Original Article

Using amphibians in laboratory studies: precautions against the emerging infectious disease chytridiomycosis

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Abstract

The African clawed frog *Xenopus laevis* is by far the most widely used amphibian species in laboratories. In the wild, *X. laevis* is an asymptomatic carrier of an emerging infectious disease called chytridiomycosis. The vector is the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), which has devastating effects on wild amphibian populations around the world. The impact of *Bd* on the metabolism of *X. laevis* has not been comprehended yet. However, even if asymptomatic, an infection is likely to affect the individual's physiology, immunology, development, reproduction and overall response to stress from a purely medical point of view, which will introduce noise and therefore increase variance within experimental groups of *X. laevis*. This could have implications on the scientific results from studies using this species. Here, we review the current knowledge on treatments of infected amphibians and propose a hygiene protocol adapted to laboratory populations and amphibian husbandry. Following the presented sanitation guidelines could further prevent the spread of *Bd* and probably of other amphibian pathogens. The sanitation guidelines will help to reduce the impact of amphibian husbandry on natural populations and must be considered a crucial contribution to amphibian conservation, as today 32% of all amphibians are considered threatened.

Keywords: Hygiene, chytridiomycosis, disinfection, Xenopus

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It is now widely recognized that research should be conducted on healthy, pathogen-free, animals and products to ensure reliable data and allow comparison and interpretation of research results. For example, infection can have either a positive or a negative impact on a trial.[1] Even subclinical infections have been pointed out to potentially confound research results.[2] As a consequence, during the 1980s, bioexclusion protocols have been developed and specific pathogen-free (SPF) animals have been bred (or requested to suppliers) to eradicate infectious diseases in animal facilities. Despite stringent procedures, several emerging and re-emerging pathogens have been recently identified in rodent laboratory colonies, including the murine norovirus.[3,4] The threat represented by emerging pathogens led to the organization of a workshop dedicated to the 'Detection, Impact and Control of Specific Pathogens in Animal Resource Facilities' in 2009. One major conclusion of this workshop was the recommendation of 'the improvement of communications of disease outbreaks and potential risks for animal models for the scientific community'.[5]

Amphibian species are widely used in developmental, cell and molecular biology, as well as in genetic and genomic research. This includes the genera *Rana*, *Bufo*, *Hyla*, *Ambystoma*, and particularly *Xenopus* spp. (*X. laevis and X. tropicalis*).[6] However, the control of pathogens and infectious diseases in amphibian facilities is rather new. Only a few breeding facilities use sentinels for the detection of diseases. Even large institutions such as the Federation of European Laboratory Animal Science Associations in Europe (FELASA) or the National Research Council *Guide for the Care and Use of Laboratory Animals*[7–9] in the USA have not yet established health monitoring recommendations for amphibians as they exist for rodents[10] and only recently a description of amphibian diseases was published.[11] As such, extensive routine health monitoring is usually not performed in amphibians, and real SPF amphibians do not exist. It has been estimated that still one-third of *X. laevis* used are taken from the wild,[12] where they are likely to encounter various pathogens. This might be especially true for several newly emerging infectious diseases including ranavirus, *Amphibiocystidium* and *Batrachochytrium dendrobatidis* (*Bd*).[11,13] *Bd* is the vector for the disease chytridiomycosis, which is known to be a proximate driver of rapid amphibian species declines and extinctions on five continents[14] (despite the still unclear origin of the disease[14–16]), and it has been placed on the OIE

Wildlife Disease List in 2001. The fungus infects keratinized epidermal cells of amphibian species and causes a hyperkeratotic and hyperplastic response of the stratum corneum and stratum granulosum.[17] The causes of death of amphibians related to *Bd* are still unknown, but may include disruption of osmoregulation and toxin release.[11,18,19] In tadpoles usually the keratinized mouthparts are infected, while the infection spreads further during and after metamorphosis (Garner, personal communication).[20]

In the wild, *X. laevis* is subclinically infected by *Bd*, carrying infection but does not develop lethal chytridiomycosis likely due to magainin, an antibiotic, antifungal, antiparasitic and antiviral, which has been found on its skin.[21] However, even if subclinical, an infection is likely to affect the physiology, immunology, development, reproduction and overall response to stress from a purely medical point of view. Hence, noise will be introduced increasing variance within experimental groups of *X. laevis*, which could have implications on the scientific results from studies using *X. laevis*.[2] This might be especially important, given the difficulty to interpret signs of illness expressed by *X. laevis*.[12] Hence, diseases might become established unrecognized and pathogenesis may develop to a high degree before ill-effects become obvious.[22] It is therefore imperative to regularly test captive populations and disinfect them if needed.

