Video Article

Reduced Itraconazole Concentration and Durations Are Successful in Treating *Batrachochytrium dendrobatidis* Infection in Amphibians

Laura A. Brannelly[1]

[1]School of Public Health, Tropical Medicine and Rehabilitation Sciences, James Cook University

Correspondence to: Laura A. Brannelly at laura.brannelly@my.jcu.edu.au

URL: https://www.jove.com/video/51166
DOI: doi:10.3791/51166

Keywords: Immunology, Issue 85, *Batrachochytrium dendrobatidis*, itraconazole, chytridiomycosis, captive assurance colonies, amphibian conservation

Date Published: 3/14/2014

Citation: Brannelly, L.A. Reduced Itraconazole Concentration and Durations Are Successful in Treating *Batrachochytrium dendrobatidis* Infection in Amphibians. *J. Vis. Exp.* (85), e51166, doi:10.3791/51166 (2014).

Abstract

Amphibians are experiencing the greatest decline of any vertebrate class and a leading cause of these declines is a fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*), which causes the disease chytridiomycosis. Captive assurance colonies are important worldwide for threatened amphibian species and may be the only lifeline for those in critical threat of extinction. Maintaining disease free colonies is a priority of captive managers, yet safe and effective treatments for all species and across life stages have not been identified. The most widely used chemotherapeutic treatment is itraconazole, although the dosage commonly used can be harmful to some individuals and species. We performed a clinical treatment trial to assess whether a lower and safer but effective dose of itraconazole could be found to cure *Bd* infections. We found that by reducing the treatment concentration from 0.01-0.0025% and reducing the treatment duration from 11-6 days of 5 min baths, frogs could be cured of *Bd* infection with fewer side effects and less treatment-associated mortality.

Video Link

The video component of this article can be found at https://www.jove.com/video/51166/

Introduction

Amphibians are currently experiencing the greatest decline in biodiversity of all vertebrate taxa[1]. Chytridiomycosis, a skin disease caused by the chytrid fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), is one of the major causes of these dramatic declines[2]. *Bd* is the worst pathogen on record for causing biodiversity loss: it infects over 600 species of amphibians and has caused declines in over 300 species, of which as many as 200 species have become critically endangered or extinct[2,3]. The amphibian chytrid fungus is not host specific and it is unclear what qualities of the host allow for *Bd* infection to progress causing severe disease. Chytridiomycosis causes death by disrupting ion channels in the skin, resulting in electrolyte loss and heart failure[4]. Also, *Bd* supernatant (containing no *Bd* zoospores) causes an immune response in tadpoles[5] leading researchers to believe *Bd* produces a toxic by-product[6].

Because many species are rapidly declining, captive assurance colonies and breeding programs are important conservation measures, and may be the only lifeline for some species. Establishing and maintaining disease free colonies is a challenge for amphibian management facilities. The current chemotherapeutic methods for treating *Bd* infections, although effective, can be harmful to the animals being treated, and no single treatment method has been successful across species and age classes of amphibians[7].

Few clinical trials of treatments for *Bd* infection have been performed[7]. The three most commonly used methods of treatment are heat therapy, chloramphenicol and itraconazole. Heat therapy is a nonchemotherapeutic treatment option that appears to have few side effects, can be used on multiple life stages, and is generally effective. In a treatment trial, Chatfield and Richards-Zawacki[8] found that 96% of the treated animals had cleared infections after being housed at 30 °C for 10 days. Although this trial was not 100% successful, it provides a baseline for others to assess heat therapy for their own species. Heat therapy, although effective for many species, is impractical for many alpine species that cannot remain at high temperatures for an extended period of time. Chloramphenicol, an antibacterial, has been used successfully to treat *Bd* in many species, and inhibits fungal growth *in vitro*. As an antibacterial, the chloramphenicol does not attack the fungus specifically but has been known to cause apoptosis in eukaryotic cells[9]. It has been speculated that chloramphenicol therapy causes apoptosis in the skin, killing intracellular sporangia, although the exact mechanism of action is unknown. Although no clinical trial has been conducted, and therefore dose and treatment time have not been assessed, current treatment protocols typically require submersion for two to four weeks[10,11], making this treatment impractical for terrestrial amphibians. Chloramphenicol has also been restricted in some countries for veterinary use because human exposure has been associated with a rare form of aplastic anemia[12].

