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The Effects of Water Volume and Bacterial Concentration on the Water Filtration Assay Used in Zebrafish Health Surveillance

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The number of zebrafish in biomedical research has increased exponentially over the past decades, leading to pressure on the laboratory animal community to develop and refine techniques to monitor zebrafish health so that suitable stocks can be maintained for research. The water filtration assay is a promising technique in which water from a zebrafish system is filtered, and the filter analyzed by PCR. In the present report, we studied how the volume of water tested and the concentration of bacterial pathogens affected test results. To do so, we used stock solutions of 3 zebrafish pathogens: *Edwardsiella ictaluri*, *Aeromonas hydrophila*, and *Mycobacterium marinum*. We used these stocks to create solutions with known concentrations of each pathogen, ranging between 10^2 and 10^7 Colony Forming Units (CFU) per ml. One, 2, and 3 L of each solution was filtered using positive pressure, and the filters were submitted to a commercial lab for PCR testing. Results were fit with a logistic regression model, and the probability of obtaining a positive result were calculated. Test sensitivity varied by organism, but in general, test results were positively correlated with the volume of the water filtered and with the concentration of bacteria in solution. We conclude that a positive result can be expected for *E. ictaluri* at 10^5 CFU per mL, *A. hydrophila* at 10^6 CFU per ml, and *M. marinum* at 10^6 CFU per mL, when 3 L of solution are filtered.

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A major goal of laboratory animal medicine is to safeguard the health of research colonies and research staff. Ideally, every animal used in research would be free of all organisms with the potential to confound research by inducing physiologic changes or causing zoonotic disease. To achieve this goal, the health status of animals should be defined and reported. As the use of zebrafish (*Danio rerio*) in research has grown, the environmental testing and technology necessary to maintain laboratory animals free from pathogenic microbial contamination has also expanded.[1,3,4,9,12–14] A novel method of testing a zebrafish colony for the presence of certain pathogens used a PCR filter assay in which water was filtered from a zebrafish system and the filters then submitted for PCR analysis.[4,19] The study demonstrated that various microbes in zebrafish system water could be identified using this method.

The present report builds on the previous study by conducting experiments designed to show whether the water filtration assay was affected by the volume of water filtered and the concentration of bacteria (*Edwardsiella ictaluri*, *Aeromonas hydrophila*, or *Mycobacterium marinum*) in the water. We selected these 3 organisms based on several factors: 1) each can cause disease in zebrafish,[5–8,17,21] 2) *A. hydrophila* and *M. marinum* have zoonotic potential,[5,7,8] 3) the necessary cultures and supplies are commercially available, and 4) expertise and equipment is available to culture the organisms. Finally, we wanted to determine if we could detect *A. hydrophila* by PCR analysis due to an outbreak of this organism in one of our colonies that routinely tested negative by other methods.

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Materials and Methods

Bacteria and Media. All bacteria were purchased from ATCC (Manassas, VA), aliquoted, and stored at –80 °C until ready to use. Trinutrient media or agar was used to grow stocks of *E. ictaluri* (ATCC 33202) and *A. hydrophila* (ATCC 7965). Middlebrook media or agar was used to grow stocks of *M. marinum* (ATCC BAA-927). Powdered samples received by ATCC were resuspended in liquid media and then plated on the appropriate agar for experiments. Six frozen *stocks* were made from the liquid culture and stored in –80C. For experiments, liquid cultures were scaled up by first plating bacteria from frozen stock onto an agar plate. A single colony was then picked and placed in 5mL of respective liquid media and scaled up to desired number of liters for filtration.

Trinutrient media was prepared inhouse using 3.0 g beef extract (Sigma Aldrich SKU B4888), 5.0 g peptone (Sigma Aldrich SKU P5905), and 1000 mL RO water. The ingredients were mixed until dissolved, and the pH was adjusted to 6.8 ± 0.2. The media was then autoclaved at 121 °C for 45 min before use and stored at room temperature. Trinutrient agar plates were produced inhouse by adding 15.0 g agar (Sigma Aldrich SKU A1296) to the above trinutrient media and autoclaving as described above. The plates were stored at 4 °C until use.

Middlebrook media was prepared inhouse using 4.7 g Middlebrook 7H9 broth base (Sigma Aldrich SKU M0178), 2.0 mL glycerol (Sigma Aldrich SKU G7893), and 900 mL DI water. The ingredients were mixed until dissolved and autoclaved

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at 121 °C for 45 min. Before use, Middlebrook media requires enrichment with a sterile filtered ADC solution after the media has been autoclaved and slightly cooled. ADC was prepared inhouse using 5.0 g bovine serum albumin fraction V (Roche SKU 10735078001), 2.0 g dextrose (Sigma Aldrich SKU D9434), 3 mg catalase (Sigma Aldrich SKU C1345), and 100 mL DI water. These ingredients were mixed until dissolved and then filter sterilized using a 0.2 µm PES membrane (Fisher, SKU 09-741-03). 100 ml of ADC enrichment solution was added aseptically to the Middlebrook media before use and stored at 4 °C. ADC-enriched Middlebrook agar plates were produced inhouse by adding 15.0 g agar (Sigma Aldrich SKU A1296) to the above media with ADC enrichment. The plates were stored at 4 °C until use.

