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Contribution to DAO Special 4 'Chytridiomycosis: an emerging disease'

Treatment of chytridiomycosis requires urgent clinical trials

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ABSTRACT: Effective and safe treatments of amphibian chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (*Bd*), are needed to prevent mortality in captive programs, reduce the risk of disease spread, and better manage the disease in threatened wild populations. *Bd* is susceptible to a range of antifungal agents and low levels of heat (>30°C) when tested *in vitro*, but there are few proven methods for clearing adult amphibians of *Bd*, and acute drug toxicity is a problem for tadpoles and juveniles. In postmetamorphic animals, heat (32 and 37°C) is the only well-supported treatment. Antifungal drugs have not undergone rigorous testing — for example, trials were small or lacked controls and thorough post-treatment testing. In addition, pharmacokinetic studies have not been performed so there are no data on blood or tissue levels of antifungal agents. However, itraconazole baths have been widely used in amphibian rescue and conservation programs and anecdotal evidence suggests that they are effective for adults and subadults. In an experimental trial with tadpoles, a low dose of itraconazole cleared *Bd* but may have been associated with cutaneous depigmentation. Fluconazole appeared safe for tadpoles as it did not cause mortality, and future attempts to find an effective dose may be worthwhile. Palliative restoration of blood sodium and potassium levels by administration of electrolyte solutions appears useful in frogs with clinical chytridiomycosis. Randomised and blinded clinical trials, which include basic pharmacological studies, are urgently needed to provide comparable evidence for the safety and efficacy of treatment options which are likely to vary with amphibian species. Priorities are to validate and optimize the use of heat and itraconazole regimes.

KEY WORDS: *Batrachochytrium dendrobatidis* · Antifungal testing · Treatment · Chytridiomycosis · Heat · Clinical trial

INTRODUCTION

The disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has spread globally, resulting in catastrophic declines of many species of amphibians (Berger et al. 1998, Skerratt et al. 2007). Spherical sporangia of *Bd* occur within superficial amphibian epidermal cells, and the infectious stage is the water-borne zoospore (Berger et al. 2005a). Treatments for frogs and tadpoles are needed to prevent mortality from chytridiomycosis in captive breeding programs, reduce the risks of disease spread associated with anthropogenic movement of amphibians, and manage chytridiomycosis in wild amphibian populations.

A treatment for chytridiomycosis could be used to clear infected and potentially infected frogs as they are taken into captivity and enable these animals to be used as a basis for captive survival-assurance colonies (Gagliardo et al. 2008). As infected tadpoles may survive and remain at sites after adults have died (Laurance et al. 1996), it is a priority to develop a treatment that would enable them to be rescued from the wild and raised to adults in captivity. Captive breeding pro-

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grams are currently the only available option to prevent extinction of some species (Mendelson et al. 2006, Gagliardo et al. 2008). The following species are thought to be extinct, or are likely to become extinct, in the wild due to chytridiomycosis and are currently only secure in captivity: the Panamanian golden frog *Atelopus zeteki* from Central America (Gagliardo et al. 2008), the Kihansi spray toad *Nectophrynoides asperginis* from Africa (Weldon & du Preez 2004), and the southern corroboree frog *Pseudophryne corroboree* from Australia (D. Hunter pers. comm.). The last known group of sharp-snouted day frog *Taudactylus acutirostris* died with chytridiomycosis in captivity in 1995. This species might have been saved if *Bd* had been detected earlier and a treatment had been available (Banks & McCracken 2002, Schloegel et al. 2006).

The ability to treat captive populations is also important for commercial use of amphibians in laboratories and in the global pet and food trade. The ability to treat chytridiomycosis in amphibians that are moved globally could reduce the risk of anthropogenic spread of the disease into naïve populations (e.g. Hong Kong may be free of *Bd*; Rowley et al. 2007). International movements of amphibians will come under increasing attention since amphibian chytridiomycosis was listed by the World Organisation for Animal Health as a notifiable disease.

Treatments may also be useful to decrease the impact of chytridiomycosis in wild amphibian populations. For example, as an emergency response at the front of the epidemic, it may be possible to treat free-living adults to allow acquired immunity to develop. There are examples in other wildlife species where treatment has apparently controlled outbreaks of infectious disease in free-living animals, e.g. sarcoptic mange (Mörner 1992). Also, as part of ongoing management, treatments could be used for frogs in areas where chytridiomycosis is endemic, especially when environmental conditions are optimal for chytridiomycosis. This may reduce the impact of the disease, potentially until host resistance evolves or pathogen virulence wanes. In stream environments, treatment would involve catching and holding frogs for individual treatment before release. In small ponds, adding antifungals to the water body may be an alternative option, although with additional risks to other organisms.

