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NOTE

Efficacy of SYBR 14/propidium iodide viability stain for the amphibian chytrid fungus *Batrachochytrium dendrobatidis*

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ABSTRACT: The amphibian chytrid fungus *Batrachochytrium dendrobatidis* is a recently described pathogen that has been implicated as a causal agent in the global decline in amphibians. Research into its biology and epidemiology has frequently involved *in vitro* experimentation. However, this research is currently limited by the inability to differentiate between viable and inviable zoospores. Stains are frequently used to determine cell viability, and this study tested a 2-colour fluorescence assay for the detection and quantification of viable *B. dendrobatidis* zoospores. The results show that the nucleic acid stains SYBR 14 and propidium iodide are effective in distinguishing live from dead zoospores, and a protocol has been optimized for their use. This viability assay provides an efficient and reliable tool that will have applications in *B. dendrobatidis* challenge and amphibian exposure experiments.

KEY WORDS: *Batrachochytrium dendrobatidis* · Amphibian chytrid fungus · Viability · Stain · Zoospore

INTRODUCTION

The amphibian chytrid fungus *Batrachochytrium dendrobatidis* is a recently described microorganism belonging to the phylum Chytridiomycota. The Chytridiomycota are a group of aquatic heterotrophic fungi with zoosporangic thalli and discharge papillae that release motile flagellated zoospores (Barr 1990). They are generally biodegraders of cellulose, chitin and keratin, although some are pathogens of vascular plants and insects (Barr 1990). *B. dendrobatidis* is the first Chytridiomycota described that infects vertebrate hosts (Berger et al. 1998, Longcore et al. 1999). It infects the keratinized mouthparts of tadpoles and the outer keratinized epidermal layers of frogs, which can cause the fatal disease chytridiomycosis in susceptible species (Berger & Speare 1998, Berger et al. 1998, Longcore et al. 1999). Chytridiomycosis has been implicated as a causal agent in the decline and extinction of amphibian populations worldwide (Laurance et al. 1996, Berger et al. 1998, Lips 2005, Skerratt et al. 2007).

Since the discovery of *Batrachochytrium dendrobatidis*, *in vitro* experimentation has played an important role in understanding its biology and epidemiology. Challenge assays have been used in the development of disinfectants and quarantine guidelines to prevent its spread (Johnson et al. 2003, Johnson & Speare 2003, Webb et al. 2007, Mendez et al. 2008) as well as the identification of environmental tolerance limits (Piotrowski et al. 2004) and the effectiveness of host immune responses (Rollins-Smith et al. 2002a,b, 2003, Johnson & Speare 2003, Harris et al. 2006, Woodhams et al. 2006a,b, Conlon et al. 2007, Woodhams et al. 2007a,b,c).

Amphibian exposure experiments conducted *in vitro* have also investigated the effects of infection on the

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survivorship of different host species (Daszak et al. 2004, Blaustein et al. 2005, Garcia et al. 2006, Vazquez et al. 2009) and how this differs with host age (Lamirande & Nichols 2002, Rachowicz & Vredenburg 2004), body size (Carey et al. 2006), immune response (Woodhams et al. 2007a), fungal strain (Berger et al. 2005, Retallick & Miera 2007) and fungal density (Carey et al. 2006). The outcome of infections has been investigated under different temperature regimes (Berger et al. 2004, Carey et al. 2006, Andre et al. 2008, Woodhams et al. 2008) and in the presence of environmental stressors (Parris & Baud 2004, Garcia et al. 2006), competitors (Parris & Cornelius 2004) and predators (Parris & Beaudoin 2004). However, this research is currently limited by the inability to confidently distinguish and quantify zoospore viability.

