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Evaluation of 5 Cleaning and Disinfection Methods for Nets Used to Collect Zebrafish (*Danio rerio*)

Chereen Collymore,[1,*†] Gina Porelli,[2] Christine Lieggi,[1,2] and Neil S Lipman[1,2]

Few standardized methods of cleaning and disinfecting equipment in zebrafish facilities have been published, even though the effectiveness of these procedures is vital to preventing the transmission of pathogenic organisms. Four chemical disinfectants and rinsing with municipal tap water were evaluated for their ability to disinfect nets used to capture zebrafish. The disinfectants included benzalkonium chloride+methylene blue, sodium hypochlorite, chlorine dioxide, and potassium peroxymonosulfate+sodium chloride for a soak time of 5 or 30 min. Disinfection effectiveness was evaluated by using an ATP-based system that measured the reduction in absolute number and percentage of relative light units. In addition, nets were cultured aerobically on blood and MacConkey agar plates to determine the number of bacteria remaining after disinfection procedures. Soaking nets in sodium hypochlorite for 30 min and in potassium peroxymonosulfate+sodium chloride for 5 or 30 min were effective means of disinfection, according to at least 90% reduction in the number of relative light units and no bacterial growth after cleaning. These results will aid facility managers, veterinarians and investigators in selecting net cleaning and disinfection protocols.

Abbreviations: RLU, relative light units; RO, reverse-osmosis–purified.

Verifying the efficacy of cleaning and disinfection procedures is essential to prevent cross-contamination between tanks of zebrafish, particularly in facilities supporting multiple investigators.[5,13] Appropriate net cleaning and disinfection reduces or eliminates organic material and kills infective agents, reducing the potential of the net to transmit pathogens.[5,14] Cleaning removes organic debris that otherwise decreases the efficacy of many disinfectants.[5,12,14] Disinfection eliminates all or most pathogenic microorganisms present on equipment.[5,12,14] This process is vital when large numbers of animals are housed in close proximity. In zebrafish facilities, nets are used frequently to capture fish from different tanks for breeding or other manipulations and are the most likely source of cross-contamination if not cleaned and disinfected correctly between uses.

ATP-based monitoring systems measure the amount of organic matter (live or dead) present on a surface or material.[3,5,12,13] ATP is detected by swabbing a test applicator across the item to be evaluated and then placing the applicator in a luminometer. The luminometer quantifies the amount of light generated when the lysis buffer (an ATP-releasing agent) reacts with luciferase (an enzyme that releases ATP) and luciferin (an ATP-activated light-producing substrate).[3] The quantity of light emitted is proportional to the amount of ATP on the surface or material tested. ATP-based monitoring systems may serve as a replacement for or can be used in addition to traditional contact agar methods (e.g., contact plates) for determining bacterial contamination.[3,5,12,13] The advantages of ATP-based monitoring systems, when compared with contact agar methods, include rapidity of analysis and results, ease of use, low cost of equipment and supplies, as well as the ability to detect a variety of potential pathogens and contaminants other than aerobic bacteria and fungi.[3,5,13] Effective cleaning and disinfection is achieved when relative light unit (RLU) values are reduced by 90% or more.[5,12] Limitations of this method include sodium hypochlorite's potential interference with detection of ATP by the luminometer.[13] Previous work demonstrated that residual sodium hypochlorite decreased ATP detection; to prevent false-negative results, multiple swabs should be used to evaluate surfaces cleaned with sodium hypochlorite. Another limitation is the method's reduced ability to detect gram-negative bacteria; false-negative results may occur due to incomplete lysis of the cell walls of gram-negative bacteria.[13] However, gram-negative bacteria frequently occur in the presence of organic debris, therefore contamination would likely still be detected by this method.[13] Given these limitations of ATP-based detection methods, we also included aerobic bacterial culture in this study.

The number of colony-forming units present after cleaning with various chemical agents can be used also to evaluate the efficacy of a cleaning and disinfection protocol.[13] Aerobic bacterial culture requires an incubation period of 48 h or more; detects live, aerobic bacteria only; and fails to determine whether a surface is free from other organic material.[3] A combination of both aerobic bacterial culture and ATP detection methods provides a comprehensive analysis of the efficacy of cleaning and disinfection procedures.