The optimal antifungal regime should be effective at eliminating *Bd* with a high safety margin for tadpoles, metamorphs, juveniles, and adults — particularly for use in valuable breeding stock of endangered species. It should be easily administered to numerous animals at one time, and be able to cure frogs in late stages of the disease. Ideally the treatment should be inexpensive and readily available, and work rapidly. A treatment that was effective after a single application would be most feasible for use in the field.

Here we review studies on *in vitro* and *in vivo* antifungal testing to assess the evidence for efficacy and safety of treatments for chytridiomycosis. No treatments have undergone rigorous testing, and we suggest a standardized approach for future trials.

EVALUATING ANTIFUNGAL EFFECTS *IN VITRO*

Routine methods used in diagnostic mycology laboratories for testing antifungal chemotherapeutics on pathogenic fungi were unsuccessful with *Bd* (Berger et al. 2009b). Due to the slow growth of *Bd*, antifungal tablet methods on agar were not useful as 1 to 2 d incubation periods did not reveal a distinct zone of inhibition, and excess diffusion of the antifungal compound occurred over longer periods. Commercial multi-well plate methods for testing drugs on yeasts involve colour changes to indicate growth, but these use RPMI as the medium, which does not support *Bd* (Berger et al. 2009b).

Three types of methods have been used for determining antifungal effects on *Bd* grown in broth cultures in 96 well plates. First, to determine exposure times and concentrations required to kill *Bd*, antimicrobial compounds were applied to fungal monolayers in wells, and then removed at various time points and new broth added (Berger 2001, Johnson et al. 2003, Webb et al. 2007). This assay is the only test for fungicidal activity on sporangia; removal of the compound allows for growth of surviving sporangia. Another advantage is that compounds can be diluted solely with water, whereas with other methods they are diluted in nutrient media, which may alter their efficacy. Second, fungistatic effects can be assessed by measuring the optical density of *Bd* broth cultures at various constant concentrations of antifungal compounds using a spectrophotometer, and a growth curve constructed (Rollins-Smith et al. 2002). Third, microscopy can be used to determine concentrations of compounds that inhibit zoospore encystment and growth (Berger et al. 2009b). This is a sensitive method that could underestimate required drug levels because concentrations that inhibit sporangia are likely to be greater than those needed to inhibit zoospores, which have no cell wall. However, this method is a useful screening test due to its simplicity in comparing different compounds at different concentrations.

The zoospore inhibition method has been used to determine the minimum inhibitory concentrations (MIC) of 9 commercially available antifungal compounds: benzalkonium chloride (<0.78 mg l^{-1}), povidone iodine (312.5 mg l^{-1}), amphotericin B (3.125 mg l^{-1}), fluconazole (<1.56 mg l^{-1}), itraconazole (<1.56 mg

l^{-1}), enilconazole (<1.56 mg l^{-1}), mercurochrome (6.25 mg l^{-1}), sodium chloride (12.5 g l^{-1}), and methylene blue (<1.56 mg l^{-1}) (Berger et al. 2009b).

To further screen compounds for anti-*Bd* effects, *in vitro* studies measuring both fungistatic and fungicidal effects should be used before doing more costly *in vivo* trials. There is variation among azole antifungals in having fungicidal or fungistatic effects against different fungal species (Anaissie et al. 2009). In addition, different levels of one compound can cause static or cidal effects. For example, in a trial that used zoospore inhibition methods to test effects of salinity, zoospores of *Bd* encysted and grew in 0.625% sodium chloride but not in 1.25% (Berger et al. 2009b), whereas 5% sodium chloride took 5 min to kill cultures containing sporangia using the method that measures fungicidal activity (Johnson et al. 2003). Although these trials provided useful information on the effects of salinity, the salt concentrations required are too high to be considered as antifungal treatments for amphibians, except perhaps for a few saline-tolerant species.

