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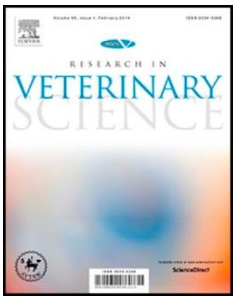

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Short communication: *In vitro* sensitivity of the amphibian pathogen *Batrachochytrium dendrobatidis* to antifungal therapeutics

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HIGHLIGHTS

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1: we report the sensitivity of *Batrachochytrium dendrobatidis* to 6 antimicrobials.
2: Voriconazole, itraconazole, and terbinafine had potent inhibitory effects.
3: Terbinafine and amphotericin B exposure killed zoospores rapidly.
4: The reported MIC and killing concentrations are useful for design of dosage regimens.

Abstract

Chytridiomycosis, a skin disease caused by *Batrachochytrium dendrobatidis*, has caused amphibian declines worldwide. Amphibians can be treated by percutaneous application of antimicrobials, but knowledge of in vitro susceptibility is lacking. Using a modified broth microdilution method, we describe the *in vitro* sensitivity of two Australian isolates of *B. dendrobatidis* to six antimicrobial agents. Growth inhibition was observed, by measurement of optical density, with all agents. Minimum inhibitory concentrations (µg/mL; isolate 1/2) were - voriconazole 0.016/0.008; itraconazole 0.032/0.016; terbinafine 0.063/0.063;

fluconazole 0.31/0.31; chloramphenicol 12.5/12.5; amphotericin B 12.5/6.25. Killing effects on zoospores were assessed by observing motility. Amphotericin B and terbinafine killed zoospores within 5 and 30 min dependent on concentration, but other antimicrobials were not effective at the highest concentrations tested (100 µg/mL). This knowledge will help in drug selection and treatment optimization. As terbinafine was potent and has rapid effects, study of its pharmacokinetics, safety and efficacy is recommended.

Key words: Batrachochytrium dendrobatidis; antifungal testing; treatment; chytridiomycosis

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Batrachochytrium dendrobatidis (*Bd*) is the cause of chytridiomycosis (Berger et al., 1998) a skin disease that has caused global amphibian population declines and extinctions (Skerratt et al., 2007). *Bd* forms round sporangia that grow within epidermal cells of amphibian skin, and infective flagellated zoospores are released through discharge tubes that protrude through the skin surface (Berger et al., 2005). Treatment of chytridiomycosis is required to manage outbreaks of disease, reduce population impacts, and reduce the risk of spread in transport. Knowledge of *in vitro* drug sensitivity will optimize treatment regimens.

Previous *in vitro* studies showed growth inhibition of *Bd* by itraconazole and fluconazole (Berger et al., 2009), but the minimum inhibitory concentrations (MIC) are unknown. Voriconazole has potent inhibitory effects against European isolates *in vitro* (Martel et al., 2011). The antibiotics chloramphenicol (10-20 µg/mL); (Poulter unpub) was florfenicol (0.5-1.0 µg/mL), and sulfonamide (8 µg/mL) were effective, but macrolides and tetracyclines were not (Muijsers et al., 2012). Caspofungin had relatively high MICs (4-16 µg/mL), varying non-significantly among isolates (Fisher et al., 2009).

Successful treatment with topical Itraconazole is reported in various amphibian species (Forzán et al., 2008, Tamukai et al., 2011, Lamirande and Nichols 2002, Une et al., 2012, Georoff et al., 2013), and tadpoles (Garner et al., 2009), but treatment failure and potential toxicity are also reported (Woodhams et al., 2012, Brannelly et al., 2012, Georoff et al., 2013). Fluconazole baths (25 µg/mL) extended the course of disease in *Litoria caerulea* but did not clear infection (Berger et al., 2009). Voriconazole topical solutions at low concentrations (1.25 µg/mL) successfully resolved infection in *Alytes cisternasii* (Martel et al., 2011). Chloramphenicol by continuous bath exposure was effective in subclinical and severe chytridiomycosis in *Litoria caerulea*, combined with electrolyte therapy (Young et al., 2012). Topical florfenicol sprays reduced burden of infection in *Alytes muletensis* but all remained infected (Muijsers et al., 2012).

Daily topical application of terbinafine (50 µg/mL) cleared infection in naturally infected *Lithobates catesbeiana*, and five other species, whereas 5 µg/mL was ineffective (Bowerman et al., 2010). *In vitro* effects were not described.