A study evaluating the efficacy of net cleaning and disinfection procedures used in a zebrafish facility showed that rinsing nets in reverse-osmosis–purified (RO) water and then soaking them for 1 h in a commercial solution containing benzalkonium chloride and methylene blue (Net Soak), rinsing again in RO

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[1]Tri-Institutional Training Program in Laboratory Animal, Medicine and Science, Memorial Sloan-Kettering Cancer, Center, The Rockefeller University, and the Weill, Cornell Medical College, New York, New York; [2]Memorial Sloan-Kettering Cancer Center and the Weill Cornell, Medical College, New York, New York.
[*]Corresponding author. Email: c.collymore@utoronto.ca
[†]Current affiliation: Division of Comparative Medicine, University of Toronto, Toronto, Ontario, Canada.

water, and drying was 96.6% effective in disinfecting the nets, according to an ATP-based monitoring system.[5]

In this study, we expanded on their results assessing the efficacy of 4 cleaning and disinfection agents commonly used in aquaculture: benzalkonium chloride+methylene blue, sodium hypochlorite, chlorine dioxide, and potassium peroxymonosulfate+sodium chloride for the cleaning and disinfection of zebrafish nets. Our aims were to validate our current 10-min cleaning protocol (compared with the 1-h soak time previously published[5]) and to evaluate other cleaning and disinfection protocols for zebrafish nets. We hypothesized that adequate disinfection, as determined by at least 90% reduction in RLU and no growth on aerobic bacterial culture, could be achieved by soaking nets in one of a variety of disinfectants for 30 min or less.

Materials and Methods

Humane care and use of animals. Tanks (2.8 to 3.5 L) holding various zebrafish stocks and strains maintained in a core facility of an AAALAC-accredited institution in compliance with the *Guide for the Care and Use of Laboratory Animals* were used to expose the nets.[6] All fish were maintained on protocols approved by Weill Cornell Medical College's IACUC. Animals were housed at a maximal density of 10 fish per liter on recirculating housing systems with mechanical and biologic filtration as well as UV disinfection (Tecniplast USA, Exton, PA, and Aquaneering, San Diego, CA), by using RO water balanced to pH 7.0 to 8.0 with sodium bicarbonate (part no SC12, Proline Water Conditioners, Aquatic Eco Systems, Apopka, FL) and a maintained at a conductivity of 600 to 1000 µS with a marine salt mixture (Instant Ocean Sea Salt, United Pet Group, Blacksburg, VA). Fish were fed an irradiated commercial pelleted diet (Zeigler Adult Zebrafish Complete Diet, Zeigler Bros, Gardners, PA) and decapsulated *Artemia nauplii* (Economy Grade Brine Shrimp, Brine Shrimp Direct, Ogden, UT) twice daily. The health status of fish is assessed biannually by testing as many as 10 fish per life-support system exposed to effluent sump water. Evaluations include a gross external exam; skin scrape, gill clip, and fin clip for microscopic examination; aerobic and anaerobic renal bacterial culture; and light microscopic evaluation of all organs after hematoxylin and eosin staining, with the addition of Luna and acid-fast stains when indicated. At the time of this study, zebrafish in this facility were considered free of known zebrafish pathogens, with the exception of nonpathogenic *Mycobacterium* spp. and low levels of *Pseudoloma neurophilia*, which were detected in select sentinel fish.

Experimental design. Nets (n = 10 per group; 110 total; 4 in., Quick-Net, Penn-Plax, Hauppauge, NY) tested were in use in 3 zebrafish holding rooms or new (never used). Prior to use in the study, all nets (except the negative control group) were disinfected by rinsing with municipal tap water, soaking for 10 min in benzalkonium chloride+methylene blue (Net Soak, United Pet Group, Jungle Laboratories, Cibolo, TX), followed by 10 min in a water conditioner and detoxifier (AmQuel Plus, Kordon, Hayward, CA), and then left to air-dry on a drying rack.