More recently, the closely related species *X. tropicalis* is replacing *X. laevis* in laboratories. In contrast to *X. laevis*, it has been reported to be severely affected by the *Bd* fungus, with more than 80% of mortality during one epizootic event.[23] These individuals were likely infected by *X. laevis* individuals.[23] Indeed, *X. tropicalis* normally lives at higher temperatures than *X. laevis*, a requirement that is not always fulfilled in laboratories and may explain *Bd* outbreaks due to a lowered ability of immune responses of *X. tropicalis* in low-temperature environments.[24] In addition, stressful captive conditions and the wide use of immunosuppressed *Xenopus* individuals largely increases the probability of developing an infection, including chytridiomycosis.[23]

The absence of clinical signs of *Bd* infection and the wide use of *X. laevis* leading to naturalization of many populations worldwide was hypothesized as fatal for global amphibian diversity.[15] The transmission to natural populations was possible, because individual frogs did either escape laboratories and breeding facilities or were liberated intentionally. Due to the unawareness of the deadly disease, precautions were not put in place. Therefore, to protect wild populations of any kind of amphibians, strict hygiene rules need to be employed to mitigate the further spread of the disease. Here in particular it is noteworthy that UV treatment of waste water before release, as is usually employed in *Xenopus* facilities, does not kill *Bd* and therefore is a potential source of *Bd* transmission.[25] In amphibian facilities with the aim to provide SPF individuals, the usually employed safety measures might be sufficient (use of safety locks, autoclaving of equipment) as these usually are effective against *Bd*. However, contamination with food has currently not been ruled out. This is particularly difficult in amphibian breeding facilities as live food needs to be provided, making autoclaving of food impossible. Further, once *Bd* has found a host the treatment becomes more delicate due to the potential negative effects of disinfectants on amphibian species. Further, many amphibian facilities are conventional animal facilities in which the risk of cross contamination is elevated as tanks often are interlinked, allowing *Bd* zoospores to be transported from one tank to another.

Here, we review the current knowledge on disinfection techniques for amphibian facilities, treatments of amphibian specimen, the effect of treatments on some amphibian species, and propose a sanitation protocol adapted to laboratory populations and amphibian husbandry.

Currently known effective disinfectants and drugs

Disinfectants have been tested on *Bd* cultures and live amphibian specimens. So far, *Bd* cultures have shown few cases of resistance to known disinfectants active against other pathogenic fungi.[25] In a series of *in vitro* tests, Johnson *et al.*[25] determined the efficacy of the following chemical disinfectants: sodium chloride, sodium hypochlorite (household bleach), potassium permanganate, formaldehyde solution, didecyl dimethyl ammonium chloride (DDAC; Path-X™ agricultural disinfectant, Nutri-Tech Solutions Pty Ltd, Yandina, Queensland, Australia), quaternary ammonium compound 128 (James Varley & Sons, St Louis, MO, USA), Mancozeb (Dithane, Dow AgroSciences, Indianapolis, IA, USA), Virkon® (DuPont, Wilmington, DE, USA), ethanol and benzalkonium chloride. The time of exposure was critical for the efficacy of most chemicals (Table 1). Webb *et al.*[26] tested three additional disinfectants, including povidone–iodine (Betadine Antiseptic Liquid, Faulding Pharmaceuticals, Adelaide, Australia), and multicomponent products containing polymeric biguanide hydrochloride, DDAC and dimethyl benzyl ammonium chloride (TriGene Virucidal Disinfectant Cleaner, Medichem International, Sevenoaks, Kent, UK) or benzalkonium chloride and biguanide (F10 Super Concentrate Disinfectant, Lomb Scientific, Taren Point, New South Wales, Australia)[27] (Table 1).