The effect of temperature on cultures of *Bd* has been tested by placing inoculated flasks in heated incubators for various periods before returning to 23°C and monitoring for presence of growth. After exposure to 30°C, half the cultures died by 8 d (Piotrowski et al. 2004). Higher temperatures killed *Bd* rapidly; broth cultures died within 4 h at 37°C, within 30 min at 47°C, and within 5 min at 60°C (Berger 2001, Johnson et al. 2003). As thermoregulation via evaporative water loss varies among amphibian species (Buttemer 1990) and temperature affects metabolic rate, *in vivo* trials assessing heat regimes in multiple species are crucial.

AMPHIBIAN TREATMENT TRIALS

Two broad strategies for treating infected amphibians are possible: (1) cure, which requires elimination of *Bd* in the epidermis; and (2) palliative care, which involves correction of pathophysiological abnormalities. These treatment strategies are complementary. Most effort has been focussed on techniques to clear *Bd* from skin. Two general approaches have been used: raising the temperature of the infected amphibians to a level that kills *Bd* and exposing infected amphibians to antifungal drugs via immersion. Palliative treatment may be effective for amphibians with severe clinical chytridiomycosis which have low plasma sodium and potassium, probably due to loss across the damaged epidermis (Voyles et al. 2007, 2009). Supplemental electrolyte treatments may be useful to correct blood electrolyte levels and prevent death (Voyles et al. 2009) while antifungals take effect and the epidermis heals.

We found 11 reports of treatment trials for chytridiomycosis involving adults, juveniles, or tadpoles which utilized disease outbreaks or experimental infections (Table 1). Some of these were reported to have been tested against *Basidiobolus ranarum* (Groff et al. 1991, Taylor et al. 1999), but were actually cases of misdiagnosed chytridiomycosis (Pessier 2002, Carey et al. 2003). The trials investigated 8 antifungal drugs or heat as treatments. Heat cleared *Bd* in 2 trials and only 3 antifungal drugs (itraconazole, miconazole, and formalin/malachite green) were reported to eradicate *Bd* (Table 1).

Treatments do not appear to be consistently successful across species (G. Marantelli unpubl. data) and may be influenced by innate resistance of hosts to chytridiomycosis, which varies among species (Ardipradja 2001). It is important to report unsuccessful attempts at treatment to build knowledge of how various species respond.

Heat

Heat was reported to eliminate *Bd* from 3 species of frogs (Table 1). Trials used experimentally infected frogs and were appropriately controlled. Temperature regimes trialled were (1) intermittent exposure to 37°C (Woodhams et al. 2003), (2) continuous exposure to 32°C for 5 d (Retallick & Miera 2007), and (3) 27°C throughout the experiment (Berger 2001, Berger et al. 2004). The high temperature (37°C) regime was used on the tropical frog *Litoria chloris* and may not be suitable for species from temperate environments. However, 32°C was used on a temperate species (*Pseudacris triserata*) and may have wider application. The lowest temperature, 27°C, resulted in a 50% cure of *Mixophyes fasciolatus* and may be useful in species more resistant to *Bd* infection or when used in combination with antifungals in more susceptible species. Some tropical, pond-adapted tadpole species can be kept in water at high temperatures (Tyler 1994), but studies are needed to determine if heat is an effective treatment for infected tadpoles.

Antifungal drugs

Of the chemical treatments, formalin and malachite green appeared to successfully treat chytridiomycosis in *Xenopus tropicalis* (Parker et al. 2002); however, the trial was uncontrolled and only 4 of the 10 survivors were tested for *Bd*. There are teratogenic and carcinogenic concerns with these compounds (Srivastava et al. 2004, Garner et al. 2009), making them dangerous for use, particularly in breeding stock of endangered frogs and to humans conducting the treatments.

Table 1. Details of clinical trials using heat or antifungal drugs to treat frogs or tadpoles with chytridiomycosis. 'Cure' refers to the authors' claims, and confidence varies with method of diagnosis and sample size. N/A: not applicable; nd: no data

