Zebrfish Embryo Disinfection with Povidone–Iodine: Evaluating an Alternative to Chlorine Bleach

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Abstract

Mycobacteriosis is a common bacterial infection in laboratory zebrafish caused by several different species and strains of *Mycobacterium*, including both rapid and slow growers. One control measure used to prevent mycobacterial spread within and between facilities is surface disinfection of eggs. Recent studies have highlighted the effectiveness of povidone–iodine (PVPI) on preventing propagation of *Mycobacterium* spp. found in zebrafish colonies. We evaluated the effect of disinfection using 12.5–50 ppm PVPI (unbuffered and buffered) on zebrafish exposed at 6 or 24 h postfertilization (hpf) to determine if this treatment is suitable for use in research zebrafish. Our results show that 6 hpf embryos are less sensitive to treatment as fewer effects on mortality, developmental delay, and deformity were observed. We also found that buffered PVPI treatment results in a greater knockdown of *Mycobacterium chelonae* and *Mycobacterium marinum*, as well as results in decreased harmful effects on embryos. Treatments of shorter (2 min vs. 5 min) duration were also more effective at killing mycobacteria in addition to resulting in fewer effects on embryo health. In addition, we compared the efficacy of a rinsing regimen to rinsing and disinfecting. Based on the findings of this study, we recommend disinfecting embryos for 2 min with buffered PVPI at 12.5–25 ppm.

Introduction

DISEASE PREVENTION in laboratory zebrafish and the maintenance of healthy stocks is a primary interest of the zebrafish research community. Diseases in laboratory zebrafish not only directly affect research due to the loss of valuable wild-type and mutant genetic lines but can also have indirect effects as chronic subclinical infections are a potential confounding source of uncontrolled experimental variance.^{1,2}

Mycobacteriosis is a common bacterial disease that affects zebrafish.^{1–4} The Zebrafish International Resource Center's (ZIRC) Diagnostic Services report that over 40% of facilities submitting specimens between 2006 and 2010 had fish diagnosed with mycobacteriosis.⁴ Several species of *Mycobacterium* have been implicated in outbreaks in zebrafish.^{1–3,5} Manifestation of mycobacterial infections in zebrafish is species specific.^{2,6–8} For example, infection with *Mycobacterium chelonae*, the most common species found in laboratory zebrafish, typically results in chronic and low-to-subclinical infections.⁶ Alternatively, *Mycobacterium marinum* causes acute and severe outbreaks that often result in obvious mortalities.⁶ Both of these manifestations have the potential to be devastating to research.

Recommendations for mycobacterial disease management in zebrafish highlight the importance of disease prevention,

as established infections are difficult and time-consuming to eradicate.² As a measure of biosecurity, facilities are encouraged to implement “eggs-only” policies, introducing only disinfected embryos from outside facilities.

Embryo surface disinfection is a common practice in fisheries and the standard disinfectant for zebrafish eggs is currently rinsing embryos with chlorine bleach. However, povidone–iodine (PVPI) has been shown to be a promising alternative as *in vitro* studies show PVPI is effective at killing *Mycobacterium* spp. that commonly infect zebrafish.⁹

Before PVPI can be recommended for use in zebrafish, an evaluation of the effect of disinfection on the health of zebrafish embryos is required. To accomplish this, we evaluated both unbuffered and buffered PVPI on wild-type zebrafish embryos at two developmental time points (6 and 24 hpf). We also tested the effect of these treatments on the survival of planktonic *M. chelonae* and *M. marinum* cultures.

Methods

Fish

All embryos used in the exposure studies were bred using AB and AB/Tübingen zebrafish obtained from both the zebrafish facility at the SUNY-ESF Center for Integrated

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Teaching and Research in Aquatic Sciences and from the Amack Laboratory at SUNY Upstate Medical University. Embryos were obtained from both paired and large group spawns and held in the E2 embryo medium¹⁰ (pH 7.4–7.5) at 28.5°C while development was monitored and staged.¹¹

Exposure

Ninety-six eggs per treatment (3 replicates of 32) were used for each concentration of PVPI (unbuffered [drugstore brand] or buffered Ovadine® [Western Chemical]) and were designated to treatment groups randomly. For all treatment solutions, the iodine concentration was verified using iodine test strips (LaMotte). The PVPI solutions were prepared fresh within 30 min of each treatment in autoclaved Milli-Q® water. A method for disinfectant exposure similar to a previous study evaluating chlorine bleach disinfection was utilized.¹² Embryos were exposed at either 6 or 24 hpf as embryo disinfection usually occurs following egg collection and screening (6 hpf) or following shipment of embryos to another facility (24 hpf).¹²