Johnson *et al.*[25] tested the efficacy of UV light, heat and desiccation on *Bd* cultures. Complete desiccation within a 3 h time window at room temperature led to the death of all *Bd* cultures, which also showed a high sensitivity to heating. The 100% death of zoospores and sporangiae occurred after 4 h at 37°C, 30 min at 47°C and 5 min at 60°C. UV light (at 1000 mW m^2 with a wavelength of 254 nm) was ineffective at killing *Bd* in culture.

Few tests were conducted on infected amphibian individuals. Banks and McCracken[28] report the inefficacy of Plistopur®, a combination of copper phosphate, acriflavin HCl and P-chlorophenoxetol (Sera, Heinsberg, North Rhine-Westphalia, Germany) followed by griseofulvin (Grisovin, Sigma Pharmaceuticals, Clayton South, Victoria, Australia) against chytridiomycosis in sharp-snouted dayfrog tadpoles. Schmidt *et al.*[29] assessed the effect of household bleach and Virkon® S on tadpole performance and zooplankton abundance in a factorial experiment. They found that bleach at a concentration of 2% killed all tadpoles of *Rana temporaria* and *Bufo bufo*. Virkon® S (10%

Table 1 Effectiveness of disinfectants on *Batrachochytrium dendrobatidis* (*Bd*) culture, their minimal effective concentration for 100% kill, time of exposure for 100% kill and the corresponding reference

Commercial product	Active compound	Min concentration for 100% kill	Time of exposure for 100% kill	References
	Sodium chloride	5%	5 min	Johnson *et al.*[25]
Household bleach	Sodium hypochlorite	1%	10 min	Johnson *et al.*[25]
	Potassium permanganate	1%	10 min	Johnson *et al.*[25]
	Formaldehyde	0.10%	10 min	Johnson *et al.*[25]
Path-XTM agricultural disinfectant quaternary ammonium compound 128	Didecyl dimethyl ammonium chloride (DDAC)	0.01%	2 min	Johnson *et al.*[25]
Dithane®	Mancozeb	0.01%	5 min	Johnson *et al.*[25]
Virkon®	Peroxygen compounds, surfactant, organic acids and an inorganic buffer system	1 g/L	20 s	Johnson *et al.*[25]
	Ethanol	70%	20 s	Johnson *et al.*[25]
	Benzalkonium chloride	1%	20 s	Johnson *et al.*[25]
TriGene virucidal disinfectant cleaner	Polymeric biguanide hydrochloride, DDAC, and dimethyl benzyl ammonium chloride	0.1 mL/L	1 min	Webb *et al.*[26]
F10 super concentrate disinfectant	Benzalkonium chloride and biguanide	0.33 mL/L	1 min	Webb *et al.*[26]
Betadine antiseptic liquid	Povidone–iodine	100 mL/L	1 min	Webb *et al.*[26]

solution) did not show any detectable negative effect on tadpoles and zooplankton, but was effective against *Bd*. Two studies analysed the efficacy of itraconazole (Sporanox®, Janssen Pharmaceutica, Beerse, Belgium). Nichols and Lamirande[30] used a 0.01% itraconazole solution in 0.6 saline over 11 days of 5 min treatments each day and successfully eliminated infection in juvenile blue-and-yellow poison dart frogs *Taudactylus acutirostris*. In subadult and adult individuals of the same species, an eight-day treatment of itraconazole baths resolved the infection.[31] Itraconazole was also successfully used in *Alytes muletensis* tadpoles, but led to depigmentation[32] (Table 1).

Amphibian husbandry protocol

Virkon® S is a broad-spectrum disinfectant, based on peroxygen compounds (potassium peroxymonosulphate), surfactant (sodium dodecylbenzenesulphonate), organic acids (sulphamic acid) and an inorganic buffer system, with known capability at killing bacteria, fungi and viruses, including *Bd*. It achieves deactivation and/or destruction of the target organism through general oxidative disruption of key structures and compounds vital to normal activity (e.g. proteins and lipids). We recommend the use of this disinfectant for equipment, because of its low environmental impact and apparently low toxicity on amphibians,[29] but lack of toxicity is not guaranteed by the manufacturer. Release to water bodies before inactivity (pale pink colour of solution) should however be avoided and disposal through sewer systems is recommended by the manufacturer. As Virkon® S is irritant to the skin and may cause serious damage to eyes, direct contact with the skin and eyes should be avoided by wearing suitable protective clothing, gloves, eye and face protection (in accordance with BS EN 166). Product label instructions and information including the precautionary statements should be read and followed before use.