For the present study, the *in vitro* potency of six antimicrobial drugs against two Australian isolates of *Bd* was assessed by determining MIC with constant exposure, and observing effectiveness of short-duration, high concentration exposure on zoospores.

Bd isolates were cultured and cryoarchived by routine methods (Berger et al., 2009). Isolate 1 was collected from a temperate region in 2009 from a tadpole of *Limnodynastes peronii* (Couta Rocks, Tasmania; CoutaRocks-Limperonii--2009- LB1). Isolate 2 is from tropical rainforest and was collected in 2010 from a tadpole of *Litoria genimaculata* (Paluma, Queensland; Paluma-Lgenimaculata-2010-MW1). Cultures were maintained in TGhL medium (8g/L tryptone, 0.5g/L gelatine hydrolysate, 1g/L lactose; Sigma-Aldrich, Australia). After 7 days growth, about 1 mL of culture was spread onto a TGhL agar plate, air-dried, sealed with parafilm and incubated at 22ºC. After 3 days, zoospores were collected by flooding the plate with up to 3 mL of TGhL medium for 15 min, counted in a haemocytometer, and diluted to approximately 10^6 zoospores/mL.

Amphotericin B (250 µg/mL solution) and chloramphenicol powder were supplied by Sigma-Aldrich. Terbinafine, fluconazole and voriconazole preparations were Lamisil AT (Novartis), Diflucan IV (Pfizer) and VFend IV (Pfizer) respectively, diluted to working concentrations in sterile single-distilled water. As itraconazole solution (Sporanox, Janssen Pharmaceutica) precipitated when it was diluted, a solution was prepared of analytic standard dissolved in dimethyl sulfoxide (DMSO, 99%; Sigma-Aldrich), and diluted to final concentration in 0.1% DMSO solution.

For each drug, 50% dilution series were prepared in 96 well flat-bottom cell culture plates (Corning Costar, USA). In the short-exposure studies, duplicate series were prepared,

and control wells contained only TGhL and distilled water. In the growth inhibition studies, 8 replicate series were prepared, with 8 positive control wells containing distilled water and TGhL only, and 8 negative controls with 0.1% F10SC disinfectant (F10 Biocare, UK) in distilled water. Positive growth controls also contained 0.1% DMSO, when assessing inhibitory effects of itraconazole dissolved in 0.1% DMSO. Finally, 50 µL of zoospore suspension (5 x 10^4 zoospores) was placed into each well of the plates.

Plates were examined immediately after preparation to confirm presence of motile zoospores and absence of clumped sporangia. For short-exposure studies, wells were examined after 5 and 30 min. Absence of motile zoospores was considered to indicate a lethal effect, with wells recorded either as killed or alive. For growth inhibition studies, plates were incubated at 21-23°C. On day 7, optical density was measured using a spectrophotometer plate reader at 492 nm, as described previously (Rollins-Smith et al., 2002), and the cultures microscopically examined. Positive controls contained a dense monolayer on the bottom of wells, and all negative controls were killed.

Statistical analysis of optical density data was performed using IBM SPSS for Windows. Mean density from the 8 wells at each concentration was determined. The MIC was defined as the lowest concentration with mean optical density +1SD, at least 90% lower than the difference between positive and negative controls. Visual examination of Q-Q plots assessed normal distribution of optical density at each concentration.

In growth inhibition tests, isolates differed minimally in sensitivity (Table 1), with no more than one dilution difference between MIC for any agent. Voriconazole and itraconazole were most potent, terbinafine and fluconazole were intermediate, while amphotericin B and chloramphenicol had the lowest potency of the tested agents.

Optical density appeared to correlate well with microscopic observations as an indicator of growth inhibition. Density readings for positive and negative controls were

normally distributed. In experimental columns, optical density occasionally deviated from normal distribution, particularly at dilution stages immediately lower than MIC.

Comparison of optical density of killed controls was previously reported as an endpoint assessment (Gibble et al., 2008, Rollins-Smith et al., 2002). In the present study, mean density slightly greater than the 95% confidence interval of the mean negative control density were occasionally observed in cultures observed to have no growth. This is attributed to apparent partial development, as the zoospores settle and increase in size, but no development occurs. This may reflect fungistatic effects, rather than rapid killing of the controls. The criterion of 90% density inhibition compared to the positive control growth was elected *a posteriori*. Variable inhibition endpoints for optical density, from 50% (Fisher et al., 2009) to 80% (Gibble et al., 2008) have been previously applied. Our method is slightly more conservative.