The viability of *Batrachochytrium dendrobatidis* zoospores in culture is difficult to determine and indices such as growth and motility are generally used. However, the use of these indicators requires observation over several weeks and an absence of growth and motility does not necessarily imply that a cell is inviable. Stains are frequently used to detect cell viability in microbiology, medical sciences and reproductive biology and they work by targeting cellular processes that change with cell death (Haugland 2002). The stains SYBR 14 and propidium iodide are nucleic acid stains that can provide a 2-colour fluorescence assay of cell viability based on their ability to penetrate cell membranes. The SYBR 14 stain passively diffuses through the membranes of all cells, binds to nucleic acids and fluoresces green with visible light excitation (Haugland 2002). Propidium iodide is a membrane-impermeable stain that can only penetrate cells when their membranes are permeabilized. Diffusion into dead and dying cells occurs as the regulation of membrane transport slows or cells lyse and the stain binds to nucleic acids making dead cells fluoresce red (Krishan 1975, Jones & Senft 1985, Haugland 2002). This paper examines the efficacy of SYBR 14/propidium iodide stains in determining the viability of *B. dendrobatidis* zoospores.

MATERIALS AND METHODS

A *Batrachochytium dendrobatidis* isolate (strain Gibbo River-Llesueuri-00-LB1) was obtained from the Australian Animal Health Laboratory (AAHL) in Geelong, Victoria, and stored at –80°C, following the protocol developed by Boyle et al. (2003). For this experiment, a sample of *B. dendrobatidis* was thawed and then grown in 10 ml culture flasks in TGhL liquid culture media (16 g tryptone, 2 g gelatin hydrolysate, 4 g lactose in 1 l deionised water) at 22°C for 4 d. A 1 ml sample of the actively growing culture was transferred to a sterile culture plate containing TGhL agar (16 g tryptone, 2 g gelatin hydrolysate, 4 g lactose, 10 g bacteriological agar in 1 l of deionised water) and allowed to grow at 22°C for 4 d. Zoospores were then harvested by flooding the culture plate with 5 ml of sterile distilled water and allowing it to stand for 1 h. This zoospore suspension was then collected and used to test the efficacy of the viability stains and to optimize stain concentrations.

To test the efficacy of the stains differentiating between live and dead zoospores, half of the resulting zoospore suspension was heat treated in an oven at 47°C for 1 h, in order to kill the zoospores (Johnson et al. 2003), while the other half was kept alive in a constant temperature room at 22°C for 1 h. Mixtures of non-heat-treated and heat-treated zoospore concentrations (presumably live or dead respectively) were then made with 0, 25, 50, 75 or 100% heat-treated zoospore suspension.

In order to confirm that the heat treatment had killed the zoospores and that those identified as dead in this experiment were non-viable, a growth experiment was conducted. A 100 µl sample of each previously prepared zoospore suspension was grown in 900 µl of TGhL liquid media in a 48-well flat-bottom culture plate, with each concentration replicated 8 times. Plates were incubated at 22°C for 7 d. The amount of growth in each well was then sampled by counting the number of zoosporangia in an ocular frame (76 × 42 µm) at 200× magnification under an inverted microscope (Axiovert 35, Zeiss).

SYBR 14 and propidium iodide (Molecular Probes) were obtained as a sperm viability kit (catalogue number L-7011). The SYBR 14 dye was supplied as a 1 mM solution in dimethyl sulfoxide (DMSO) and was diluted 1:100 with 1X tris-buffered saline (TBS, pH 7.4). The propidium iodide was supplied as an aqueous 2.4 mM solution and was used undiluted. To stain zoospores, 50 µl of each suspension mixture was added to 8 wells in a 96-well plate and left for 20 min so that zoospores settled to the bottom of the well. A 6 µl sample of the diluted SYBR 14 dye was added to each well and allowed to incubate at room temperature for 10 min. A total of 2 µl of undiluted propidium iodide was then added to each and again left to incubate at room temperature for 10 min. To quantify the stained zoospores, the number of zoospores fluorescing red and green within an ocular frame (26 × 17 µm) were counted at 400× magnification under an inverted microscope with mercury lamp fluorescence (HBO 50 W/AC, Osram) and blue light 450 to 490 nm excitation (450 to 490 nm exciter filter, FT 510 nm chrome beam splitter, LP 520 nm barrier filter). The percentage of zoospores that were fluorescing red was then deter-

mined. A chi-square goodness-of-fit test was used to determine whether the observed percentage of red zoospores was significantly different from the expected percentage of heat-treated and presumably dead zoospores in each suspension mixture.