Itraconazole, an azole antifungal, is the most widely used for treatment of *Bd* infection in amphibians in captivity. The most widely used protocol is bathing animals in a 0.01% itraconazole solution for five minutes a day for 11 consecutive days. This protocol was derived from standards used for mammalian administration, and, while effective for amphibians, it is not based on clinical trials in amphibians[11,13]. This protocol can cause negative side effects in certain species, especially in the genus *Rana* and in tadpoles and recent metamorphs[11]. Tadpole treatment

Copyright © 2014 Journal of Visualized Experiments

has been successful using a much lower concentration (0.0005%) of itraconazole, but was associated with depigmentation[14]. This same concentration was trialed on recent metamorphs and found to be unsuccessful at treating infection[15]. Because there is risk of negative side effects with itraconazole treatment, some practitioners have used one half the usual concentration (0.005%) for 11 consecutive days and found this to be successful in curing *Bd* infections[16].

The present study is a summary of a clinical treatment trial using itraconazole to treat *Bd* infection[15]. The protocol describes an attempt to find the lowest dose and treatment time that is both safe and effective for treating *Bd* infected animals with itraconazole. Animals used in this study were recent metamorphs of gulf coast toads, *Incilius nebulifer*.

Protocol

Ethics Approval: This study and its methods were approved by Tulane University's Animal Use and Care Committee (protocol no. 0407).

1. Testing for *Bd* Infection

1. Swabbing for *Bd*
    1. Swab animals with a sterile cotton swab five times on the ventrum, five times on each thigh, each side, and each limb for a total of 45 strokes. Swabbing is a noninvasive method of sampling for *Bd* on amphibian skin.
    2. Gently rotate the swab during and between strokes to ensure the greatest amount of DNA is gathered on the swab.
    3. Store swabs in 1.5 ml microtubes at -20 °C.
2. DNA Extraction and qPCR assay
    1. Extract genomic DNA from the swabs using a commercially available DNA isolation kit, in accordance with the instructions for animal tissue.
    2. Analyze the extracted DNA using quantitative real time PCR (qPCR) following Boyle *et al.*[17], with the following modifications:
    3. Dilute DNA extraction samples 1:10 with double deionized water.
    4. Add 0.7 µl of bovine serum albumin (BSA) to prevent PCR inhibition.
    5. Include an internal positive control in each sample to ensure PCR inhibition was not affecting results.
    6. Run samples with a positive and negative control and a series of dilution standards to estimate zoospore (infection) load.

2. Inoculation

1. Culture *Bd*
    1. Prepare tryptone broth by adding 10 g tryptone to 1 L reverse osmosis or deionized water, autoclaved (121 °C for 40 min) and allowed to cool. Once cooled to 23 °C, add 1 ml penicillin-streptomycin.
    2. Grow *Bd* strain (JEL411 from J. Longcore, isolated from Guabal, Panama from a *Phyllomedusa lemur*) in a tryptone broth for seven days at 23 °C.
2. Transfer the broth culture to tryptone and agar plates.
    1. To make plates, add 10 g tryptone and 10 g agar to 1 L reverse osmosis or deionized water and autoclave (121 °C for 40 min).
    2. Once the autoclaved mixture is cool enough to touch, but before it congeals, pour into Petri dishes, 1/4-1/3 full, in a laminar flow hood.
    3. When agar has congealed and cooled completely, add 0.5 ml *Bd* broth to each plate and spread evenly around the plate. Allow to dry.
    4. Once the plates are inoculated with *Bd* zoospores, seal plates with Parafilm.
    5. Allow plates to incubate at 23 °C for five to seven days. Check zoospore movement to ensure viability before inoculation.
3. Flood plates for zoospore inoculation solution
    1. After the five to seven days of *Bd* incubation and growth, flood each plate with 5 ml of aged tap water and allow zoospores to enter the media for 10 min.
    2. Pour the zoospore suspension into a portable container.
    3. Estimate zoospore concentration with a hemocytometer.
    4. Dilute mixture to a concentration of 1 x 10^6 per 3 ml with aged tap water.
4. Inoculate animals
    1. In individual 50 ml containers, inoculate each animal by pouring 3 ml inoculum mixture over its ventrum. Allow extra inoculum to drip into the base of the inoculation container.
    2. Each animal will remain in inoculation container for 24 hr to ensure infection.
    3. After 24 hr of inoculation, return animals to individual disinfected terraria.
    4. Allow infection to build for two weeks. Confirm infection with swab and qPCR results, and begin treatment.
    5. For *Bd*-negative controls, flood *Bd*-free agar plates with 5 ml aged tap water, and mock inoculate the animals using the same methods as above.