Embryo exposures were carried out by placing 32 embryos at 6 or 24 hpf in a 50-mL conical tube where the bottom was replaced with a 50- μ m mesh screen held in the E2 medium. This tube was transferred to treatment and rinse solutions as illustrated in Fig. 1. For each treatment solution (unbuffered PVPI: 12.5, 25, and 50 ppm; buffered PVPI: 12.5, 25, and 50 ppm), three treatment groups were run concurrently with embryos from the same clutch: 2-min exposure, 5-min exposure, and 5-min control (no iodine) exposure. During each exposure, the 32 embryos in the conical tube were transferred from the E2 medium, to a PVPI treatment solution for 2 or 5 min, to three separate sterile Milli-Q water rinses (conical tubes were fully lowered into beakers containing 35 mL of sterile Milli-Q and lifted out of solution, total rinse time lasted ~5 s), and to the fresh E2 medium (pH 7.4–7.5).

Toxicity evaluation

Following exposures, embryos were individually loaded into wells containing 300 μ L of E2 medium (pH 7.4–7.5) in a

sterile 96-well plate so that embryos from the three concurrent exposures were incubated in the same 96-well plate. Following exposures, 6 hpf embryos were monitored at 30 min postexposure and daily up to 5 dpf. Twenty-four hpf embryos were monitored at 30 min and 5 h postexposure as well as daily up to 5 dpf. Monitoring included observation of mortality, developmental delay, and deformity as in previous studies.^{12,13} Once embryos reached 5 dpf, they were euthanized in a solution of 300 mg/L MS222 buffered to a pH of 7.5.

Culture

An *in vitro* exposure of *M. chelonae* and *M. marinum* to the PVPI treatment solutions used in this study was also carried out. These exposures were carried out in triplicate following a previously described method⁹ for *M. chelonae* cultures and similarly for *M. marinum*; however, for *M. marinum*, the initial suspension of cells was prepared by inoculating sterile water with *M. marinum* freshly cultured on solid-phase Middlebrook 7H10 (MB 7H10) agar supplemented with hemin. Also, following exposures, solid-phase MB 7H10 agar supplemented with hemin was used, and plates were incubated for 14 days before colony counts.

An additional evaluation of disinfection *in vitro* was carried out to simulate an actual zebrafish embryo disinfection event. For this, $N=32$ embryos at 6 hpf were placed in a sterile 30-mL culture flask in E2 medium (pH 7.4–7.5). This flask was then inoculated with enough broth culture to result in a final concentration of 1.0×10^6 colony forming units (CFU)/mL of *M. chelonae*. This was intended to simulate an embryo shipment to another facility, in spawn water containing bacteria. This flask was then incubated overnight at 28.5°C at 50 rpm on a shaker incubator to emulate courier transport and bacterial incubation during this period. An additional uninoculated flask was prepared and incubated as a control.

The following morning at 10:30 am (a time point when an overnight express courier option would be received), the contents of the flask were emptied into a 50-mL conical tube with the bottom replaced with 50- μ m mesh and were taken

FIG. 1. Schematic of the embryo disinfectant exposure experiment.

through the disinfection exposure similar to the embryos undergoing a 5-min exposure to either 25 ppm unbuffered PVPI or a control of sterile water. Samples (1 mL) from the original E2 medium (pH 7.4–7.5) from the flask and final E2 medium (pH 7.4–7.5) solutions were taken and prepared for plating. Samples were diluted to 10^{-1} , 10^{-2} , and 10^{-3} . One hundred microliters of each dilution was plated in triplicate on Middlebrook agar plates using a sterile spreader and incubated at 28.5°C for 7 days. Following incubation, colony counts were performed. This experiment was performed in triplicate.

Statistics

To determine the difference between disinfection treatments on embryo health at different developmental stages, the following analysis was carried out using R 3.1.0¹⁴ and R Studio.¹⁵ Embryo mortality, developmental delay, and deformity up to 5 dpf following disinfection treatment were recorded and entered in a spread sheet and saved as text files for analysis. Descriptive statistics were obtained using the psych package¹⁶ and data normality and equal variances were assessed using the stats package¹⁴ and car package,¹⁷ respectively.

