

# Additional disinfectants effective against the amphibian chytrid fungus *Batrachochytrium dendrobatidis*

R. Webb\*, D. Mendez, L. Berger, R. Speare

Amphibian Disease Ecology Group, School of Public Health, Tropical Medicine and Rehabilitation Sciences,  
James Cook University, Townsville 4811, Australia

**ABSTRACT:** Chytridiomycosis, a disease contributing to amphibian declines worldwide, is caused by the fungus *Batrachochytrium dendrobatidis*. Identifying efficient and practical disinfectants effective against *B. dendrobatidis* is important to reduce the spread of the disease both in the wild and captivity. Previous studies identified a range of suitable disinfectant strategies. We evaluated the suitability of 3 additional disinfectants: two of these (TriGene Virucidal Disinfectant Cleaner and F10 Super Concentrate Disinfectant) are mixtures of chemicals and one (Betadine Antiseptic Liquid) contains a single active ingredient, povidone iodine. The disinfectants were tested using a range of concentrations for 1, 5 and 10 min to determine their ability to kill *B. dendrobatidis in vitro*. The measure of effectiveness was 100% kill of zoospores grown in multiwell plates. All disinfectants had a 100% efficacy at concentrations recommended by the manufacturers. The lowest concentrations capable of 100% kill after exposure for 1 min were 0.1 ml l<sup>-1</sup> for TriGene, 0.33 ml l<sup>-1</sup> for F10 and 100 ml l<sup>-1</sup> for Betadine. TriGene is the most effective disinfectant yet to be found, and both TriGene and F10 are more effective than various disinfectants tested in previous studies. TriGene and F10 are considered suitable for use in the field, as only small amounts of concentrate are needed.

**KEY WORDS:** *Batrachochytrium dendrobatidis* · Disinfect · TriGene · F10 · Betadine · Amphibian disease

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## INTRODUCTION

The amphibian chytrid fungus *Batrachochytrium dendrobatidis* is the cause of chytridiomycosis, a disease responsible for declines of amphibian populations in Australia, New Zealand, North America, Central America, South America and Europe (Berger et al. 1998, Mutschmann et al. 2000, Bosch et al. 2001, Fellers et al. 2001, Waldman et al. 2001, Bonaccorso et al. 2003, Burrowes et al. 2004, Lips et al. 2006, Puschendorf et al. 2006). Protocols to prevent the spread of chytridiomycosis by human activities include disinfection of equipment used with amphibians or that has had contact with contaminated water bodies (Australian Government Department of Environment and Heritage 2006). Disinfectants effective against *B. dendrobatidis* are also used

in laboratories and in captive amphibian facilities. Johnson et al. (2003) investigated the efficacy of 8 disinfectants against *B. dendrobatidis*, and showed acceptable levels of activity for 4, with those containing didecyl dimethyl ammonium chloride (DDAC) being active at the lowest concentration (1 ml l<sup>-1</sup>). The most commonly used disinfectant appears to be bleach at concentrations of between 1 and 4% (approximately 0.45 to 1.8 g sodium hypochlorite l<sup>-1</sup>), a much higher range of concentrations than for DDAC products. In the field, researchers often need to transport disinfectants to distant sites, and, consequently, volume and weight are important considerations. Disinfectant stock solutions can be diluted to the desired concentration with stream or pond water, and the more active a disinfectant is, the smaller the required volume of stock solution.

\*Email: rebecca.webb1@jcu.edu.au

F10 Super Concentrate Disinfectant (Health and Hygiene Pty.) is a wide-spectrum disinfectant designed for use in the presence of animals. It is used in zoological parks as well as veterinary and agricultural situations<sup>1</sup>. F10 contains 54 g benzalkonium chloride l<sup>-1</sup> and 4 g polyhexamethylene biguanide hydrochloride l<sup>-1</sup>. TriGene Virucidal Surface Disinfectant Cleaner (Medichem International Marketing) is recommended for disinfecting hard surfaces and instruments. The active ingredients of the product are: dodecylamine sulphamate, octyldecyl dimethyl ammonium chloride, poly (hexamethylene) biguanide hydrochloride, 4 nonyl phenyl- $\omega$ -hydroxyl-poly (oxyethylene) and ethanol<sup>2</sup>. Betadine Antiseptic Liquid (Faulding Healthcare Pty.) is a commonly used topical antiseptic. The disinfectant contains 10 % povidone-iodine, which is equivalent to 1 % available iodine (Anonymous 1998).

The present paper reports the efficacy of these 3 disinfectants in killing *Batrachochytrium dendrobatidis* *in vitro*.