3. Treatment Regimen

1. Prepare treatment bath
    1. Add 2.50 ml aqueous itraconazole (1% Sporanox Oral Solution) to 1 L Amphibian Ringer's Solution (0.0025% itraconazole - the lowest effective treatment concentration).

Copyright © 2014 Journal of Visualized Experiments

1. Amphibian Ringer's Solution recipe: 1 L reverse osmosis or deionized water, 6.6 g sodium chloride, 0.15 g potassium chloride, 0.15 g calcium chloride, and 0.2 g sodium bicarbonate. Autoclave solution (121 °C for 40 min) and allow to cool to room temperature.
2. For 0.01% itraconazole treatment concentration, add 10.00 ml aqueous itraconazole (1% Sporanox Oral Solution) to 1 L Amphibian Ringer's Solution
3. For 0.005% itraconazole treatment concentration, add 5.00 ml aqueous itraconazole (1% Sporanox Oral Solution) to 1 L Amphibian Ringer's Solution.

2. For *Bd*-positive and *Bd*-negative controls, bathe animals in Amphibian Ringer's Solution with no itraconazole.

2. Treatment
   1. Place individual amphibians in disinfected 50 ml containers with 35 ml of the treatment solution.
   2. Ensure animals are fully covered for five minutes by gently swirling the container.
   3. Return animals to disinfected terraria. Repeat for six days.

3. Ensure treatment success
   1. Test animals for *Bd* infection on the day treatment commences.
   2. Test animals for *Bd* infection one week after treatment finishes, and once a week for four weeks to ensure infection has cleared.

4. *Bd* as a Biohazard

1. Disinfect individual terraria weekly.
   1. Bathe the terraria and treatment containers in a 10% bleach solution bath.
   2. Rinse each bleached container twice with running water.
   3. Allow containers to dry for 24 hr before reuse.

2. Wear clean nitrile gloves when handling animals to prevent cross contamination of *Bd* pathogen. Change gloves between handling each animal.
3. Disinfect all liquid waste prior to disposal by bringing the solution to 10% bleach.
4. Autoclave all solid waste that is potentially exposed to *Bd* prior to disposal.

Representative Results

Itraconazole treatment was considered successful if all animals were *Bd*-negative four weeks after treatment ended. Before treatment commenced, two weeks after inoculation, all *Bd*-inoculated animals were infected, with a mean zoospore load of 1,121.7 (±17.34), while four weeks after treatment ended, the *Bd*-positive control animals had a mean zoospore load of 16,765.12 (±14643.08).

Treatment of *Incilius nebulifer* (n = 15) was successful using baths in 0.0025% itraconazole concentration for five-minutes a day for six days. This low concentration dosage of itraconazole treatment did not differ significantly in mortality when compared to the *Bd*-positive controls (Cox regression: $\chi_{2,1}$ = 2.17, exp(b) = 20,500, p = 0.339) (**Figure 1B**), many of whom survived until the end of the trial with *Bd* infection.

Baths of 0.0025% itraconazole for 11 days, and both 0.005% and 0.0025% itraconazole for six days were all found to be effective and safe treatment options (**Figures 1A** and **1B**).The recommended treatment concentration and half that concentration for a treatment length of 11 days significantly increased mortality when compared to the *Bd*-positive controls (Cox regression: 0.01% itraconazole $\chi_{2,1}$ = 35.68, exp(b) = 307.54, p < 0.01; 0.005% itraconazole $\chi_{2,1}$ =16.17, exp(b) = 9.40, p < 0.01) (**Figure 1A**). The 0.01% itraconazole treatment for 11 days caused 100% mortality in *I. nebulifer*, and half that concentration (0.005% itraconazole for 11 days) caused 60% mortality (**Figure 1A**). Necropsy and histopathology for a small subset of animals that died during treatment were conducted. No animal in this study died of chytridiomycosis.