The following 9 experimental groups were evaluated for cleaning and disinfection efficacy: benzalkonium chloride (less than 1%) and methylene blue (Net Soak) for soaks of 5 and 30 min; 2% sodium hypochlorite (Ultra-Clorox, The Clorox Company, Oakland, CA) for soaks of 5 and 30 min; 1:18:1 chlorine dioxide (Clidox-S, Pharmacal Research Labs, Waterbury, CT) for soaks of 5 and 30 min, 1% potassium peroxymonosulfate+sodium chloride (Virkon Aquatic, EI DuPont de Nemours, Wilmington, DE) for soaks of 5 and 30 min; and rinse and air-dry only. Positive and negative control groups were included also.

For all experimental groups, each net was inserted into a zebrafish tank for approximately 2 min of exposure to the fish and tank water, mimicking the time and process used to capture fish. The same 10 tanks holding a similar density of fish were used throughout the study for net exposure. Nets were inserted into tanks at approximately the same time after feeding on each study day. Immediately after exposure, each net underwent ATP detection testing. Nets were then rinsed under hot, high-flow municipal tap water for 30 s to remove organic debris, soaked for the prescribed time (5 or 30 min) in 1 of 4 chemical disinfectants, placed in the water conditioning and detoxifying solution for 10 min, and air-dried for 30 min by hanging the net on a drying rack. ATP testing then was repeated and aerobic bacterial culture performed. The nets that were rinsed and air-dried only were inserted into a zebrafish tank as described, subjected to ATP detection testing, rinsed under running tap water for 30 s, and then allow to air-dry for 30 min prior to a second ATP detection test and aerobic bacterial culture. Nets in the positive control group were evaluated for aerobic bacterial culture only immediately after removal from the zebrafish tank. Nets in the negative control group were subjected to aerobic bacterial culture immediately after removal from the plastic packaging (they were unused prior to testing).

For ATP detection, the RLU obtained for each experimental group prior to and after disinfection were compared. For aerobic bacteria detection, nets serving as positive and negative controls were used to compare disinfection efficacy, because only one sample was taken. We defined effectiveness as at least 90% reduction in RLU and no growth on aerobic bacterial culture after treatment.[3,5,12]

Net disinfectant solutions. Disinfectant solutions were prepared 15 min before use. The benzalkonium chloride+methylene blue solution was prepared by adding 18 mL of the concentrated solution to 14 L of RO water in a large bucket (20-Quart Little Giant Flat Back Bucket, Miller Manufacturing, Glencoe, MN). The sodium hypochlorite solution was prepared by adding 2 L of 6.15% bleach to 6150 mL of RO water in a large bucket. The chlorine dioxide solution was prepared by mixing 10 L of the base and activator in a commercially available preparation system into a large bucket. The potassium peroxymonosulfate+sodium chloride solution was prepared by placing a single (37 g) packet of powder into 1 gallon of RO water in a large bucket. The water conditioning and detoxifying solution was prepared by adding 9 mL of the concentrated solution to 14 L of RO water in a large bucket. All solutions were prepared at room temperature (22 °C).

ATP detection. One moistened ATP collection swab (Ultrasnap ATP Test, Hygiena USA, Camarillo, CA) was rolled along the entire inside surface of each net to collect each sample. All the swabs were read within the recommended 2-h time limit according to the manufacturer's recommended process. A luminometer (Hygiena USA) was used to determine the number of RLU generated.

Aerobic bacterial culture. To obtain aerobic bacterial cultures, nets were inserted into 24 mL of sterile water (Vedco, St Joseph, MO) contained within a sterile 6-oz plastic container (Berry Plastics, Evansville, IN) until the entire net surface was submerged for 10 s and then removed. The containers were closed and transported to a microbiologic lab (Laboratory of Comparative Pathology, Memorial Sloan-Kettering Cancer Center–Weill Cornell Medical College, New York, NY) at room temperature for processing. The maximal time between sample collection and processing was 30 min. A 1-µL sample, collected after the sterile water containers were vortexed for 10 s, was placed onto the center of both a blood agar (BBL TSAII

5% SB, Becton Dickinson, Sparks, MD) and a MacConkey agar (BBL MacConkey 5% SB, Becton Dickinson) plate by using a 1-µL loop (VWR International, Radnor, PA). The sample then was streaked over the plate in a zigzag pattern by using a 1-µL loop. Plates were incubated for 48 h at 37 °C. After incubation, the total number of colonies on each plate was determined and reported as no. of cfu/µL.