In the case of data with a normal distribution and equal variances, an analysis of variance (ANOVA) was performed to separately compare mortality, developmental delay, or deformity between disinfection treatments using the stats package.¹⁴ In the scenario of a significant ANOVA result ($p < 0.05$), Tukey comparison *post hoc* testing was performed using the agricolae package.¹⁸ If data were non-normally distributed or had unequal variance, a Kruskal–Wallis rank sum test was used to compare mortality, developmental delay, or deformity between disinfection treatments using the stats package.¹⁴ In the case of a significant result ($p < 0.05$), *post hoc* testing for pairwise multiple comparisons of the ranked data was performed using the PMCMR package.¹⁹ Visualization of data was then carried out as a clustered bar graph using the sciplot package.²⁰

Analyses of PVPI disinfection on *M. chelonae* and *M. marinum* were analyzed using R 3.1.0¹⁴ and R Studio.¹⁵ Descriptive statistics were obtained using the psych package¹⁶ and data normality and equal variances were assessed using the stats package¹⁴ and car package,¹⁷ respectively. A one-way ANOVA was performed using the stats package and Tukey *post hoc* comparisons were carried out using the agricolae package.¹⁸ Data were visualized as a clustered bar chart using the sciplot package.²⁰

To compare the effect of PVPI disinfection to a rinsing regimen, resulting colony counts on serial dilution plates were entered into a spreadsheet and the average colony count from the original flask as well as from the final E2 solution was calculated for each replicate for both the disinfection treatment and the control treatment. A percent survival was calculated for each replicate as [(E2 average colony count/Flask average colony count) $\times 100$]. These survival values were then analyzed using R 3.1.0¹⁴ and R Studio.¹⁵ Descriptive statistics were obtained using the psych package,¹⁶ and data normality and equal variances were assessed using the stats package¹⁴ and car package,¹⁷ respectively. An independent two-group *t*-test was carried out to compare the survival between the disinfection treatment and control treatment using the stats package.¹⁴

Results

Effects of PVPI disinfection on embryo health

Results from the disinfectant embryo exposures show that for 6 hpf embryos disinfected with unbuffered PVPI, a significant increase in embryo mortality was only observed for the 50 ppm-5-min PVPI treatment compared to the control (0 ppm) embryos [F(12,41)=4.46, $p < 0.001$] (Fig. 2). There was no significant effect of unbuffered PVPI treatment on developmental delay or deformity for any of the treatments at 6 hpf.

Disinfection treatments with buffered Ovadine resulted in no significant difference in mortality, developmental delay, or deformity for any of the treatments tested compared to 6 hpf control embryos (Fig. 2). When comparing PVPI treatments of the same concentration and duration between unbuffered and buffered PVPI solutions for 6 hpf embryos, there was a significant difference in embryo mortality for the 50 ppm-5-min treatment [F(12,41)=4.46, $p < 0.001$] (compare Fig. 2a and 2c).

For disinfection treatment of 24 hpf embryos, effects on embryo health were observed for 25 and 50 ppm treatments relative to controls (Fig. 2). Unbuffered PVPI treatments resulted in a significant increase in mortality for 25 ppm-5-min, 50 ppm-2-min, and 50 ppm-5-min treatments compared to control 24 hpf embryos [$\chi^2(12) = 34.24$, $p < 0.001$] (Fig. 2). Also, there was significantly greater embryo deformity observed for the 25 ppm-2-min treatment compared to control 24 hpf embryos [F(12,47)=2.148, $p < 0.05$]. There was no significant effect on developmental delay observed for any of the unbuffered PVPI treatments compared to 24 hpf control embryos. Buffered PVPI treatments of 24 hpf embryos resulted in significantly greater mortality for both 50 ppm treatments (2 and 5 min) compared to control 24 hpf embryos [$\chi^2(12) = 34.24$, $p < 0.001$] (Fig. 2). There were no significant developmental delay or deformity effects for buffered PVPI treatment of 24 hpf embryos. When comparing PVPI treatments of the same concentration and duration between unbuffered and buffered PVPI solutions for 24 hpf embryos, there was significant difference in embryo health effects for 25 ppm treatments. Specifically, the unbuffered PVPI solution resulted in significantly higher embryo deformity for the 25 ppm-2-min treatments [F(12,47)=2.148, $p < 0.05$], as well as significantly higher embryo mortality for the 25 ppm-5-min treatment compared to the buffered treatments [$\chi^2(12) = 34.24$, $p < 0.001$] (compare Fig. 2b and 2d). No other significant differences were observed when comparing the two PVPI solutions.

Effect of PVPI disinfection on *Mycobacterium* spp.

All PVPI treatments of *M. chelonae* and *M. marinum* in culture resulted in lower survival than control treatments. Generally, knockdown of survival increased as PVPI concentration increased and knockdown of *M. chelonae* was more variable than *M. marinum* (Fig. 3).