Copyright © 2014 Journal of Visualized Experiments

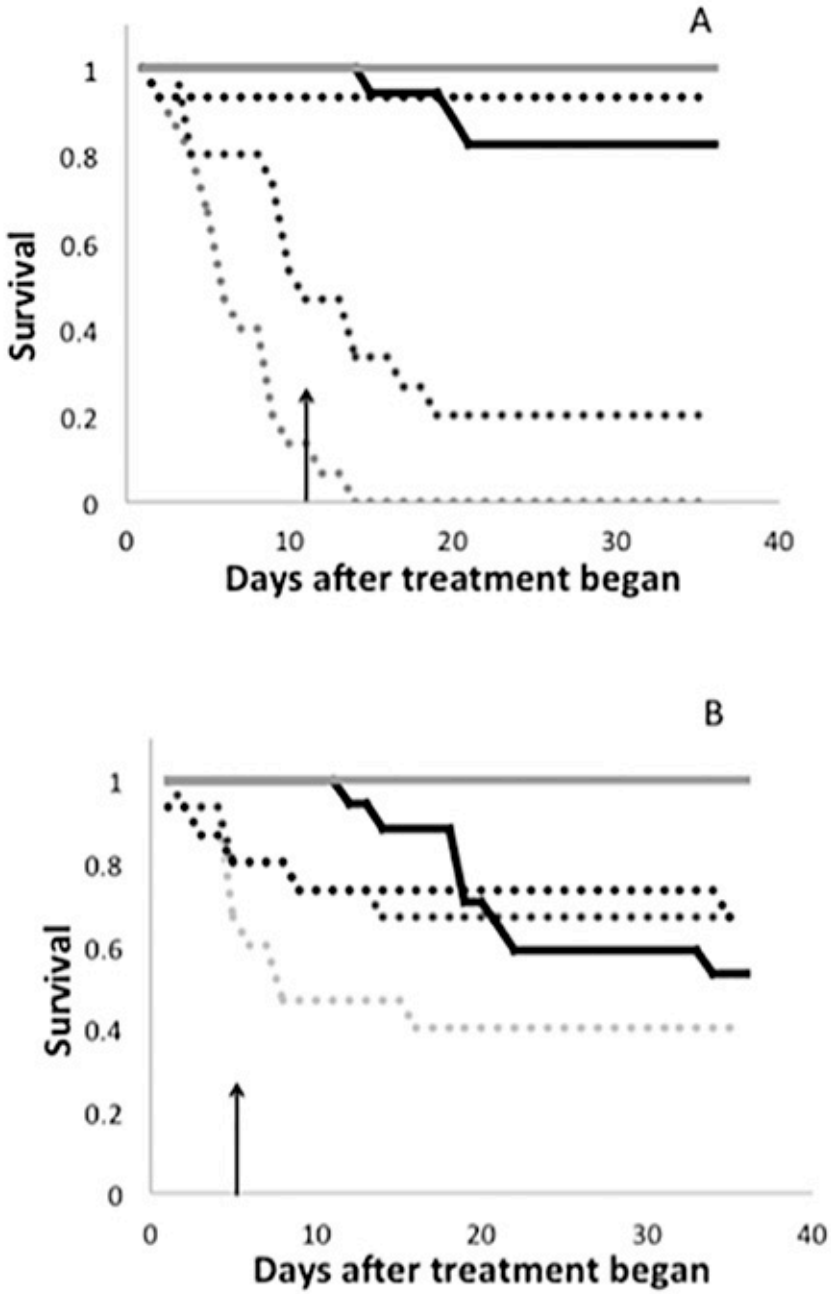

Figure 1. Differences in survival across itraconazole treatments[15]. (**A**) *Incilius nebulifer:* 11 d treatment (n = 15 for each itraconazole concentration of 0.01, 0.005, and 0.0025%, n = 17 for the *Bd*-positive control group and n = 9 for the *Bd*-negative control group). (**B**) *I. nebulifer*: 6 d treatment (n = 15 for each itraconazole concentration of 0.01, 0.005 and 0.0025%, n = 17 for the *Bd*-positive control group and n = 9 for the *Bd*-negative control group). Black arrows indicate the last day of treatment. Solid lines indicate survival of control groups (black = *Bd*-positive, gray = *Bd*-negative). Broken lines indicate survival of itraconazole treatment groups (light gray dots = 0.01%, medium gray dots = 0.005%, black dots = 0.0025% itraconazole). (This figure has been modified from Brannelly *et al.*[15], Figure 1B and 1C). Click here to view larger image.

