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NOTE

Sodium hypochlorite denatures the DNA of the amphibian chytrid fungus *Batrachochytrium dendrobatidis*

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ABSTRACT: *Batrachochytrium dendrobatidis*, an aquatic amphibian fungus, has been implicated in many amphibian declines and extinctions. A real-time polymerase chain reaction (PCR) TaqMan® assay is now used to detect and quantify *B. dendrobatidis* on amphibians and other substrates via tissue samples, swabbing and filtration. The extreme sensitivity of this diagnostic test makes it necessary to rigorously avoid cross-contamination of samples, which can produce false positives. One technique used to eliminate contamination is to destroy the contaminating DNA by chemical means. We tested 3 concentrations of sodium hypochlorite (NaOCl) (1, 6 and12%) over 4 time periods (1, 6, 15 and 24 h) to determine if NaOCl denatures *B. dendrobatidis* DNA sufficiently to prevent its recognition and amplification in PCR tests for the fungus. Soaking in 12% NaOCl denatured 100% of DNA within 1 h. Six percent NaOCl was on average 99.999% effective across all exposure periods, with only very low numbers of zoospores detected following treatment. One percent NaOCl was ineffective across all treatment periods. Under ideal, clean conditions treatment with 6% NaOCl may be sufficient to destroy DNA and prevent cross-contamination of samples; however, we recommend treatment with 12% NaOCl for 1 h to be confident all *B. dendrobatidis* DNA is destroyed.

KEY WORDS: Chytridiomycosis · Denaturation · Field hygiene · PCR · Diagnostic test

INTRODUCTION

Chytridiomycosis, caused by the highly virulent fungus *Batrachochytrium dendrobatidis*, has caused declines of natural amphibian populations and deaths in captive populations around the world (Berger et al. 1998, Pessier et al. 1999, Lips et al. 2006, Rachowicz et al. 2006, Skerratt et al. 2007). *B. dendrobatidis* is a highly transmissible pathogen requiring proper hygiene protocols, including disinfection of equipment, to prevent its spread (Speare et al. 2004). Many disinfectants are very effective at killing *B. dendrobatidis* on a range of substrates (Johnson et al. 2003, Webb et al. 2007). However, for researchers collecting and processing samples for diagnostic PCR, killing *B. dendrobatidis* is not enough; it is necessary to destroy its DNA to prevent cross-contamination of samples that may lead to false positives.

DNA-based PCR tests are now commonly used diagnostic tools to detect *Batrachochytrium dendrobatidis* due to their sensitivity and specificity (Annis et al. 2004, Boyle et al. 2004, Hyatt et al. 2007). When using diagnostic PCR, it is important to prevent contamination of samples. Even very low levels of DNA contamination on sampling equipment can create false positives. Recent papers have raised concerns regarding the generation of false positives via shared equipment (Kirshtein et al. 2007, Woodhams et al. 2007). While it is now standard practice to disinfect field equipment with bleach, >70% ethanol or other treatments to

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prevent the transfer of live *B. dendrobatidis* among individual amphibians and sites (Johnson et al. 2003, Speare et al. 2004, Webb et al. 2007), these protocols may not denature *B. dendrobatidis* DNA. This would make the results of diagnostic PCR unreliable. Dipping instruments in alcohol and burning off the residue is an effective sterilisation technique and may render *B. dendrobatidis* DNA undetectable by PCR. However, this has not yet been tested and flaming is an impractical solution for larger field tools such as collection trays and animal enclosures and equipment that would be destroyed by flames such as fabrics and plastics.