Active ingredient Dosage, duration	Species, stage	Type of trial, temperature (°C)	Zoospore dose, treatment commenced	Treated vs. untreated No.	Mortality (%)	Diagnostic test	Cure	Comment	Source
Formalin & malachite green									
10 mg l^{-1} & 0.8 mg l^{-1}, 30 min bath on 3 alternate days, repeated after 8 d	*Hymenochirus curtipes*, adult	Natural infection, 20–22	N/A	135 vs. 130	25 vs. 74	Histology	No	Misdiagnosed as *Basidiobolus ranarum*; mortality rate by 24 d; brief mention of histology	Groff et al. (1991)
25 mg l^{-1} & 0.1 mg l^{-1}, 24 h bath on 4 alternate days	*Xenopus tropicalis*, adult	Natural infection, 20–22	N/A	15 vs. 0	33 vs. N/A	Histology	Yes	Assessed mainly on clinical signs; 4/10 tested	Parker et al. (2002)
Benzalkonium chloride									
2 mg l^{-1}, 30 min bath on 3 alternate days, repeated after 8 d	*Hymenochirus curtipes*, adult	Natural infection, 20–22	N/A	135 vs. 130	10 vs. 74	Histology	No	Misdiagnosed as *Basidiobolus ranarum*; mortality rate by 24 d; brief mention of histology	Groff et al. (1991)
4 mg l^{-1}, 30 min bath on 3 alternate days, repeated after 8 d	*Hymenochirus curtipes*, adult	Natural infection, 20–22	N/A	135 vs. 130	16 vs. 74	Histology	No	Misdiagnosed as *Basidiobolus ranarum*; mortality rate after 24 d; brief mention of histology	Groff et al. (1991)
1 mg l^{-1}, 3 or 7 d bath	*Litoria caerulea*, juveniles	Experiment, 16–20	5×10^4, Day 19	18 vs. 14	100 vs. 100	Histology	No	Time till death delayed	Berger et al. (2009b)
1 mg l^{-1}, 3 h bath for 3 d, repeated after 5 d[a]	*Mixophyes fasciolatus*, tadpoles	Experiment	Unknown	56 vs. 57	N/A	Histology	No	Tested 18 d after last treatment; treatment reduced prevalence	McInnes (1999)
Copper sulphate									
1 mg l^{-1}, 30 min bath on 3 alternate days, repeated after 8 d	*Hymenochirus curtipes*, adult	Natural infection, 20–22	N/A	135 vs. 130	30 vs. 74	Histology	No	Misdiagnosed as *Basidiobolus ranarum*; mortality rate by 24 d; brief mention of histology	Groff et al. (1991)
Itraconazole									
1 microbead orally 9 d	*Bufo baxteri*, adult	Natural infection	N/A	nd	nd	Survival	Unknown	Reported as successful. Reversible side effects: dark skin, abdominal oedema, hunching	Taylor et al. (1999)
(Sporanox)[b] 100 mg l^{-1}, in 0.6% saline, 5 min bath, 11 d	*Dendrobates tinctorius*, juvenile	Experiment, 20–25	Unknown, Day 14	3 vs. 3	0 vs. 100	Histology	Yes	Tested at 63 d	Nichols et al. (2000)
(Itrafungol) 0.5–1.5 mg l^{-1}, 5 min bath, 7–21 d	*Alytes muletensis*, tadpoles	Experiment	5×10^3 (4× in 2 wk), Day 14	54 vs. 12	N/A	TaqMan® PCR	Yes	Tested 1 wk after last treatment; 75–100% untreated tadpoles PCR positive; cutaneous depigmentation in treated tadpoles	Garner et al. (2009)

Table 1 (continued)