Finally, in addition to determining the efficacy of these stains, the stain concentrations that allowed the greatest differentiation between red and green were identified through a visual assessment of fluorescent intensity with different concentration combinations of SYBR 14 and propidium iodide. Cell monolayers were created by growing 25 µl of the actively growing culture in 175 µl of fresh liquid media in each well of a 96-well plate at 22°C for 7 d. Following growth, 150 µl of active culture media was removed from each well, leaving a 50 µl layer of culture media covering the surface. Combinations of SYBR 14 (2, 4, 6, 8 and 10 µl) and propidium iodide (1, 2 and 3 µl) volumes were each added to 8 wells, with SYBR 14 added first and left to incubate for 10 min, and then propidium iodide was added followed by another 10 min incubation period. Each well was then observed under an inverted microscope at 400× magnification and were given scores out of 5 for intensity of green and red zoospore fluorescence and the intensity of any background fluorescence (0 = no fluorescence; 5 = bright fluorescence). The concentration combination that provided the highest average zoospore fluorescence and the lowest average background fluorescence was considered optimal.

RESULTS

The growth experiment confirmed that heat treatment was effective in killing zoospores by demonstrating that no growth occurred in the 100% heat-treated suspension (Fig. 1). The number of zoospores counted following growth in the 0 to 75% heat-treated mixtures also approximated the proportion of cells added to each group, relative to one another (Fig. 1). In addition, all motile zoospores were found to stain green and motility was observed in stained cells for 4 h, at which point the experiment was terminated. No motile zoospores were seen to stain red.

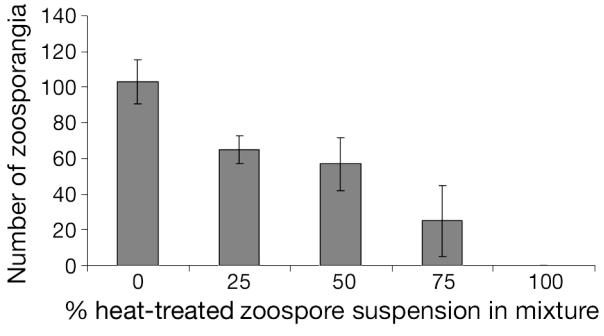

Fig. 1. *Batrachochytrium dendrobatidis*. Mean (± SE) zoosporangia counted after 7 d growth at 22°C in zoospore suspension mixture containing various proportions of heat-treated (and presumably dead) zoospores

Zoospore mixtures containing no heat-treated zoospores (0% heat-treated) still stained 12 ± 6% of zoospores red (average ± SE) and this was assumed to be due to the collection of some dead zoospores from the culture plate at the harvesting stage. Therefore, the number of red zoospores counted in each well of the remaining mixtures was corrected for the 12% of dead zoospores in the original zoospore suspension. Following this correction, the mean proportions of red zoospores counted in each well were similar to the proportion of heat-treated zoospores added to the mixtures (Table 1). A chi-square goodness of fit test determined that there was no significant difference between the observed and expected proportions of red zoospores in each group (25% mixture $\chi^2_7 = 3.67$; 50% mixture $\chi^2_7 = 12.20$; 75% mixture $\chi^2_7 = 6.00$), indicating that the stains were accurately distinguishing live from dead zoospores.

The testing of differing stain concentrations established that optimal staining was achieved with 6 µl of the 1:100 diluted SYBR 14 and 2 µl of undiluted propidium iodide. These concentrations produced brightly stained zoospores that were clearly distinguishable as either green or red with little background fluorescence (Fig. 2). Incubation times of 10 min between the additions of each stain were found to be appropriate for achieving optimal stain differentiation.