The antimicrobial properties of sodium hypochlorite (NaOCl) are well known and it is widely used as a disinfectant in medicine (Eventov et al. 1998), endodontics (Gomes et al. 2001), water treatment and around the home. Archaeologists and forensic scientists have discovered its usefulness for destroying contaminating DNA prior to PCR amplification of target DNA from teeth and bones (Kemp & Smith 2005). NaOCl has also been used to denature pathogens and allergens. Sehulster et al. (1981) found that NaOCl eliminates Hepatitis B antigenicity; Matsui et al. (2003) reduced the immunogenicity of a cat allergen, and Martyny et al. (2005) denatured the fungal allergen *Aspergillus fumigatus* on environmental substrates, significantly reducing its recognition by ELISA. We experimentally tested 3 concentrations of NaOCl over 4 exposure periods to determine its effectiveness at denaturing *Batrachochytrium dendrobatidis* DNA as determined through a real-time PCR assay.

MATERIALS AND METHODS

We maintained *Batrachochytrium dendrobatidis* culture in TGHL broth (16 g tryptone, 4 g gelatine hydrolysate, 2 g lactose, 10 g agar, and 1000 ml distilled H_2O) in 75 cm^2 tissue culture flasks (Sarstedt) at 20°C. After 4 d, the flask bottom was scraped with a cell scraper to dislodge all encysted zoospores and zoosporangia. The entire cell suspension (~30 ml) was emptied into a 50 ml centrifuge tube and centrifuged at 1100 *g* for 5 min at 4°C. The supernatant, containing the reproductive zoospores, was then transferred to a clean tube and the pellet, consisting mostly of zoosporangia, was discarded. The tube containing the supernatant was gently inverted 2 or more times to ensure mixing. Three separate aliquots of the suspension were then removed and counted on a haemocytometer. The concentration of zoospores was then diluted to 4.0×10^5 zoospores ml^{-1} in TGHL broth. We added 200 µl of this suspension to each well of a 96-well plate and the first 5 columns of a second 96-well plate. A visual inspection of the wells using an inverted microscope confirmed that all wells contained similar numbers of active zoospores. The 96-well plates were then placed in a 20°C incubator.

After 3 d, microscopic examination confirmed that there was considerable growth in each well, with mature zoosporangia and active zoospores. We removed the broth from each well and replaced it with 200 µl of 1 of 3 NaOCl concentrations; 1, 6 and 12%, or a control (TGHL). Each NaOCl dilution at each exposure period had 7 replicates and the control group had 10 replicates.

In order to prevent *Batrachochytrium dendrobatidis* in the control group from continuing reproduction, controls were processed immediately after addition of the TGHL. The 200 µl of TGHL was removed and 200 µl of a dilute saline (DS) solution added (Boyle et al. 2003). The bottom and sides of each control well were scraped 16 times, and the corner where the sides meet the bottom of the well was scraped 4 times with a sterile wooden dowel. The 200 µl aliquot of DS was then removed and placed in a 1.5 ml Micro tube (Sarstedt). A second 200 µl aliquot of DS was then added to the well and the process was repeated. All samples were immediately centrifuged at 16 100 *g* for 3 min to form a pellet of *B. dendrobatidis*. Most of the supernatant (280 µl) was removed and discarded and the tube placed in a –60°C freezer.

Following 1, 6, 15 and 24 h of exposure of *Batrachochytrium dendrobatidis* to NaOCl, the procedure detailed above for the control group was repeated with the 3 NaOCl dilution treatments. As with the TGHL in the controls, the NaOCl solution was removed and discarded from each well prior to the addition of DS. Before the addition and removal of the NaOCl solution, a visual inspection of each well was conducted to assess fungal condition. Quantitative real-time TaqMan® (Applied Biosystems) PCR assays were run on all samples using a Rotor-Gene™ 6000 (Corbett Life Sciences) as described by Boyle et al. (2004) with some modifications. In order to test for possible inhibition by residual NaOCl, a repeat triplicate analysis was performed on 4 samples containing the highest concentration of NaOCl (12%), thus the most likely to inhibit, and 4 controls containing no NaOCl by incorporating the TaqMan® Exogenous internal positive control (IPC) (0.6x Exo IPC Mix, 0.6x Exo IPC DNA) into the assay. Inhibition is indicated by threshold cycle (Ct) values significantly higher than those obtained for the negative control.