Active ingredient Dosage, duration	Species, stage	Type of trial, temperature (°C)	Zoospore dose, treatment commenced	Treated vs. untreated No.	 Mortality (%)	Diagnostic test	Cure	Comment	Source
Miconazole nitrate (Conofite lotion)									
100 mg l^{-1}, in 0.6% saline, 5 min bath, 8 d	*Dendrobates tinctorius*, juvenile	Experiment, 20–25	Unknown, Day 14	3 vs. 3	0 vs. 100	Histology	Yes	Side effects; tested at 63d	Nichols et al. (2000)
Trimethoprim-sulfadiazine (Tribrissen)									
1 g l^{-1}, 5 min bath, 11 d	*Dendrobates tinctorius*, juvenile	Experiment, 20–25	Unknown, Day 14	3 vs. 3	66 vs. 100	Histology	No	Time till death delayed by treatment; light positive on histology at 63 d post-infection in survivors	Nichols et al. (2000)
Fluconazole (Diflucan)									
25 mg l^{-1}, 3 or 7 d bath	*Litoria caerulea*, juvenile	Experiment, 16–20	5×10^4, Day 19	18 vs. 14	100 vs. 100	Histology	No	Time till death delayed by treatment	Berger et al. (2009b)
6.6 mg l^{-1}, 6 h bath, 7 d[c]	*Mixophyes fasciolatus*, tadpole	Experiment	Unknown	56 vs. 57	N/A	Histology	No	Tested 18 d after last treatment; treatment reduced prevalence	McInnes (1999)
Heat									
37°C, 8 h × 2 d	*Litoria chloris*, juvenile	Experiment, 13.5–23.2	1.5×10^4, Day 12	10 vs. 30	0 vs. 97	Histology, clinical signs	Yes	Survived 5 mo post-treatment	Woodhams et al. (2003)
32°C, 5 d	*Pseudacris triserata*, adult	Experiment, 20	8×10^4, 1.2×10^5, Day 93	9 vs. 7	33 vs. 57	RT-PCR	Yes	All untreated survivors infected; tested at 172 d	Retallick & Miera (2007)
27°C, 98 d	*Mixophyes fasciolatus*, juvenile	Experiment, 17 & 23	1×10^3, Day 14	8 vs. 15	50 vs. 100	Histology, whole skin mount	No	All 4 survivors at 27°C uninfected	Berger (2001) Berger et al. (2004)
Copper phosphate, acriflavine HCl, P-chlorophenoxetol (Plistopur)									
Bath, 3 d	*Taudactylus acutirostris*, tadpole	Natural infection, 17–24	N/A	5 vs. nd	100 vs. nd	Histology	No	Died 6–28 d post-metamorphosis	Banks & McCracken (2002)
Methylene blue									
3 mg l^{-1} & 6 mg l^{-1} bath, 3 d and 3 d[d]	*Mixophyes fasciolatus*, tadpole	Experiment	Unknown	56 vs. 57	N/A	Histology	No	Tested 18 d after last treatment; treatment had no effect	McInnes (1999)

[a]Benzalkonium chloride tadpole toxicity trial: (1) 10 mg l^{-1} for 3 h, high mortality rate; (2) 2 mg l^{-1} for 24 h, high mortality rate; (3) 2 mg l^{-1} for 3 h, lower mortality rate (2/7); and (4) 1 mg l^{-1} for 3 h × 6 d, no deaths. [b]Itraconazole (Sporanox) tadpole toxicity trial: 3.3 mg l^{-1} for 3, 6, or 24 h, high mortality rate (McInnes 1999). [c]Fluconazole (Diflucan) tadpole toxicity trial: (1) 3.3 mg l^{-1} for 24 h × 3 d, 3.3 mg l^{-1} for 6 h × 3 d; and (2) 6.6 mg l^{-1} for 6 h × 7 d, no mortality. [d]Methylene blue tadpole toxicity trial: (1) 12 mg l^{-1} continuous for 3 d, flush tank, then 24 mg l^{-1} continuous for 3 d, high mortality rate; and (2) 3 mg l^{-1} continuous for 3 d, flush tank, then 6 mg l^{-1} continuous for 3 d, no mortality.

Several treatment trials suggest that itraconazole can clear infected tadpoles and frogs (Table 1), although further studies are required to confirm its efficacy and safety. The azoles are widely used in human and veterinary medicine as antifungal agents and they work by inhibiting the synthesis of ergosterol, a major component of fungal cell membranes (Marichal et al. 1999). Itraconazole concentrates and persists in keratinizing tissues for several weeks after cessation of treatment in mammals (Riviere & Papich 2009). Therefore, it has a possible advantage over other azole drugs in treating chytridiomycosis because in amphibians *Bd* grows only within keratinising epidermal cells (Marantelli et al. 2004). Of the initial treatment trials with azole antifungals, both itraconazole and miconazole successfully treated experimentally infected post-metamorphic *Dendrobates tinctorius* in a controlled trial (Nichols et al. 2000). However, this experiment involved a small sample size of 3 frogs per group, and treated animals were judged to be free of infection at 63 d post-treatment by histology — a test with low sensitivity (Hyatt et al. 2007). Despite these limitations, the results of this trial have been used as a basis for the common use of itraconazole baths for treatment of chytridiomycosis in post-metamorphic zoo animals and in amphibian conservation programs (Forzan et al. 2008, Gagliardo et al. 2008, Pessier 2008). In these situations, anecdotal reports suggest that itraconazole treatment has been successful. However, multiple treatment cycles are sometimes necessary to clear all infected animals of infection when monitored with TaqMan® real-time PCR (Pessier 2008). A recent controlled trial successfully used itraconazole to clear *Bd* from experimentally infected *Alytes muletensis* tadpoles (Garner et al. 2009). This trial used substantially lower doses of itraconazole than previously tested on frogs (0.5 to 1.5 mg l^{-1} compared with 100 mg l^{-1}; Table 1).