Husbandry practices

When setting up a new facility, all equipment should be properly sterilized before use. If uncertainty remains, sterilization should be performed, so that the facility can be considered pathogen free. If facility users move among several breeding rooms or outdoor sites, the sterilization or disinfection protocols should be applied. When handling amphibians, disposable, powder-free gloves should be used and dumped in biological hazard garbage. However, there are some reports on negative effects of certain gloves, leading to mortalities in larval and adult amphibians.[33,34] In addition, we advise that equipment should be regularly (every week) disinfected or sterilized within the housing place as a general precautious principle.

The entry to any amphibian husbandry should be protected by a safety lock. Despite the fact that chytridiomycosis does not pose a threat to humans (no zoonoses) and therefore regulations do not (yet) require an elevated security of facilities, a safety lock is highly recommendable to avoid any exit or entry of *Bd* and infected amphibians to and from any breeding facility. Laboratory coats and shoes should be provided for each person working in the breeding facility, for visitors disposable paper shoe protection is recommended. Shoes should be disinfected at entry and exit using a footbath or cushions soaked with either 1% Virkon® S solution (10 g Virkon® S in 1 L of water; renewed every day) or any other available surface disinfectant (renewed at least once per week). Hands and laboratory coats need to be disinfected when exiting the facility.

Reception, quarantine and diagnostics

Whenever possible, amphibians captured in the wild should be housed individually. Preventing co-housing of amphibians during collection limits contacts and disease transmission among animals. As amphibians bred in captivity are not systematically tested for *Bd*, no one can be sure

that new individuals purchased or exchanged with other laboratories are *Bd* negative. Any new individual entering a laboratory facility should therefore be placed in quarantine and tested negative before being included with the rest of the group. Individuals should be regularly tested for *Bd* using, i.e. the realtime polymerase chain reaction Taqman assay published earlier.[17] With this assay the accurate detection and quantification of one zoospore in a diagnostic sample is possible, using an MGB probe and two specific primers, ITS-1 and 5.8S. Generally, there are two possibilities for achieving reliable test results: (i) testing all individuals at regular intervals (i.e. every 6 months) and (ii) keeping a sentinel population, which receives water from all the tanks in the breeding facility and test the individuals once per month as suggested for fishes.[35] A sentinel population will reduce the costs and increase the probability of detecting even low concentrations of infections and should be preferred, but which tank contains infected individuals will remain unclear. Once infection is detected, it is not sufficient to treat only the individual and disinfect its tank, but all equipment and tanks it could have come in contact with. Hence, a complete disinfection or sterilization of tanks, equipment and water in the location where *Bd* has been detected is recommended.

Sterilization of equipment

A standard sterilization protocol for equipment, shoes and coats comprise the following steps. A fresh 1% Virkon® S solution (10 g/L) should be prepared every day and the solution should not be used when the colour is not at least a medium pink. A spray bottle can be used to squirt Virkon® S solution on all used equipment, which might have been in contact with water and wait for 5 min before re-use, and preferably until the equipment has dried (no rinsing required). Small material in contact with amphibians (e.g. scalpels, scissors, etc.) should be immersed in a 1% Virkon® S solution and stored in disposable plastic bags or in a special storage room to assure that they cannot be re-contaminated. After sterilization of equipment, hands should be disinfected with an antiseptic. Recommendations of local Occupational Health and Safety specialists should be sought to adapt the choice to local situations. Finally, laboratory clothes should be disinfected by washing them at 60°C and any disposable items (gloves, bags, etc.) should be collected in biological waste containers.