Examination of the data made it clear that some results did not require statistical hypothesis testing. When results were not certain by examination, hypotheses were tested using 1-way ANOVA to compare differences among exposure periods within NaOCl treatments, and Bonferroni-adjusted *t*-tests

were used to determine whether groups of NaOCl treatments that did not differ significantly among themselves differed significantly from controls. All statistical analyses were performed using STATISTICA 7.1 (StatSoft).

RESULTS

The 1% NaOCl solution did not differ significantly in effectiveness across the 4 exposure periods (ANOVA, $F = 0.777$, df = 3, 27, p = 0.519), and comparison with the control treatment indicated that it did not denature *Batrachochytrium dendrobatidis* DNA effectively enough over any of the 4 exposure periods for use as a denaturing agent ($t = -0.340$, df = 36, p = 0.736, Fig. 1). Only very low numbers of zoospores were detected in the 6% NaOCl treatment (Fig. 1). These did not differ among exposure period treatments (ANOVA, $F = 0.531$, df = 3, 27 p = 0.665) and were significantly reduced compared to controls ($t = 3.088$, df = 36, p = 0.004). The 12% NaOCl treatment was 100% effective and denatured all *B. dendrobatidis* DNA across all exposure periods (Fig. 1). No inhibition from the 12% NaOCl was detected with the internal positive controls (mean Ct value 12% NaOCl = 28.12, mean Ct value control = 28.61, $t = -0.985$, df = 6, p = 0.363). Visual inspection of each well prior to the removal of NaOCl revealed that zoosporangia and zoospores from the 6 and 12% NaOCl treatments were severely fragmented and completely unrecognisable. The 1% NaOCl treatment wells contained shrivelled and shrunken but recognizable zoospores and zoosporangia.

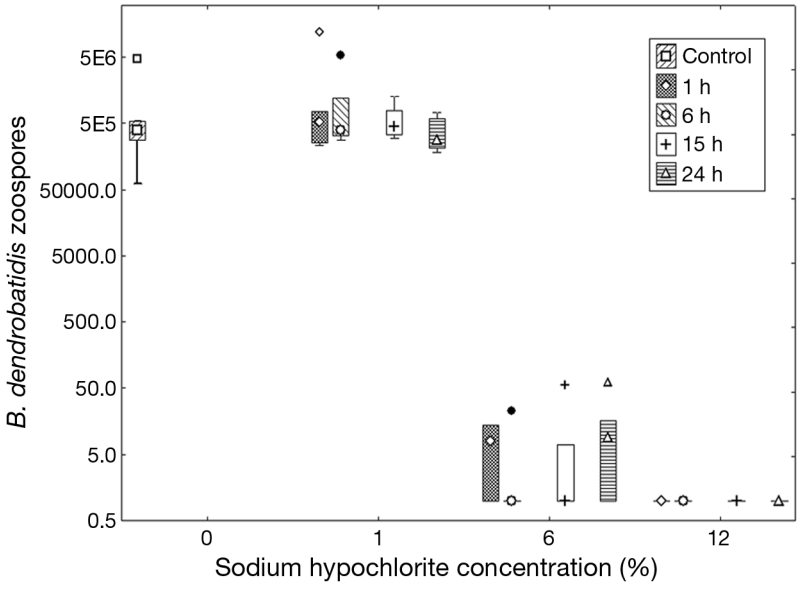

Fig. 1. *Batrachochytrium dendrobatidis*. Number of zoospores detected in controls and at 3 concentrations of sodium hypochlorite (NaOCl) after varying periods of exposure. Symbols within boxes represent the median; boxes represent the upper and lower quartiles; whiskers represent the non-outlier spread; symbols above boxes represent the outliers