## MATERIALS AND METHODS

The experiment followed Protocol 2 of Johnson et al. (2003), except for minor modifications as given in the following description. Two *Batrachochytrium dendrobatidis* strains were used, one isolated from *Litoria caerulea* (Rockhampton-Lcaerulea-99-LB-1) and another from *L. rheocola* (Mt Misery-Lrheocola-05-LB-1) (Berger et al. 2005b), depending on which strain was producing the most zoospores at the time. Zoospores were placed in 96-well plates to ensure that all wells contained cultures of approximately the same age. Zoospores were collected from the cultures by 2 methods. Either an agar plate supporting an active culture was flooded with tryptone, gelatin hydrolysate, lactose (TGhL) broth, left for 30 min and then the broth collected, or a 4 d old culture in TGhL broth was centrifuged at 2000 rpm (690  $\times$  g) for 2 min and the supernatant was collected. The resulting zoospore/broth solution was then diluted with broth until the zoospore concentration was 4  $\times$  10<sup>5</sup> zoospores ml<sup>-1</sup>, as determined by a haemocytometer count, placed in wells and grown for 4 to 6 d at 21°C. The 3 disinfectants, TriGene, F10 and Betadine, were diluted with sterile water. Sterile water was placed in the control wells. All were tested against plates containing *B. dendrobatidis* cultures of 4 to 6 d of age, with exposure times of 10, 5 and 1 min. Concentrations recommended by the manufacturer were used as the starting point, and the disinfectants were diluted until *B. dendrobatidis* zoospo-

rangia survived. For each concentration 8 replicates were done. Plates were checked every 3 to 4 d for at least 2 wk to detect growth of sporangia and presence of motile zoospores. From each inactive well containing intact zoosporangia, 20  $\mu$ l was transferred to fresh media after 2 wk instead of after 4 wk as in the original protocol of Johnson et al. (2003), since wells which were inactive for 2 wk did not show growth after this time. For a treatment to be considered effective, it had to result in 100% kill of all 8 replicates. If liquid was transferred from these wells to fresh broth, then the additional wells had to have been inactive for the treatment to be considered effective.

## RESULTS

All 3 disinfectants had 100% killing effect on zoosporangia at concentrations recommended by the manufacturers (Table 1). Betadine was only effective at much higher concentrations (100 ml l<sup>-1</sup>) of stock solution than the other 2 disinfectants. F10 and TriGene were effective at 0.33 ml l<sup>-1</sup> (1:3000) and 0.1 ml l<sup>-1</sup> (1:10 000), respectively. At the lowest concentration longer exposure times changed efficacy for F10, which was only effective at 0.28 ml l<sup>-1</sup> if exposure was for at least 10 min.

Table 1. Killing effect of 3 disinfectants on *Batrachochytrium dendrobatidis* zoosporangia *in vitro*

Disinfectant concentration	Exposure time (min)	100% effective?
<b>F10</b>		
4 ml l <sup>-1</sup> (1:250)	1, 5, 10	Yes
1 ml l <sup>-1</sup> (1:1000)	1, 5, 10	Yes
0.33 ml l <sup>-1</sup> (1:3000)	1, 5, 10	Yes
0.28 ml l <sup>-1</sup> (1:3500)	10	Yes
0.28 ml l <sup>-1</sup> (1:3500)	1, 5	No
0.25 ml l <sup>-1</sup> (1:4000)	1, 5, 10	No
<b>Betadine</b>		
Full strength <sup>a</sup>	1, 5, 10 <sup>a</sup>	Yes
500 ml l <sup>-1</sup> <sup>b</sup>	1, 5, 10 <sup>a</sup>	Yes
100 ml l <sup>-1</sup> <sup>c</sup>	1, 5, 10 <sup>b</sup>	Yes
50 ml l <sup>-1</sup> <sup>d</sup>	1, 5, 10 <sup>c</sup>	No
<b>TriGene</b>		
50 ml l <sup>-1</sup> (1:20)	1, 5, 10	Yes
2 ml l <sup>-1</sup> (1:500)	1, 5, 10	Yes
1 ml l <sup>-1</sup> (1:1000)	1, 5, 10	Yes
0.2 ml l <sup>-1</sup> (1:5000)	1, 5, 10	Yes
0.1 ml l <sup>-1</sup> (1:10000)	1, 5, 10	Yes
0.09 ml l <sup>-1</sup> (1:11000)	1, 5, 10	No

<sup>a</sup>10% povidone iodine; <sup>b</sup>5% povidone iodine; <sup>c</sup>1% povidone iodine; <sup>d</sup>0.5% povidone iodine

<sup>1</sup>www.healthandhygiene.net/index.php?mainid=1. Accessed 15 January 2007

<sup>2</sup>www.medichem.co.uk/trigene-dis-safety.html. Accessed 28 March 2006

## DISCUSSION

The commercially available disinfectants F10, Betadine and TriGene were effective disinfectants for *Batrachochytrium dendrobatidis* when used for 1 min at the concentrations recommended by the manufacturers. The 3 products tested can be considered disinfectants where *B. dendrobatidis* may be present. Of the 3 disinfectants, TriGene was active at the lowest concentration, being effective at a dilution of 0.1 ml l<sup>-1</sup>, followed by F10 at a dilution of 0.33 ml l<sup>-1</sup> and then Betadine at a dilution of 100 ml l<sup>-1</sup>. It is recommended that either TriGene or F10 be used as a disinfectant in situations where *B. dendrobatidis* may be present. The concentration of F10 recommended by the manufacturers for the disinfection of fungi, in general, is 2 ml l<sup>-1</sup> for 15 min, or, in the case of fungal spores, 4 ml l<sup>-1</sup> for 30 min<sup>1</sup>. For TriGene, a concentration of 10 ml l<sup>-1</sup> is recommended for general cleaning, and 20 ml l<sup>-1</sup> if organic material is present<sup>2</sup>. However, this study demonstrates that much lower concentrations and exposure times of both these chemicals are sufficient to kill *B. dendrobatidis* *in vitro*. Since organic material such as that in soil may interfere with, and reduce, the efficiency of these disinfectants (Wilson & Margolin 2003), higher concentrations should be used in the