There are, however, concerns with the safety of itraconazole, which has caused deaths in metamorphs (Pessier 2008) and tadpoles even when low doses were used (3.3 mg l^{-1} for 3 h; McInnes 1999). The deaths occurred shortly after exposure to the itraconazole solution and it is unclear if toxicity was due to the drug itself or to the vehicle used to make itraconazole soluble. The recent trial of low dose itraconazole in *Alytes muletensis* tadpoles did not cause death, but may have been associated with cutaneous depigmentation; the implications of this need to be determined before itraconazole can be recommended for tadpoles (Garner et al. 2009). Itraconazole has been associated with changed liver function and hypokalemia during long-term use in humans (Tucker et al. 1990). Because of the frequent use of itraconazole in captive amphibian programs, it is crucial that the minimum effective dose is determined and that the efficacy and safety of treatment be confirmed in multiple amphibian species.

Fluconazole is water soluble (Riviere & Papich 2009) and appears to be safe in tadpoles, but trials with low and moderate doses in infected tadpoles and post-metamorphs did not clear *Bd* (McInnes 1999, Berger et al. 2009b; Table 1). Repeating these trials with a higher dose may be worthwhile.

Some treatments for chytridiomycosis (e.g. benzalkonium chloride) that were trialled at levels above the effective *in vitro* levels were ineffective *in vivo* (Groff et al. 1991). This may be due in part to protection of *Bd* by its intracellular location and possibly from the modification of the epidermal cells that occurs with *Bd* infection (Berger et al. 2005a), or to limited uptake of antifungal drug by the keratinizing host cells.

Basic pharmacokinetic data are not available on any of the drugs that have been used for treatment. Typically, antifungal agents are applied by immersing the amphibian in the solution with the assumption that the drug will be absorbed into the skin to attack the zoosporangia. Antifungal drugs are also likely to be absorbed into the bloodstream of amphibians. However, there are no data on skin or blood absorption or even on the effect of antifungal agents on intracellular zoosporangia.

Treatment of pathophysiological abnormalities

Since amphibians with clinical chytridiomycosis have severe blood electrolyte abnormalities, particularly hyponatremia and hypokalemia, oral electrolyte supplements are likely to be useful as supportive care (Voyles et al. 2009). In that study (op. cit), electrolyte treatment with 12% Whitaker-Wright solution (113.0 g NaCl, 8.6 g $MgSO_4 \cdot 7H_2O$, 4.2 g $CaCl_2$, 1.7 g KCl l^{-1} distilled water = 100% stock solution; Wright & Whitaker 2001) was administered via stomach tube every 4 to 6 h once severe clinical signs appeared. In some cases treatment continued up to approximately 36 h before frogs died. This treatment regime led to increased activity levels and slightly prolonged survival; however, testing plasma electrolyte levels during prolonged treatment may be necessary to avoid overdosing (Voyles et al. 2009). A less risky alternative may be to replace the water in enclosures of sick frogs with an isotonic or hypotonic amphibian electrolyte solution, although we assume that absorption of electrolytes through the skin will be compromised by severe chytridiomycosis. Also, isotonic concentrations for ill frogs will be lower than for healthy frogs, and dehydration could result if hypertonic baths are used. Wright & Whitaker (2001) reviewed the use of electrolyte baths for ill amphibians. For treating chytrid-

iomycosis, further trials with earlier administration of varied electrolyte doses and routes of administration, especially bath treatments, are needed.

PRACTICAL AND HYGIENE ASPECTS FOR TREATMENT

Treatments for chytridiomycosis may fail for a variety of practical reasons including poor hygiene, treatment methodology, and treatment formulation. Whether in a clinical setting or conducting clinical trials, the following recommendations should be followed:

(1) Since treatment may not be 100% effective, it is necessary to house frogs individually or in small groups to prevent cross-infection. Strict hygiene procedures are needed to prevent transmission between tubs — e.g. dedicated instruments for each tub, changing gloves between tubs.

(2) As *Bd* can survive in soil and water (Johnson & Speare 2003), simple enclosures with minimal or disposable or easily disinfected substrates should be used. Enclosures/water/substrates should be changed or disinfected concurrent with each session of antifungal treatment. Animals should go into a *Bd*-free enclosure after each treatment.

(3) For bath treatments we recommend agitating the treatment solution to ensure contact of the topical medication with all skin surfaces.