DISCUSSION

The use of stains to target cellular processes that change with cell death can resolve the uncertainty associated with determining cell viability. The results of the present study show that live and dead *Batrachochytium dendrobatidis* zoospores can be differenti-

Table 1. Mean proportion (±SD) of red zoospores counted following cell viability staining of heat-treated zoospore suspension mixtures where 12% of red zoospores were disregarded from each well to account for dead zoospores collected at harvesting

% of heat-treated zoospores	Mean % of red zoospores (±SD)
0	0
25	28 (±13)
50	62 (±14)
75	77 (±10)
100	100 (±0)

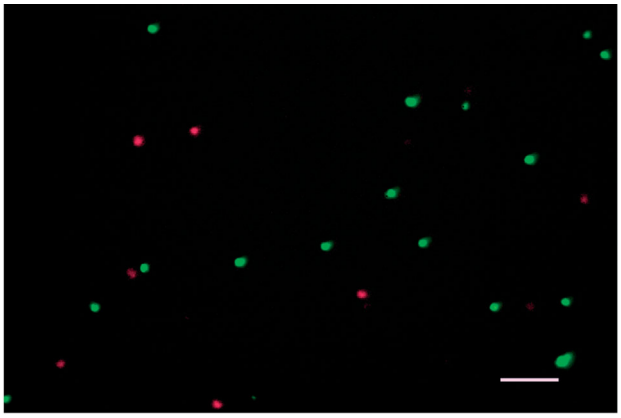

Fig. 2. *Batrachochytrium dendrobatidis.* Photomicrograph of zoospores following cell viability staining with SYBR 14 and propidium iodide. Live zoospores stain green; dead zoospores stain red. Scale bar = 20 µm

ated with the use of a SYBR 14/propidium iodide 2-colour fluorescent assay and a protocol has been optimized for this use. This assay provides a simple and time-efficient tool that will assist in the quantification of viable zoospores and will have a range of applications.

The availability of a viability stain for *Batrachochytium dendrobatidis* zoospores will prove particularly valuable for assessing the outcome of challenge assays. The capacity to distinguish live from dead cells following exposure to a stressor will allow survival rates to be quantified for comparison between treatments. This method removes the need to rely upon indices of cell viability such as growth and motility and will result in more reliable outcomes. The quantification of viable zoospores may also prove useful in amphibian exposure experiments that require zoospores to be harvested from actively growing cultures. Determining the proportion of viable, and therefore infective, zoospores harvested prior to exposures will allow the desired dosage to be calculated. It will also enhance repeatability in future studies and may improve the accuracy of infection outcome predictions. This will be particularly important in experiments exposing amphibians to multiple batches, strains or isolates of *B. dendrobatidis*, where differences in the proportion of viable zoospores in each harvest may confound results.

In addition to distinguishing live from dead cells for quantification, staining of live zoospores may also provide novel ways of observing motility, dispersal, growth and survival. SYBR 14 binds to nucleic acids and fluoresces, without interfering with cellular processes, and this study suggests observation of live zoospores could be made for at least 4 h following staining. However, because both SYBR 14 and propidium iodide are nucleic acid stains, caution should be taken when utilizing these stains in situations where DNA may be disrupted or denatured, such as in the presence of alcohols or oxidizing agents (Overton & McCoy 1994, Phe et al. 2007). Damage or changes to the structural integrity of nucleic acids will limit the stain's binding ability, resulting in reduced fluorescent signal (Hamilton et al. 1980, Overton & McCoy 1994, Phe et al. 2007). The inclusion of internal positive controls in experiments will allow these reduced signals to be identified.

Despite this limitation, the use of viability stains in *Batrachochytium dendrobatidis* research will improve the efficiency, reliability and repeatability of future *in vitro* experimental outcomes. The identification of methods to control the spread and impact of *B. dendrobatidis* infection is essential for the prevention and reversal of amphibian declines and the availability of the SYBR 14/propidium iodide viability assay can facilitate this process.

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