DISCUSSION

The present study found that 12% NaOCl can be effectively used to denature 100% of even very high densities of *Batrachochytrium dendrobatidis* within 1 h. Similarly, 6% NaOCl was extremely effective, with a mean of only 6 to 13 zoospore equivalents detected across all exposure periods (Fig. 1). This represents a mean reduction from the controls of 99.999%. As the PCR assay can detect as little as 1 zoospore (Boyle et al. 2004), a minimum of ca. 100 000 contaminant zoospores on average would need to be present for a false positive result following treatment with 6% NaOCl. In practice, the number of *B. dendrobatidis* zoospores contaminating equipment is likely to be a fraction of the levels examined in the present study. For example, the highest level of *B. dendrobatidis* reported on a swab sample from an infected amphibian is 545 000 zoospores on a *Mixophyes fasciolatus* (Hyatt et al. 2007). Any contamination of equipment is likely to be at levels far below those of severely infected amphibians. It is possible then that a 6% NaOCl solution can be used to denature DNA sufficiently on clean equipment to prevent false positives through PCR. However, as field equipment often contains soil, plant material and other particulates which may either shield *B. dendrobatidis* or decrease the efficiency of NaOCl (LeChevallier et al. 1988), it is far safer and advisable to use 12% NaOCl to prevent cross-contamination. Prior to soaking in NaOCl, equipment should be cleaned to reduce the negative impact of any attached particles. The 1% NaOCl solution did not effectively denature *B. dendrobatidis* zoospores at any of the exposure periods and should not be used to prevent sample contamination.

Most household bleach products contain between 4.00 and 6.15% NaOCl, and the most common concentration of commercially available bulk NaOCl is 12%, making the concentrations tested here readily available to most researchers. Exactly how NaOCl deactivates microorganisms has never been experimentally shown (Gomes et al. 2001). However, it is believed that cell

death is a result of oxidation of sulfhydryl groups and amino acids on the exterior of the cell by OCl^- (Eventov et al. 1998). NaOCl destroys DNA in a similar manner through oxidative damage (Ohnishi et al. 2002, Kemp & Smith 2005), resulting in the breakdown of DNA into segments shorter than that recognized by the PCR assay (Prince & Andrus 1992).

Because PCR is now the preferred diagnostic test for chytridiomycosis (Hyatt et al. 2007), it is crucial to minimise contamination so that results are comparable and reliable. Just as great care should be taken in the laboratory during processing of samples to prevent contamination, equal care must be taken in the field during the collection of samples. When sampling amphibians for infection, each individual should be captured with a separate pair of gloves or plastic bag. If individuals are captured in a bag, a fresh pair of gloves should be worn during each swabbing and each swab should be housed individually. These precautions should be sufficient to prevent cross-contamination of DNA during field surveys where the amphibians are captured by hand and returned to their natural habitat following swabbing. However, as researchers move from documenting occurrence and prevalence of chytridiomycosis into more manipulative experimental work, the risk of contaminating samples through shared contact presents an important problem. For example, reusing experimental equipment such as containers, enclosures, sorting trays and nets in conjunction with PCR can result in contamination of samples. Also, equipment used to search for *Batrachochytrium dendrobatidis* in the environment, such as filter holders and tubing, could cross-contaminate samples (Kirshtein et al. 2007). As NaOCl is toxic, great care should be taken to avoid release or spillage of bleach into water bodies, drains or drainages when in the field. Used bleach that needs to be disposed of should always be carried back to the lab or, if absolutely necessary, spread onto a flat surface such as a paved road to evaporate. NaOCl evaporates quickly and breaks down to water, oxygen and table salt (NaCl). However, other chemicals may be added during production, particularly in household cleaning products (Clarkson & Moule 1998).

Bleach already forms a part of many researchers' tool kit for the purposes of disinfection. Our experiment shows that NaOCl can also be used to denature *Batrachochytrium dendrobatidis* DNA to prevent its recognition by PCR and reduce the likelihood of cross-contamination of samples.

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