(4) The formulation of medications may affect their efficacy and toxicity. For instance, itraconazole is commonly used in amphibian conservation programs based on commercial oral solutions formulated for humans (Sporanox Oral Solution®, Janssen-Cilag EMEA) or veterinary use (Itrafungol®, Janssen Animal Health). Solutions have also been compounded from other itraconazole formulations, such as oral capsules. Hence the formulation must be stated and kept constant if possible.

DESIGNING CLINICAL TRIALS

Clinical trials have 2 general aims: to determine the efficacy of a treatment regime and to detect adverse effects of the treatment. Clinical trials for use of new drugs in humans are divided into 4 phases. Phase I trials determine safety, tolerability, and pharmacokinetics of a drug in a small group of healthy subjects. Phase II trials determine efficacy and safety in a small group of typical cases. Randomised controlled or comparative trials (RCT) provide the best form of evidence in this phase. Phase III are larger RCT trials, often involving several testing centres, to further test efficacy and safety in patients. Post-marketing surveillance is sometimes referred to as Phase IV and involves ongoing monitoring for adverse effects (pharmacovigilance) to detect any rare or long-term adverse effects over a much larger patient population. This is currently best practice and treatment trials in amphibians should aim for similar standards although we recognise that this is not always feasible. Here we focus on chemotherapeutics, but testing the safety and efficacy of heat will follow similar principles.

For the treatment of chytridiomycosis, no Phase I trials including pharmacokinetics have been performed for antifungal drugs. Although the drugs being used are not new, there are no data on their use in amphibians and Phase I trials are needed. Monitoring of drug concentrations in serum and skin after topical and other routes of administration will be useful in understanding the pharmacokinetics. Single or multiple ascending dose studies test for adverse side effects. If a dose is safe, a new group of subjects is then given higher levels to reach the maximum tolerated dose. When a compound is evaluated in amphibians, toxicity testing can be done by initially giving small groups of animals, e.g. 3 per group, doses at the *in vitro* minimum inhibitory concentration and double and triple that dose or exposure time. In evaluating safety of heat treatments, the temperature tolerance of adults and tadpoles will vary greatly among species depending on their natural habitat.

Interspecific differences in tolerance to antifungal treatments have occurred in fish (Intorre et al. 2007) and are also expected in amphibians. No criteria have been established to assess safety of antifungal agents in amphibians and most studies have used direct observation with crude indices, such as death (McInnes 1999), to evaluate safety. More subtle clinical signs can be missed if animals are not systematically and regularly examined. Thus the minimum standards to evaluate safety of antifungal agents in adult amphibians should include regular direct observation, weight change, growth, and survival. For tadpoles, regular direct observation, survival, growth, progression to metamorphosis, weight at metamorphosis, and growth after metamorphosis should be the minimum assessment for adverse effects of treatment. Histopathology or clinical pathology can also be used to evaluate subtle tissue effects.

Some Phase II trials have been carried out for chytridiomycosis, but no Phase III trials have been conducted and there is no systematic ongoing collection of data on adverse effects.

Treatment trials for *Bd* can be accomplished using infection experiments or when treating spontaneous cases or outbreaks. Phase II clinical trials in humans are usually randomised, double blind, and placebo-controlled or compared to currently accepted therapy.

For Phase II trials in amphibians, the method of randomisation should be stated in the protocol. Blinding can be done by having different individuals administering treatment and performing animal care and assessment, and by using individual identification that does not indicate treatment. An alternative to a placebo treatment for the controls is to compare the test treatment with an accepted alternative treatment — a randomised comparative rather than a controlled trial.

To calculate the number of individuals required to demonstrate treatment efficacy, it is important to consider the desired statistical power, the acceptable Type I error, and the size and precision of the expected effect. Such estimations can be difficult because rates of infection, mortality, and spontaneous clearance can differ according to the species tested, age, exposure dose, isolate of *Bd*, and experimental conditions (Berger et al. 2009a). There is currently no standard experimental infection model. However, in previous studies with species of moderate to high susceptibility, 8 to 10 animals per group were sufficient for initial trials (eg Woodhams et al. 2003, Berger et al. 2004).