Itraconazole and voriconazole had potent inhibitory effects (Table 1). The observed MIC of voriconazole (0.008-0.0016 µg/mL) is consistent with the 0.00625-0.0125 µg/mL range previously described (Martel et al., 2011). Fluconazole was less potent (MIC 0.31 µg/mL), and this may explain its failure to treat chytridiomycosis in amphibians in a clinical trial when used topically at 25 µg/ml (Berger et al., 2009). Further trials with higher exposure rates may be valuable. For these agents, zoospores remained motile after 30 min at the highest concentrations tested (100 µg/mL). Short-duration topical exposure will not kill zoospores at the skin surface, even at concentrations greatly exceeding the MIC, and treatment efficacy will depend on persistence of adequate drug concentrations in the skin. This may contribute to the observed failure of short-duration itraconazole therapy in some instances (Georoff et al., 2013, Woodhams et al., 2012). Our data suggest that the frequency of itraconazole application, in addition to the applied concentration, is important to the clinical outcome. High potency of itraconzole and voriconazole support these drugs as

treatment choices, but the lack of rapid effect means that systemic therapy may be more appropriate than topical application.

Chloramphenicol was also inhibitory but with lower potency (Table 1). The observed MIC (12 µg/mL) is similar to a previous unpublished reported MIC of 10-20 µg/mL (Poulter unpub). Partial inhibition was observed below the stated MIC, but its significance is unknown. No effect on motility was observed after 30 minutes of high-concentration exposure. Severe chytridiomycosis in *L. caerulea* was treated by continuous exposure to 20 µg/mL chloramphenicol for 28 days (Young et al., 2012), which is only slightly greater than the *in vitro* MIC. Due to its low potency, this agent is a poor candidate for intermittent application, and topical concentrations lower than 20µg/mL are unlikely to be of clinical benefit.

Zoospore motility ceased after 5 min of exposure to terbinafine (6.25-12.5 µg/mL) and amphotericin B (50 µg/mL), and 30 min at lower concentrations of terbinafine (3.12 µg/mL) and amphotericin B (12.5 µg/mL). No difference was detected between isolates. Bowerman et al., 2010 report successful treatment of chytridiomycosis using topical terbinafine at 50-100 µg/mL, well above the MIC (0.063 µg/mL) and slightly greater than that required to kill zoospores within 5 min. This rapid effect is likely to contribute substantially to the therapeutic outcome when intermittent topical therapy is used, as prolonged drug retention at the site of infection may be less important. Terbinafine is thus a strong candidate for further trials of intermittent topical treatment. However, further work is required to assess the lethal concentrations of this drug against sporangia, which may be more resistant. Amphotericin B was included as a model fungicidal agent; previous studies indicate it is too toxic for clinical use in amphibians (Martel et al., 2011).

Evaluation of optical density was chosen for determination of the study endpoint, as it was expected to provide a more quantitative evaluation than direct examination alone and

appears sensitive in comparing growth. However, the high starting zoospore density required was difficult to achieve. We suggest microscopic examination is an easier method for MIC screening and our observations suggest similar results are achieved (data not shown).

To optimize treatment regimes, pharmacokinetic studies and clinical trials are needed to examine absorption, and maintenance of drug concentration in the infected skin over time, and correlation with clinical outcome (Berger et al., 2010). The data presented in this study will aid in the interpretation of the clinical relevance of observed drug concentrations.

This study helps with selection of antifungal agents for clinical trials. Terbinafine is potent and apparently fungicidal to zoospores at low concentrations, and there is one report of it being effective and safe in a range of species (Bowerman et al., 2010). Therefore, we suggest further work is warranted to optimize its use, and compare with more widely used treatments.

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Conflict of interest statement

None of the authors have a financial or personal relationship with other people or organizations which could inappropriately influence or bias the content of this paper.

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Table 1: Minimum Inhibitory Concentrations of formulations against *Batrachochytrium dendrobatidis*, resulting in at least 90% inhibition compared with positive controls.

	Minimum Inhibitory Concentrations (µg/mL)					
	Amphotericin	**Chloramphenicol**	**Terbinafine**	**Fluconazole**	**Voriconazole**	**Itraconazole**
Isolate 1*	12.5	12.5	0.063	0.31	0.016	0.031
Isolate 2**	6.25	12.5	0.063	0.31	0.0078	0.016

* Limperonii- CoutaRocks-2009- LB1

** Lgenimaculata- Paluma-2010-MW1