For *M. chelonae* exposures, unbuffered (drugstore brand) PVPI treatments were most effective for 2-min exposures compared to 5-min exposures. Based on average bacterial knockdown, the most effective treatments were 50 ppm-2 min, 25 ppm-2 min, and 12.5 ppm-2 min (less than 2% survival), which were more effective than 5 min at the same concentration. The next most effective treatments were 25 ppm-5 min

FIG. 2. Results of the embryo disinfectant exposures displaying the percent deformity (white bars), developmental delay (gray bars), and mortality (black bars) for (A) 6 hpf embryos exposed to unbuffered PVPI, (B) 24 hpf embryos exposed to unbuffered PVPI, (C) 6 hpf embryos exposed to buffered PVPI, and (D) 24 hpf embryos exposed to buffered PVPI. Significant differences between treatment concentration/durations are indicated by an asterisk. Significant differences between PVPI solutions for the same treatment are indicated by a plus-sign. hpf, hour postfertilization; PVPI, povidone-iodine.

and 50 ppm-5 min (less than 20% survival). The least effective treatment, resulting in the greatest bacterial survival, was the 12.5 ppm-5-min treatment, which resulted in significantly more survival of *M. chelonae* than all the other unbuffered PVPI treatments [$F(23,466)=4.012$, $p<0.05$].

All buffered (Ovadine) treatments were equally effective (less than 0.1% survival for all treatments) as there was no significant difference between treatment concentrations or durations ($p>0.05$). All PVPI treatments of *M. marinum*

resulted in less than 0.1% survival and there were no significant differences found between treatment concentrations or durations ($p>0.05$).

When unbuffered and buffered (Ovadine) PVPI solutions are compared, the only difference observed was for the *M. chelonae* treatment of 12.5 ppm for 5 min ($p<0.05$), which was previously shown to be the least effective PVPI treatment of *M. chelonae*. This treatment was also the only treatment where, after comparing *M. chelonae* and *M. marinum* survival for the same PVPI treatment, differences were observed between species ($p<0.05$).

Embryo mock-disinfection results

For the embryo mock disinfection, before disinfection, the flasks, both control and treatment, originally inoculated and incubated overnight with *M. chelonae* contained an average 1.8×10^5 – 2.0×10^5 CFU/mL. Following treatments (rinsing and PVPI disinfection, or rinsing alone), the amount of *M. chelonae* remaining in the E2 medium containing embryos decreased. For the control treatment, which underwent three rinses in sterile water, the resulting E2 medium contained 3.0×10^3 CFU/mL *M. chelonae* based on plate counts. For the disinfection treatment, which consisted of a 25 ppm unbuffered PVPI for 5-min treatment, the resulting E2 medium contained 0 CFU/mL (SD=0 CFU/mL) *M. chelonae* based on plate counts.

FIG. 3. Results from the *in vitro* PVPI disinfection displaying the percent knockdown of *Mycobacterium chelonae* (white) and *Mycobacterium marinum* (gray). Significant differences between treatments are indicated by an asterisk.

Discussion

The results from this study show that zebrafish embryos can tolerate disinfection with PVPI at concentrations/

durations that are effective at killing mycobacteria. We found that similar to our previous study,⁹ PVPI is effective at killing mycobacteria at 12.5–50 ppm PVPI at both 2- and 5-min treatment durations. Generally, results from our evaluation of PVPI disinfection on embryo health show that effects on embryo health occur at higher concentrations and/or treatment durations of PVPI for both 24 and 6 hpf embryos.

The use of a buffered PVPI solution (Ovadine) resulted in more consistent knockdown of planktonic *M. chelonae* and *M. marinum* in medium, as well as less embryo mortality and deformity compared to unbuffered treatments. This result is intriguing as buffering of chlorine bleach solutions has also been shown to enhance disinfection, although this buffering has been shown to have a negative effect on embryo health.¹²

The pH of both buffered and unbuffered 50 ppm PVPI solutions was subsequently measured. Unbuffered PVPI had a pH of 5.97, 5.78, and 5.60 for 12.5, 25, and 50 ppm solutions, respectively. Unbuffered PVPI was unstable requiring three repeats of measurements. Buffered PVPI had a pH of 6.27, 6.01, and 5.70 for 12.5, 25, and 50 ppm solutions, respectively, and did not require multiple measurements. The stability of pH as well as acidity may have influenced the impact of PVPI solutions on zebrafish embryo health. The less variable knockdown of *M. chelonae* and *M. marinum* following buffered PVPI treatment was likely due to the different additives and composition of the PVPI solution and the iodine-complexing properties and the subsequent effect on the concentration of free molecular iodine available for binding.²¹