Statistical analysis. The results were not normally distributed, according to the Shapiro–Wilks normality test. Log transformation did not normalize the data. The Kruskal–Wallis test was used to compare data between groups, followed by a posthoc Bonferroni test to identify the groups that were significantly different from one another. The Kruskal–Wallis test was used to determine differences in percentage reduction of RLU between groups, followed by posthoc Bonferroni tests to identify groups that were significantly different from one another. The Wilcoxon matched-pairs signed-ranks test was used to compare RLU data before and after cleaning within groups. A P value of 0.05 was considered significant. Data were analyzed by using statistical analysis software (Stata Software, StataCorp, College Station, TX).

Results

ATP detection. All disinfection agents at both time points yielded statistically significant ($P < 0.05$) reductions in absolute RLU values after comparison of pre- and posttreatment values (Figure 1). No RLU were detected when nets were exposed to sodium hypochlorite 2% for 30 min. Potassium peroxymonosulfate+sodium chloride for 5 or 30 min resulted in 90% and 92%, respectively, reductions in absolute RLU values (Figure 2). The decrease in RLU after cleaning was statistically significant ($P < 0.05$) for the nets that were rinsed and air-dried only; however only a 53% reduction in RLU was achieved. None of the disinfection groups, including the rinse and air-dry group, differed significantly from another after treatment.

Aerobic bacterial culture. The positive-control nets had an average of 10 CFUs/µL on blood agar and 2.2 CFUs/µL on MacConkey agar (Figure 3). No bacterial growth was observed after treatment, including rinsing and air-drying only.

Discussion

Multiple factors must be considered when determining the best cleaning and disinfecting procedures for equipment in a zebrafish facility. These include pathogens of concern, type and frequency of equipment used, husbandry practices, system configuration, and facility utilization. In addition, there are disinfectant-specific considerations, including required contact time, effective pH and concentration, presence and effect of organic matter, amount required, toxicity, disposal issues, and cost.[5,10,14] According to our results, 3 chemical agents adequately disinfected zebrafish nets, as determined by a greater than 90% reduction in RLU and no growth on aerobic bacterial culture.

Benzalkonium chloride +methylene blue reduced RLU readings by only 85% in 30 min. This value is less than that previously described when a 1-h soak in this solution reduced RLU by 96.6%.[5] In addition, we found that 5 min of contact time in this solution reduced RLU by only 71%. However, we did not detect aerobic growth after using this solution. Furthermore, the commercial benzalkonium chloride +methylene blue formulation (Net Soak) maintains net pliability, which may reduce disruption of the fish's skin or mucus layer.[5] A disadvantage is its reported ineffectiveness against *Mycobacterium* spp. and variable activity against *Pseudomonas* spp. and some viruses.[14] The manufacturer recommends changing the solution weekly to maintain its effectiveness.

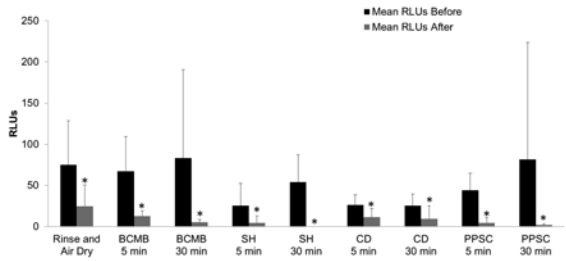

Figure 1. RLU (mean ± 1 SD; n = 10 nets per group) before and after implementing each cleaning and disinfection protocol with either benzalkonium chloride+methylene blue (BCMB), sodium hypochlorite (SH), chlorine dioxide (CD), or potassium peroxymonosulfate+sodium choride (PPSC). *, Value significantly ($P < 0.05$, Wilcoxon matched-pairs signed-rank test) different as compared with precleaning values. Postcleaning RLU values did not differ significantly between disinfectants.