Discussion

Using this protocol we were able to safely and effectively reduce the treatment dose and time of itraconazole treatment for *Bd* in amphibians. The lowest treatment dose and time (0.0025% for six days) was a successful treatment for subclinically infected *I. nebulifer*. The effectiveness of both a lower dose and treatment time demonstrate the need for clinical treatment trials for *Bd* infection treatments, as there are currently no safe and effective treatments to be used for all species and age classes of amphibians.

The cause of death resulting from itraconazole treatment is unknown at this point[11]. Necropsy and histopathology were conducted on a small subset of individuals that died during or shortly after the treatment period in the Brannelly *et al.*[15] study. Results of these analyses were

inconclusive as to the cause of death, although it is clear that no animal died of chytridiomycosis. Moderate autolysis occurred between death and specimen preservation, which may have affected our ability to detect subtle differences between treatment groups.

Although this protocol supports the existence of one safe and effective treatment regimen, more trials like this one need to be underway. A second azole treatment, voriconazole, has been demonstrated to be effective in at least one species[18]. Voriconazole is more stable in a water solution than itraconazole and has been demonstrated to be safe for tadpole use[18]. Although a single treatment dose of itraconazole and voriconazole are similar in pricing, voriconazole is much more expensive for purchase than itraconazole, leading researchers and captive managers to choose itraconazole more regularly. At this time, only one published study has demonstrated the effectively of voriconazole[18]. To demonstrate efficacy, more species need to be trialed.

Azole antifungals represent one type of antifungal chemotherapy that inhibit fungal growth[13]. There are other treatment methods that aim to kill the fungal pathogen rather than just reduce growth. *Bd* is killed *in vitro* at temperatures above 28 °C and heat therapy aims to kill *Bd* in the epidermis. Terbinafine hydrochloride, an alternative antifungal that has been successful at treating *Bd* infection in some species[19], works by causing lysis of the fungal cells. Antifungals that directly attack the pathogen could be more successful treatments, and more work needs to be done to test promising treatment options for *Bd* infection.

Itraconazole treatment has been shown to be effective in subclinically infected animals, but treatment of clinical chytridiomycosis has largely been unsuccessful. Only one study to date[20] has successfully treated terminally ill amphibians, and the treatment involved an aggressive combination of electrolyte therapy and chemotherapy. Successful treatment options for terminally ill animals are needed, especially for critically endangered animals in captive colonies where protection of every individual is essential for conservation of the species.

Although reducing the dose and treatment time was successful for *I. nebulifer*, the treatment has not been trialed on other species or animals with clinical infections. Some species and life stages are intolerant of itraconazole treatment, so before applying a large-scale treatment regime, captive managers should trial treatment on a small subset of individuals to determine safety and effectiveness. Testing the treated animals for at least four weeks after the completion of treatment is necessary to ensure eradication of low level infection[7], and should be implemented after every treatment regimen.

Establishing safe and effective treatment options for *Bd* infections in captive assurance colonies area priority for amphibian conservation efforts. Maintaining *Bd* free colonies is essential to the success of conservation efforts and captive colonies may be the only lifeline for species on the brink of extinction.

Disclosures

The author declares no competing financial interests.

Acknowledgements

I would like to thank C.L. Richards-Zawacki for help conceptualizing the experimental design and assisting with materials, A. Pessier for help establishing a treatment regimen and necropsy analyses, M.W.H. Chatfield, M.J. Robak, K. Tasker and D. Lenger for help in the laboratory and support and M. McFadden and P. Harlow at the Taronga Zoo for use of their space during filming. The project was partially funded by Louisiana Department of Wildlife and Fisheries through the Louisiana Environmental Education Commission grant awarded to L.A. Brannelly and by Life Technologies.