Similar to our previous study,⁹ we found that shorter durations of disinfection resulted in a more consistent decrease in survival of *M. chelonae*. This could be attributed to the amount of available bactericidal iodine (e.g., hydrated iodine [I₂], hypiodous acid [HOI], and iodine cation [H₂OI⁺]), as there are several potential reactions of molecular iodine in water (e.g., hydrolysis, dissociation, protonation, complex formation, and disproportionation) that could limit availability over time as more reactions are able to occur.²²

Shorter durations of PVPI exposures had less of an impact on embryo health as embryo mortality was increased significantly for 6 hpf embryo treatment with unbuffered 50 ppm-5 min compared to 50 ppm-2 min as well as 24 hpf embryo treatment of 25 ppm-2 min compared to 25 ppm-5 min. An exception to this pattern was with 24 hpf embryos treated with unbuffered 25 ppm PVPI for 2 min. In this study, we observed an increase in embryo deformity compared to controls and this was not observed for 5-min-treated embryos, although this may be due to more 5-min embryos dying compared to 2 min.

While 6 and 24 hpf embryos cannot be compared directly as they are undergoing different developmental processes and subsequent variable natural early mortality, for 24 hpf embryos, instances of significantly increased mortality compared to 24 hpf control embryos occurred at 25–50 ppm (unbuffered PVPI). For buffered PVPI at 24 hpf, 25 ppm treatments are an option as increased embryo mortality was only observed for 50 ppm treatments. Whereas for 6 hpf embryos, instances of significantly higher mortality compared to control 6 hpf embryos occurred only at 50 ppm (unbuffered PVPI). This is similar to Kent *et al.*,¹² supporting the recommendation to, when possible, treat embryos at an earlier time point.

We also evaluated the role that rinsing embryos have in preventing mycobacterial spread through a mock-disinfection experiment. We found that rinsing embryos that were incubated with *M. chelonae* resulted in a decrease in planktonic bacteria; however, an average of 3.0×10^3 CFU/mL bacteria persisted in the embryo medium. Although rinsing reduced the amount of planktonic bacteria dramatically, they were not eliminated and could still pose an infection risk. The rinsing followed by disinfection resulted in no culturable mycobacteria in the embryo water. Thus, the diluting effect of rinsing coupled with disinfection with PVPI is very effective at reducing the risk of mycobacterial contamination in embryo cultures.

Generally, we recommend disinfection with at least 12.5 ppm PVPI. Similar to Kent *et al.*,¹² we recommend disinfecting embryos from a outside facility or from a population with a known infection with a higher (25–50 ppm) disinfectant concentration, as potential biosecurity risk would outweigh the health effects on the first generation. Following generations could then be treated with a lower concentration. Although the potential effects of PVPI disinfection on fine developmental or physiological processes are not known, caution might be warranted in toxicological and behavioral studies until additional studies are conducted.

We only evaluated the effects of PVPI disinfection on wild-type and AB/Tübingen embryos that were not dechorionated. Also, the regular practice of pronase-mediated chorion removal may also influence the effect of disinfection on embryo health. Other genetic lines of zebrafish may be more or less sensitive to disinfection with PVPI. These factors as well as facility water hardness, pH, and conductivity should all be considered when adopting a disinfection protocol.

Also, to be consistent with our previous culture studies,⁹ we utilized Milli-Q water for diluting our PVPI concentrates as well as for rinse solutions. The usage of alternative rinse solutions (i.e., E2 medium, autoclaved system water) may result in different embryo health effects and future studies should consider evaluating these solutions. Finally, as previously discussed,^{9,12} these methods contribute to good biosecurity and, as such, are likely to reduce the spread of microorganisms. However, they are less likely to prevent the spread of intraovum pathogens (e.g., *Pseudoloma neurophilia*)²³ and a holistic approach, including additional disease prevention and control measures, should be taken in addition to embryo disinfection.

In conclusion, we have demonstrated that PVPI is effective for killing *Mycobacterium* spp. in media and that these same concentrations and durations are not harmful to zebrafish embryo health. We also demonstrate that while the practice of rinsing embryos results in decreased bacteria counts, disinfection is much more effective. We also showed that treatment with buffered PVPI (Ovadine) relative to unbuffered results in greater bacterial knockdown, as well as increased embryo survival.

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Disclosure Statement

No competing financial interests exist.

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