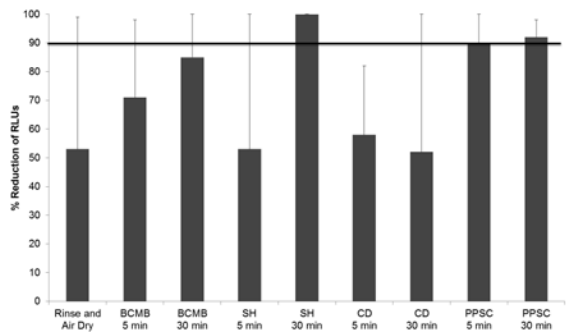

Figure 2. Percentage reduction in RLU (mean ± 1 SD; n = 10 nets per group) after net cleaning and disinfection compared with beforehand with either benzalkonium chloride+methylene blue (BCMB), sodium hypochlorite (SH), chlorine dioxide (CD), or potassium peroxymonosulfate+sodium choride (PPSC). The sodium hypochlorite for 30 min and potassium peroxymonosulfate+sodium chloride for 5 and 30 min groups reduced RLU values by at least 90% (horizontal line) of our criterion for adequate disinfection. There were no differences between groups, according to the Kruskal–Wallis test.

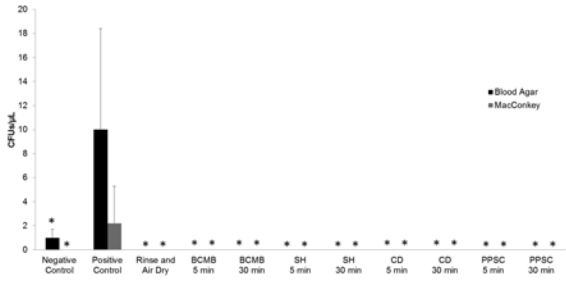

Figure 3. Number (mean ± 1 SD; n = 10 per group and media) of aerobic bacterial colonies at 48 h after cleaning and disinfection with either benzalkonium chloride+methylene blue (BCMB), sodium hypochlorite (SH), chlorine dioxide (CD), or potassium peroxymonosulfate+sodium choride (PPSC). *, Value significantly ($P \leq 0.05$, Kruskal–Wallis test followed by Bonferroni test) different from that for the positive control.

Soaking in sodium hypochlorite (2%; Ultra-Clorox) for 30 min effectively reduced the number of RLU (100% reduction). No bacteria grew on aerobic culture, but soaking for 5 min reduced RLUs by only 53%. Chlorine-based agents have a broad spectrum of antimicrobial activity.[5,14] A major advantage is that sodium hypochlorite has been shown to be effective against *Mycobacterium marinum* at 20 min of contact time.[10] Sodium hypochlorite also is effective against fungi, such as *Aphanomyces* spp., and viruses, such as infectious pancreatic necrosis virus.[4,7] A major

disadvantage is the extreme toxicity of chlorine-based products to fish, in that as little as 0.25 mg chlorine /L can be toxic.[1] In addition, decreased pH leads to increased free chlorine and thus increased toxicity.[1,5,14] To neutralize chlorine, nets should be soaked in 4 mg/L sodium thiosulfate per 1 ppm of chlorine used before they are dried and reused.[14] To prevent accidental exposure, chlorine solutions should be clearly labeled and stored in a sealed container; ideally, they should not be stored in the holding room. Chlorine-based disinfectants are harsh to human skin and mucous membranes, are corrosive to metal,[5,14] and their efficacy is reduced in the presence of organic matter.[4,14] In addition, 5 min of contact time only reduced RLUs by 53% and it would not be expected to eliminate *Mycobacterium* spp.[10] Sodium hypochlorite solutions must be reconstituted daily to remain effective, according to the manufacturer's recommendation.

