *et al.* 2009). In susceptible amphibian species *B. dendrobatidis* invades skin epithelium (Van Rooij *et al.* 2012) and is able to cause hyperplasia and hyperkeratosis of the epidermis (Berger *et al.* 1998; Pessier *et al.* 1999). These changes attribute to a critical impairment of the normal functioning of the amphibian skin leading to dehydration, electrolyte imbalance, and cardiac arrest (Berger *et al.* 1998; Voyles *et al.* 2007, 2009; Marcum *et al.* 2010; Brutyn *et al.* 2012). Fast and reliable detection of *B. dendrobatidis* is therefore of the greatest importance. The most reliable techniques for detecting *B. dendrobatidis* are based on detecting and quantifying the amount of *B. dendrobatidis* DNA present in a sample (Boyle *et al.* 2004; Hyatt *et al.* 2007; Kirshtein *et al.* 2007; Walker *et al.* 2007). Although these methods can accurately detect and quantify the number of *B. dendrobatidis* genomic equivalents (GE) present in samples, no distinction is made between viable and dead cells of *B. dendrobatidis*. While this is sufficient for the purpose of screening for the presence of *B. dendrobatidis*, fast and selective quantification of viable *B. dendrobatidis* cells without the need for

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culturing would be a major advantage for other purposes. Stockwell *et al.* (2010) already developed a technique to discriminate viable from dead *B. dendrobatidis* zoospores. However, the major drawback of this technique is the lack of specificity towards *B. dendrobatidis* since all cells with a compromised cell membrane will be stained. One method that has proven effective for selective quantification of viable cells is the use of the DNA intercalating dye ethidium monoazide (EMA) in conjunction with RT-PCR (Nogva *et al.* 2003; Rudi *et al.* 2005; Nocker & Camper 2006; Delgado-Viscogliosi *et al.* 2009). The aim of this study is to develop a technique that allows quantification of viable *B. dendrobatidis* cells present in a sample by combining EMA treatment with the RT-PCR described by Boyle *et al.* (2004). Furthermore the application of the developed EMA RT-PCR for the determination of the *B. dendrobatidis* killing capacity of a disinfecting agent is presented.

Materials and methods

Strain & culture conditions

The *Batrachochytrium dendrobatidis* strain JEL423 used in this study was kindly provided by Dr J. Longcore. This strain was isolated from Lemur leaf frogs (*Phyllomedusa lemur*) involved in a mass mortality event (El Copé, Panama, 2004). Strain JEL423 was grown in TGhL broth (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose per litre distilled water) in 25 cm^2 flasks at 20 °C for 5 d.

For collection of zoospores, TGhL agar plates (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, 10 g bacteriological agar per litre distilled water) were inoculated with a 2 ml aliquot of 5-d-old broth culture, and incubated for 5–7 d at 20 °C. Zoospores were collected by flooding each plate with 2 ml distilled water followed by collection of the fluid. The zoospores were washed three times in distilled water by centrifugation (1200 rpm, 20 °C, 2 min). The concentration of zoospores per millilitre was determined with a haemocytometer. Heat treatments (85 °C, 15 min) of aliquots of zoospore suspensions were carried out to obtain dead zoospores. Successful killing of the zoospores was confirmed by plating the heat-treated zoospores on TGhL agar plates and checking for absence of growth during 10 d by light microscopy. Sporangia of *B. dendrobatidis* were harvested by gently scraping the inside of a 25 cm^2 flask that contained a 2-d-old broth culture.

EMA treatment and RT-PCR sample preparation

EMA (Sigma–Aldrich Inc., Bornem, Belgium) was dissolved and diluted in dimethyl formamide (Sigma–Aldrich Inc., Bornem, Belgium) to a concentration of 1 mg ml^{-1} and stored at −20 °C in 1.5 ml lightproof microcentrifuge tubes (Greiner Bio-One GmbH, Frickenhausen, Germany). For the optimization of the EMA protocol, a zoospore suspension containing approximately 10^7 zoospores per millilitre was prepared. During the optimization of the EMA protocol, different EMA treatment concentrations and light exposure times were tested. The tested EMA treatment concentrations were 10, 25, and 50 μg ml^{-1}. The effect of presence of TGhL broth during EMA treatment was assessed by adding a volume of TGhL broth equal to half the sample volume, while the same volume of sterile distilled water was added to the controls. The tested light exposure times were 1 and 5 min (500 W halogen light, 20 cm distance between samples and light). Samples were cooled on ice during incubation to avoid overheating. Samples were washed by centrifugation (5000 rpm, 5 min, 20 °C) followed by resuspension of the pellet in 25 μl HPLC water. DNA extraction of these resuspended pellets was carried out by adding 100 μl Prepman Ultra (Applied Biosystems, Foster City, USA) and heating them to 100 °C for 10 min. All samples were diluted 1:10 in HPLC water in order to minimize PCR inhibition, and stored at −20 °C until further use. Details on the number of included samples per experiment can be found in the specific experiment Subsections 2.3, 2.4, and 2.5. RT-PCR assays were performed on a CFX96 Real Time System (Biorad, Hercules, California, USA) with amplification conditions, primer, and probe concentrations according to Boyle *et al.* (2004). Every sample was run in triplicate in the RT-PCR assay. The method described by Hyatt *et al.* (2007) using the TaqMan Exogenous Internal Positive Control Reagents was used to make sure that PCR inhibition did not affect the RT-PCR results. RT-PCR signals (Ct-values) are converted to GE based on standards containing DNA of 1000, 100, 10, 1, and 0.1 *Batrachochytrium dendrobatidis* genomic equivalents which are prepared as described by Boyle *et al.* (2004). The GE values of the EMA treated samples are considered as the viable fraction of *B. dendrobatidis* cells, while the GE values of the untreated samples are considered as the sum of both viable and dead *B. dendrobatidis* cell fractions. With these assumptions both viable and dead fractions of *B. dendrobatidis* cells in a sample can be calculated.

In experiments described in Subsections 2.3, 2.4, and 2.5 a final EMA concentration of 25 μg ml^{-1} was used. EMA treated samples were incubated shielded from light in 24 well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) for 10 min, followed by incubation in visible halogen light for 5 min. A volume of TGhL broth equal to half the sample volume was added for its protective effect on viable *B. dendrobatidis* organisms during EMA treatment.

Discrimination between viable and dead Batrachochytrium dendrobatidis *zoospores in mixed samples*

A zoospore suspension containing approximately 1.7×10^6 zoospores per millilitre was prepared.

Mixed samples composed of viable and dead *B. dendrobatidis* zoospores were prepared. These samples had different ratio's of viable and dead zoospores ranging from 0 to 100 % viable zoospores and 100–0 % dead zoospores respectively. Three replicates of each ratio were prepared. A 200 μl aliquot of each sample was treated with EMA according to the optimized protocol described in Subsection 2.2. A 200 μl aliquot of each sample without EMA treatment was included as reference. The GE values for the EMA treated and untreated samples were used to determine the number of present viable and dead zoospores in each sample.

Discrimination between viable and dead Batrachochytrium dendrobatidis *zoospores at different zoospore concentrations*

Ten-fold serial dilutions of a zoospore suspension (ranging from 10^6 to 10^1 *B. dendrobatidis* zoospores per millilitre)