References

1. Stuart, S. N., *et al.* Status and trends of amphibian declines and extinctions worldwide. *Science.* **306** (5702), 1783-1786, 10.1126/science.1103538 (2004).
2. Skerratt, L. F., *et al.* Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth.* **4**, 125-134, 10.1007/s10393-007-0093-5 (2007).
3. Wake, D. B., Vredenburg, V. T. Colloquium paper: are we in the midst of the sixth mass extinction? A view from the world of amphibians. *PNAS.* **105**, 11466-11473, 10.1073/pnas.0801921105 (2008).
4. Voyles, J., *et al.* Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. *Dis. Aquat. Org.* **77** (2), 113-118, 10.3354/dao01838 (2007).
5. Ramsey, J. P. Amphibian immune defenses against *Batrachochytrium dendrobatidis*: A war between host and pathogen. PhD Dissertation (2011).
6. McMahon, T. A., *et al.* Chytrid fungus *Batrachochytrium dendrobatidis* has nonamphibian hosts and releases chemicals that cause pathology in the absence of infection. *PNAS.* **110** (1), 210-215, 10.1073/pnas.1200592110 (2013).
7. Berger, L., Speare, R., Pessier, A., Voyles, J., Skerratt, L. F. Treatment of chytridiomycosis requires urgent clinical trials. *Dis. Aquat. Org.* **92**, 165-174, 10.3354/dao02238 (2010).
8. Chatfield, M. W. H., Richards-Zawacki, C. L. Elevated temperature as a treatment for *Batrachochytrium dendrobatidis* infection in captive frogs. *Dis. Aquat. Org.* **94** (3), 235-238, 10.3354/dao02337 (2011).
9. Karbowski, M., *et al.* Free radical-induced megamitochondria formation and apoptosis. *Free Rad. Biol. Med.* **26**, 396-409, 10.1016/S0891-5849(98)00209-3 (1999).
10. Bishop, P. J., *et al.* Elimination of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* by Archey's frog *Leiopelma archeyi*. *Dis. Aquat. Org.* **84** (1), 9-15, 10.3354/dao02028 (2009).
11. Pessier, A. P., Mendelson, J. *A Manual For Control of Infectious Diseases in Amphibian Survival Assurance Colonies and Reintroduction Programs*. (February) IUCN/SSC Conservation Breeding Specialist Group: Apple Valley, MN (2009).

Copyright © 2014 Journal of Visualized Experiments

12. Page, S. W. Chloramphenicol 1. Hazards of use and the current regulatory environment. *Aus. Vet. J.* **68** (1), 1-2**,** 10.1111/j.1751-0813.1991.tb09826.x (1991).
13. Yoon, H.-J., Seo, M. H., Kim, J.-K. *Pharmaceutical formulations for itraconazole*. Patent (2005).
14. Garner, T. W. J., Garcia, G., Carroll, B., Fisher, M. C. Using itraconazole to clean *Batrachochytrium dendrobatidis* infection, and subsequent depigmentation of *Alytes muletensis* tadpoles. *Dis. Aquat. Org.* **83**, 257-260, 10.3354/dao02008 (2009).
15. Brannelly, L. A., Richards-Zawacki, C. L., Pessier, A. P. Clinical trials with itraconazole as a treatment for chytrid fungal infections in amphibians. *Dis. Aquat. Org.* **101** (2), 95-104, 10.3354/dao02521 (2012).
16. Jones, M. E. B., Paddock, D., Bender, L., Allen, J. L., Schrenzel, M. S., Pessier, A. P. Treatment of chytridiomycosis with reduced-dose itraconazole. *Dis. Aquat. Org.* **99** (3), 243-249, 10.3354/dao02475 (2012).
17. Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T., Hyatt, A. D. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis. Aquat. Org.* **60** (2), 141-148, 10.3354/dao060141 (2004).
18. Martel, A., *et al*. Developing a safe antifungal treatment protocol to eliminate *Batrachochytrium dendrobatidis* from amphibians. *Med. Mycol.* **49** (2), 143-149, 10.3109/13693786.2010.508185 (2011).
19. Bowerman, J., Rombough, C., Weinstock, S. R., Padgett-Flohr, G. E. Terbinafine hydrochloride in ethanol effectively clears *Batrachochytrium dendrobatidis* in amphibians. *J. Herpetol. Med. Surg.* **20** (1), 24-29, 10.5818/1529-9651-20.1.24 (2010).
20. Young, S., Speare, R., Berger, L., Skerratt, L.F. Chloramphenicol with fluid and electrolyte therapy cures terminally ill green tree frogs (*Litoria caerulea*) with chytridiomycosis. *J. Zoo Wildlife Med.* **43** (2), 330-337 (2012).

Copyright © 2014 Journal of Visualized Experiments