Chlorine dioxide (Clidox-S) has previously been used in aquaculture, but it did not reduce RLU counts effectively[14] even though no aerobic bacterial growth was observed. The elevated RLU indicate the persistence of organic material, which could serve as a niche in which bacteria could persist. Alternatively, if sufficient quantities were present, this residual organic material might neutralize the disinfectant. The manufacturer recommends reconstituting the solution every 14 d. We do not recommend using chlorine dioxide for disinfecting zebrafish nets, given that it has the same disadvantages as sodium hypochlorite but without the same efficacy.[4,5,14]

Potassium peroxymonosulfate+sodium chloride (Virkon Aquatic) was highly effective at reducing RLU at both 5 and 30 min. It also eliminated aerobic bacterial growth. This chemical disinfectant was the only one to reduce RLU by 90% at 5 min. Potassium peroxymonosulfate+sodium chloride is EPA-registered in all states except California and is considered environmentally friendly.[8-11,14] Unlike other disinfectants, this compound is nontoxic to zebrafish and humans.[8-11,14] The liquid solution is not corrosive to equipment.[8-11,14] Although reported to be effective against a wide variety of pathogens including fungi and viruses, potassium peroxymonosulfate+sodium chloride is not effective against *Mycobacterium marinum* at the manufacturer's recommended concentration.[8-11,14] Another disadvantage is that potassium peroxymonosulfate+sodium chloride is more expensive than are other disinfectants.[14] The manufacturer recommends replacing the solution every 7 d to maintain efficacy.

Surprisingly, rinsing and air-drying for 30 min effectively reduced RLU and prevented aerobic bacterial growth. New York City municipal tap water contains chlorine which may explain some of the efficacy observed in the study. Although not as effective when compared with the disinfectants, these results suggest that rinsing and drying may also be important for reducing organic contamination and aerobic bacteria on nets. Rinsing is important for removing organic matter, which serves as a niche in which bacteria can grow.[14] Waterborne bacteria are likely highly sensitive to desiccation.

Caution should be used when interpreting our current results, given that this study did not evaluate the effectiveness of the disinfectants against known zebrafish-specific pathogens. Some bacteria, such as *Mycobacterium marinum*, form biofilms, which may reduce disinfectant efficacy.[8] Investigations have been conducted to evaluate the efficacy of disinfectants on infectious agents in vitro.[8-11] However, these studies did not evaluate disinfectant effectiveness in the presence of biofilm. In the event of an infectious disease outbreak, contact times may need to be extended or cleaning prior to disinfection may need to be more vigorous.

We used a different ATP-based monitoring system than that used in other studies.[5,12,13] Therefore the quantity of RLU we measured may not correlate directly with published results. Each institution should establish its own standards when evaluating disinfection protocols.[13] This goal can be accomplished by conducting validation tests to compare soiled and disinfected surfaces in various facility areas or on equipment pre- and postprocedure.[12]

The incubation temperature we used here for aerobic bacterial culture was higher than that recommended for culturing aquatic bacterial pathogens, which are best cultured at 25 °C.[2] Furthermore because many aquatic pathogens grow slowly, aerobic aquatic cultures should be held and evaluated after 5 d or more.[2] This is especially true for aquatic *Mycobacterium* species, which require more than 48 h to grow. Although we incubated the cultures at 37 °C and evaluated them at 48 h, there is no reason to suspect that the disinfectants' efficacy in killing bacterial species growing at a higher temperature would be markedly different than that for those growing at a lower temperature and more slowly.

The findings of this study suggest that disinfectant contact times that are shorter than was previously reported may be sufficient for sanitizing nets used for zebrafish. A municipal tap water rinse followed by soaking in sodium hypochlorite (Ultra Clorox) for 30 min or in potassium peroxymonosulfate+sodium chloride (Virkon Aquatic) for 5 or 30 min, followed by 10 min in a water conditioner and detoxifier and 30 min of drying time, were all effective. These results likely will assist facility managers, veterinarians, and investigators in selecting a suitable net cleaning and disinfection protocol.

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