

## **A protocol for treating chytrid (*Batrachochytrium dendrobatidis*) -infected frogs**

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**Please note – if you use this protocol we would like you to inform us of the species used and the result by sending an email to Phil Bishop (details above)**

### **Summary**

Chloramphenicol has been shown to be lethal to *Batrachochytrium*. The concentration required (20ppm) is comparable to that achieved in serum (veterinary and clinical practice) when inhibiting sensitive pathogenic bacteria. This result (the use of a well-characterised antibacterial compound to kill a fungal pathogen) was discovered empirically.

A detailed treatment protocol is shown below. This protocol may be modified by further simplification, but this is the simplest protocol we have so far validated empirically.

The frogs used in the protocol trials were wild-caught in late spring/early summer or raised from frogspawn laid in middle spring.

### **Species**

Two of the species involved were:

*Litoria ewingii*, the Brown tree frog (an Australian native, naturalised in NZ)

*Litoria raniformis*, Southern bell frog (another Australian native, naturalised in NZ)

Naturally infected members of a third species, *Leiopelma archeyi*, were also investigated. *L. archeyi* is a critically endangered NZ terrestrial frog.

### **Housing**

Frogs were housed individually in plastic containers.

As the frogs were partially immersed in the treatment, it is important that the frog spends a significant proportion of the time in the treatment solution, rather than on the walls of the container. As a general rule, the larger the frog,

the less likely for it to spend time on the walls. Ventilation holes were drilled in the tops and sides of the boxes so as to allow stacking of boxes.

*L. ewingi* were either in ~500mL round lidded pots or 500mL flat, oblong boxes that contained ~10-20mL of frog-water.

*L. raniformis* were kept in 4L lidded boxes with ~100-200mL of frog-water. The general idea is to have about 10 times the volume of treatment solution compared to the volume of the frog (*L. ewingii* is ~2.0g and *L. raniformis* ~20g).

Frog-water is 0.5g/L of SafeStart (from VitaPet/MasterPet in Lower Hutt NZ), a fresh water conditioner for aquaria that is obtainable from the supermarket. It is claimed to remove chlorine and chloramine from tap water and to detoxify heavy metals such as zinc and copper. It also produces a synthetic slime coat. SafeStart contains electrolytes to allow fish (or frogs) to maintain their electrolyte balance. Similar commercial products are no doubt available in the UK/US etc. Distilled water was used to make up the frog water.

The bases of the pots and boxes were lined with fabric cut from yellow ScotchBrite antibacterial dish-cloths. Prolonged standing on a plastic surface can cause inflammation of the frogs' feet and the cloths prevent this. Before using any other substitute cloths, check they are not toxic; we suspect some dish-cloths available in NZ are not good for frogs.

The frogs were kept in a constant temperature room at 19-20°C and ~80-90% humidity, with a light:dark cycle of 12h:12h.

Small frogs were fed 1-2 times a week with wax-moth larvae; *L. raniformis* were usually fed with cockroaches and/or waxmoths. Crickets were occasionally fed.

If not undergoing treatment, frogs were kept moist. The ScotchBrite cloths are helpful in keeping a moist substrate. Frogs were routinely cleaned once per week.

## **Infection**

In preliminary experiments we tested the sensitivity of *Batrachochytrium* to chloramphenicol in liquid and agar-solidified mTGhL medium. 10ppm chloramphenicol will kill chytrids in mTGhL after 4 days. The chloramphenicol doesn't simply inhibit growth; it kills the chytrids. This was shown by sampling the liquid culture and plating on chloramphenicol-free medium.

Frogs were experimentally infected by bathing them in a suspension of *Batrachochytrium* zoospores (strain JEL 197) in artificial pondwater for 4-8h. The zoospores were obtained by flooding 4 day cultures on mTGhL medium. The presence of zoospores was determined by binocular microscopy (lots of very active zoospores) and counted by haemocytometer (zoospores become very quickly inactive, due to attachment to glass). Zoospores were used at a concentration of 100,000/mL.

After 1-2 weeks the frogs were swabbed and their infection level ascertained by quantitative real-time PCR as per the method of Boyle et al., 2004 (using a Roche LC2 LightCycler). As a general rule, *L. ewingii* is more easily infected than *L. raniformis*. The frequency of infection of *L. ewingii* was ~100%. *L. raniformis* was ~60%.

Infected frogs were randomly assigned into the treatment protocol or to non-treatment maintenance.

### **Treatment**

*L. ewingii* were kept in 10mL and *L. raniformis* in 100mL.

Treated frogs were kept in 20ppm Chloramphenicol (Sigma C0378).

This solution was made at 10X strength, 200mg/L, in hot water (DON'T try to dissolve it in any, even tiny, amount of ethanol; ethanol is very toxic).

Each day, the chloramphenicol solution surrounding the frog was poured off (into a Virkon suspension) and freshly diluted solution added.

Every 2-3 days the frog was momentarily removed from the box while the box was washed in copious amounts of very hot water and a clean ScotchBrite mat added.

Frogs were swabbed every 7-10 days in order to quantify their infection level.

Frogs determined to be chytrid-free from two consecutive swabs were left untreated for ~2 weeks and re-swabbed to check for recurrence of chytrids.

Initial trials were made on a small number (2 or 3) of frogs with solutions of 5 and 10ppm chloramphenicol to test the sensitivity or otherwise of the frogs to this compound. Subsequently, further frogs were added to the treatment when the safety of chloramphenicol had been determined.

Our present protocol employs treatment for 2-4 weeks, swabbing, then continuing for one further week even if the frog is determined to be chytrid free by PCR. We have used this protocol with 31 *L. raniformis* and 30 *L. ewingii*. All treated frogs were cleared of infection and remain clear of infection. Untreated frogs remained infected; in many cases the infection became more symptomatic and led to the death of a number of *L. ewingii* showing classic *Batrachochytrium* pathology. We have noticed no adverse effects of treatment; frogs remain vigorous and retain their appetites. We have treated some frogs with chloramphenicol for in excess of three months to test for any adverse effects without finding any.

### **Other information**

We have also exposed infected frogs to a concentration of chloramphenicol insufficient to kill chytrids for a period of several months in order to determine if resistance to chloramphenicol readily emerges. At the end of this period, frogs were cured without difficulty using the standard protocol. This result suggests that resistance does not readily emerge. This is what might be expected from the use of chloramphenicol in veterinary and clinical practice, where resistance to chloramphenicol is rarely reported.

Chloramphenicol is readily absorbed and readily equilibrates through organ systems. It can be degraded by the liver and by micro-organisms. This is the

rationale of changing the treatment solution each day (comparable to injecting a patient).

It is potentially possible to overdose with chloramphenicol (grey baby syndrome due to the liver insufficiency of newborns). However, this is usually reversible by reducing or stopping the treatment. Given that the chytrid is killed by 10ppm, there is no need to exceed 20ppm in the treatment and exceeding this may be hazardous.

As amphibians are notorious for their species-specific reactions and susceptibilities, If treating a new species of amphibian, about which nothing is known of its response, caution would suggest trying out the response of 1 or 2 frogs to the treatment first. If the trial frogs are OK after 24 or 48h, then treatment could be extended to others. If the initial trial frogs fall sick, the treatment could be discontinued. We have no reason to anticipate any ill effects, having seen none in the three species we treated.

In clinical practice, chloramphenicol is without adverse effects, with the exception of grey baby syndrome in newborns and an extremely rare genetic sensitivity.