field than those that are effective *in vitro*. In Table 2 the recommended concentrations of the 3 disinfectants tested here, as well as Virkon (Antec International) and DDAC are twice the minimum effective concentrations for an exposure of 1 min. It is also recommended that equipment be cleaned prior to disinfection. According to the Material Safety Data Sheets of the manufacturers, F10 and TriGene are non-toxic, non-irritant, non-corrosive and biodegradable<sup>1,2</sup> (ChemWatch 2006). In contrast, bleach (sodium hypochlorite), a commonly used disinfectant, is considered a dangerous and hazardous chemical (ChemWatch 2006). Bleach is also toxic to aquatic organisms and may cause long-term damage to aquatic environments (ChemWatch 2006). In previous studies, DDAC was determined to be highly effective at low concentrations (1 ml l<sup>-1</sup>) (Johnson et al. 2003). Since DDAC can be toxic to aquatic invertebrates and fish at concentrations of 1 ppm (Farrell et al. 1998), care is needed to avoid local contamination of water bodies when used in the field. TriGene and F10 are also effective at concentrations of <1 ml l<sup>-1</sup>. Smaller volumes of these concentrates would need to be taken into the field, saving space, expense and reducing the risk of release into the environment.

A range of strategies is now available to be used in different situations, particularly for field work, laboratory research, captive husbandry and for instruments used repeatedly on frogs (Table 2). In conclusion, TriGene and F10 are preferable for use in the field over the previously recommended DDAC products, as the former are active at much lower concentrations and appear to have no record of environmental toxicity. Any of these 3 disinfectants is recommended instead of bleach due to their efficacy at lower concentrations and less hazardous qualities. Research on the efficacy of disinfectants in the field would complete the results from the laboratory studies. However, evaluating the viability of *Batrachochytrium dendrobatidis* in natural samples cannot currently be done owing to overgrowth by other saprophytic fungi and bacteria that out compete *B. dendrobatidis* in culture media (Johnson & Speare 2005).

F10 has been used to treat fungal infections in reptiles and birds<sup>1</sup>, but there does not appear to be data on its safety for amphibians. The zoosporengia of *Batrachochytrium dendro-*

Table 2. Disinfectant options for the amphibian chytrid for various purposes in amphibian research and husbandry. Apart from the data on F10 and TriGene from this study, all data are based on Johnson et al. (2003). DDAC: didecyl dimethyl ammonium chloride

Purpose	Disinfectant and recommended concentration (double minimum effective concentration in most cases)
<b>Field use</b>	
Nets, boots, other equipment	TriGene Viricidal Surface Disinfectant Cleaner (0.2 ml l <sup>-1</sup> ) F10 Super Concentrate Disinfectant (0.7 ml l <sup>-1</sup> ) DDAC (2 ml l <sup>-1</sup> ) 4% bleach (1.8 g l <sup>-1</sup> sodium hypochlorite)
Instruments (scales, scissors, calipers)	70% ethanol (700 ml l <sup>-1</sup> ) wipes or liquid
<b>Laboratory use</b>	
Cultures, disposable equipment	70% ethanol (700 ml l <sup>-1</sup> ) Virkon (2 g l <sup>-1</sup> ) TriGene Viricidal Surface Disinfectant Cleaner (0.2 ml l <sup>-1</sup> ) F10 Super Concentrate Disinfectant (0.7 ml l <sup>-1</sup> ) DDAC (2 ml l <sup>-1</sup> ) 4% bleach (1.8 g l <sup>-1</sup> sodium hypochlorite)
Amphibian carcasses	Heat (60°C for 30 min) 70% ethanol (700 ml l <sup>-1</sup> )
<b>Captive husbandry</b>	
General cleaning and sterilising inanimate tanks and enclosures	TriGene Viricidal Surface Disinfectant Cleaner (0.2 ml l <sup>-1</sup> ) F10 Super Concentrate Disinfectant (0.7 ml l <sup>-1</sup> ) 4% bleach (1.8 g l <sup>-1</sup> sodium hypochlorite) Virkon (2 g l <sup>-1</sup> ) Heat (60°C for 30 min)

*batidis* in infected frogs are intracellular, surrounded by modified intracellular structures, possibly explaining why a fungus so sensitive to antifungal agents *in vitro* is so difficult to cure *in vivo* (Berger et al. 2005a). Research into the safety and efficacy of F10 as a potential treatment for chytridiomycosis of amphibians is needed.

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