Topical treatment of affected amphibians

Itraconazole is a triazole antifungal drug usually used for the treatment of systemic fungal infections of dogs, cats and humans and comes as capsules or a liquid solution. It is effective against all filamentous fungi, dimorphic fungi and yeasts, such as blastomycosis, histoplasmosis, aspergillosis and cryptococcosis. It may also be used against some yeast and dermatophyte (ringworm) infections. Triazoles are a subgroup of the azole group of drugs. These drugs are fungistatic at the concentrations used systemically and fungicidal at the concentrations that may be achieved topically. The mechanism of action is through the disruption of the oxidative enzymes of the fungal organism.[36] Itraconazole is a potent cytochrome P450 3A4 isoenzyme system (CYP3A4) inhibitor and may increase plasma concentrations of drugs metabolized by this pathway. Detailed handling instructions and precautions are provided by the manufacturer and should be consulted before use.

Treatment of individual amphibians and tadpoles need to take into consideration potential negative effects of the different drugs (Table 2). So far, itraconazole promises the

Table 2 Effectiveness of disinfectants in treatments of live animals (*in vivo*) and their side-effects, their minimal effective concentration for 100% kill, time of exposure for 100% kill, the days of treatment (*N* days), the species and life stage, and the corresponding reference

Commercial product	Active compound	Test	Min concentration	Time of exposure	*N* days	Species	Side-effects	References
Sporanox	Itraconazole	*In vivo*	0.5 mg/L	5 min	7 days	*Alytes muletensis* (tadpoles)	Depigmentation	Garner *et al.*[32]
Sporanox	Itraconazole	*In vivo*	0.01%	5 min	8 days	*Taudactylus acutirostris* (adult)	None	Nichols and Lamirande[30]
Sporanox	Itraconazole	*In vivo*	0.1%	5 min	11 days	*Ambystoma mexicanum* (adult) *Potymotyphlus kaupii* (adult)	None	Forzán *et al.*[37]
Plistopur®	Copper phosphate, acriflavin HCL and P-chlorophenoxetol	*In vivo*	Not effective			*Taudactylus acutirostris* (adult)	None	Banks and McCracken[28]
Grisovin	Griseofulvin	*In vivo*	Not effective			*Taudactylus acutirostris* (adult)	None	Banks and McCracken[28]
Household bleach	Sodium hypochlorite	*In vivo*	2%	Added once a week	21 days	*Rana temporaria*, *Bufo bufo* (tadpoles)	Killing of tadpoles	Schmidt *et al.*[29]
Virkon®	Peroxygen compounds, surfactant, organic acids and an inorganic buffer system	*In vivo*	10 g/L	Added once a week	21 days	*Rana temporaria*, *Bufo bufo* (tadpoles)	None	Schmidt *et al.*[29]

highest efficacy.[30–32] It is therefore recommended that *Bd*-infected individuals be treated using itraconazole by being bathed in an itraconazole solution. Forzán *et al.*[37] adapted the itraconazole treatment of Nichols and Lamirande[30,31] using a 0.1% solution in 0.6% saline for 5 min treatments each day over a period of 11 days and reported no side-effects. Garner *et al.*[32] reduced the number of treatments to seven days (Table 2), but reported depigmentation of tadpole skin in their species.[32] The latter treatment is preferable; as it reduces the number of times individuals are handled and stressed, but side-effects need to be taken into account and the individuals need to be closely observed during the treatment. A second test for *Bd* should be performed two weeks after the end of the topical treatment to exclude the possibility that re-infection has occurred.

Conclusion

We proposed a husbandry protocol for equipment sanitation and topical treatment for affected amphibians and showed that precaution needs to be taken when working with captive amphibians to ensure research with *Bd*-free amphibians and avoid environmental contamination. We proposed an antifungal treatment successful against *Bd* infection based on current knowledge. Following the guidelines may reduce the impact that chytridiomycosis may have on results from experiments using infected amphibians. Not knowing the infectious status of amphibians used in the laboratory is likely to affect the outcome of any kind of research, and experimental outcomes may potentially be flawed.

Further, our general recommendations to run experimental amphibian facilities should help to avoid transmission of diseases, especially that of *Bd*, the vector for chytridiomycosis. The spread of chytridiomycosis had been largely accidental, with a devastating effect on many amphibian species. Following the sanitation guidelines outlined here could further prevent the spread of *Bd* and probably of other amphibian pathogens. The sanitation guidelines will help to reduce the impact of amphibian husbandry on natural populations and must be considered a crucial contribution to amphibian conservation, as today 32% of all amphibians, about 2100 species, are considered threatened.[38,39]

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