Criteria for monitoring infected amphibians in treatment trials should include morbidity, mortality, and clearance rates. To determine clearance rates, the TaqMan® real-time quantitative PCR test is the most sensitive tool available (Boyle et al. 2004, Hyatt et al. 2007, Skerratt et al. 2008) and is essential to show infection has been eliminated. Because infected frogs may remain aclinical and histology of toe-tip clips is less sensitive than PCR, assessment of clinical signs and microscopy should no longer be used to prove elimination of *Bd* (L. Skerratt et al. unpubl. data). To increase confidence that susceptible amphibians are clear of infection, we suggest conducting 3 PCR tests, with each test at least 1 wk apart, starting the week after the last treatment. Resistant individuals, however, may remain chronically infected with light infections that have a low probability of detection, even with the above testing regime (L. Skerratt et al. unpubl. data). If tadpoles metamorphose within the treatment time, observations and testing during the month following metamorphosis may be a sensitive method of detection, as recently metamorphosed frogs in captivity appear to be highly susceptible (Berger et al. 1998, Marantelli et al. 2004).

Amphibians in treatment trials should be similar in age, size, genetic background, and history. If experimental frogs are heterogenous, they should be matched between groups. Positive control frogs that are infected but untreated are needed to determine infection, mortality, and spontaneous clearance rates. Uninfected negative controls are also useful to assess the effect of captive husbandry on the test species.

The virulence of *Bd* differs with temperature, host species and age, fungal isolate (and possibly passage history), and dose (Ardipradja 2001, Lamirande & Nichols 2002, Berger et al. 2004, 2005b, Woodhams et al. 2007). The success of a treatment will probably depend on the burden of *Bd* and severity of disease, which is also affected by the time that treatment commences post infection. All of the above factors need to be kept as consistent as possible when comparing trial results. Ultimately, treatments are needed for clearing lightly infected, sub-clinical frogs as well as for saving those with clinical disease.

Another aspect to consider, and perhaps exploit, is that the distribution of *Bd* in tadpoles follows the presence of keratin, which varies in stages of late development (Marantelli et al. 2004). Tadpoles are only infected in their mouthparts until they near metamorphosis, when infection may develop on skin of the feet. During metamorphosis, the infection on the mouth is lost, and for a brief period infection occurs solely on the feet before it spreads over the body (Marantelli et al. 2004). Bath treatment to attempt to stop the redistribution to the feet via the waterborne zoospores could be effective in clearing tadpoles. However, this has not been trialled.

Treatments that have been demonstrated to be effective in captive individuals can then be tested in wild amphibians. Mark-recapture studies will allow repeat testing of treated and control individuals. Matched, untreated populations should be monitored to assess effects on prevalence of *Bd* and amphibian abundance.

SUMMARY AND CONCLUSIONS

Most antifungal agents appear to be effective at killing *Bd in vitro*, but it appears *Bd* is quite difficult to kill on the host. To screen potentially therapeutic compounds for chytridiomycosis, *in vitro* studies should be performed before embarking on more costly *in vivo* trials.

Heating (32 and 37°C) has been demonstrated as effective against chytridiomycosis in 2 amphibian species (Woodhams et al. 2003, Retallick & Miera 2007), and should be tested and optimized in a range of species. In some situations (e.g. for field use) a drug treatment may be more convenient. Although itraconazole and formalin/malachite green baths both appear to be effective treatments for post-metamorphic frogs (Nichols et al. 2000, Parker et al. 2002), these trials did not meet RCT standards and toxicity is an issue, particularly for formalin/malachite green. Successful treatment of infected tadpoles of one species has been reported in a controlled trial using itraconazole, but may have been associated with depigmentation (Garner et al. 2009).

Some antifungal drugs are toxic to amphibians at therapeutic levels (McInnes 1999). Toxicity may differ with life stage, with tadpoles being more sensitive, and probably with species. Effects on survival and reproduction need to be considered. Fluconazole appeared safe for tadpoles and could be trialled at higher doses compared with previous attempts (McInnes 1999). Combining heat and antifungals may be useful in developing safe treatments.

Assessing the use of electrolyte supplements delivered by various routes will be important for managing late stage disease.

Randomised controlled trials, with adequate sample sizes and cure documented by PCR testing are needed to test treatment protocols for different life stages (tadpole, metamorph, adult) in multiple species. Currently, we do not know if the therapeutic response from one member of a genus can be used to predict the response of other members of that genus.

Trials are a priority for threatened species in conservation programs. Phase I trials on pharmacokinetics and safety are required, better designed Phase II trials are needed, and Phase IV trials (ongoing monitoring of treatments) would be ideal before an adequate evidence base will be available to ensure the most efficacious, safe, and feasible treatments are used.

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