Determining defined colony forming units of bacteria. Defined concentrations of bacteria were produced by first determining the number of colony-forming units (CFU) per mL of a liquid bacterial culture that was diluted to a standardized optical density (OD) when measured at a wavelength of 600 nm (OD_{600}) (Thermo Scientific Genesys 20). To do this, liquid cultures were grown in 5 mL culture of media overnight (for *E. ictaluri* and *A. hydrophila*) or for 7 d (for *M. marinum*), in 15 mL culture tubes shaken at 225 rpm at 30 °C. The OD_{600} of the liquid culture was measured and normalized with the appropriate media to achieve an OD_{600} of 0.1. This normalized OD_{600} was considered to be the 10^{-1} sample. This sample was then used to create further dilutions (10^{-2} to 10^{-10}). Each of these dilutions (100 µL) were then plated in triplicate and spread evenly onto agar and incubated at 30 °C. The number of CFU were counted after approximately 24 h (for *E. ictaluri* and *A. hydrophila*) or 168 h (for *M. marinum*) of incubation. Plates containing over 600 CFU were too crowded for a reliable CFU count and were excluded from consideration. Results from the triplicates of the remainder CFU dilutions were averaged to determine the average CFU/mL corresponding to the 0.1 OD_{600} dilution (defined as 10^{-1}).

Filtration. Liquid cultures of bacteria were grown in a 5 mL culture of media overnight (for *E. ictaluri* and *A. hydrophila*) or 7 d (for *M. marinum*), in 15 mL culture tubes shaken at 225 rpm at 30 °C. The OD_{600} of each liquid culture was determined, and samples were then diluted with appropriate media to reach an OD_{600} of 0.1. Based on the previously acquired CFU data, serial dilutions were made by taking a calculated aliquot of each bacterial culture and adding it to reverse osmosis (RO) water to create the desired CFU/mL (10^2 to 10^7). Samples with known concentrations of one of the test bacteria were prepared in volumes of 1, 2, and 3 L, each in triplicate. Samples were filtered immediately after the bacteria were added to the RO water. These steps were repeated 3 times for each type of bacteria at each concentration and at each volume (see Figures 1, 2, and 3). For example, for *E. ictaluri* at 10^5 CFU/mL, a total of 9 samples were prepared: 3 each at total volumes of 1, 2, and 3 L. A new filter was used for each sample.

Samples were filtered using vacuum through a 0.2 µm polyethersulfone (PES) membrane (Fisher, SKU 09-741-03). The filtration device was attached to a 1L receptacle, requiring that it be emptied once or twice when filtering larger volumes of water. After all water for a given sample had been filtered, the filter was removed from the filtration device, placed in a conical tube, and shipped to IDEXX Biolanalytics (Columbia, MO) for PCR testing for *E. ictaluri* or *M. marinum*, or to Charles River (Wilmington, MA.) for *A. hydrophila*.

Statistics. The data were fitted with a logistic regression model. The logistic regression model provides a predictive analysis for dichotomous (binary) data. It is used to explain the relationship between one dependent binary variable (in this case positive or negative PCR results) and one or more independent variables (in this case, volume of sample and concentration of bacteria).

The model can be described as:

$$\text{Logit}(E(Y)) = b0 + b1\,X1 + b2\,\log(X2 + 1) \quad - \text{ EQUATION ONE}$$

where logit function is defined as: logit(x) = log(x/(1−x)), Y is binary variable, that is Y = 1 if the test result is positive, Y = 0 if it's negative, and X1 is the volume of water (L), X2 is the concentration of the bacteria (CFU/mL). We used the logarithm of X2 since the range of the concentration of the bacteria is large compared with the volume of water. The additional constant term inside the logarithm is to avoid infinity at X2 = 0, that is, the negative control.

We also performed modelling based on the Akaike information criterion (AIC). In particular, we fitted the model with the interaction between X1 and X2

$$\text{logit}(E(Y)) = b0 + b1\,X1 + b2\,\log(X2 + 1) + b3\,X1 * \log(X2 + 1) \quad - \text{ EQUATION TWO}$$

and computed the AIC of model EQUATION ONE and EQUATION TWO. The model with smaller AIC was selected.

Finally, we also assessed 3 different models for *E. ictaluri*, *M. marinum* and *A. hydrophila* separately. The dataset includes the results obtained from 57 filters tested for *E. ictaluri*, 48 with *M. marinum* and 48 for *A. hydrophila*. The model was fitted and selected using the glmnet package (version 3.0 to 2) in R (version 3.6.2).

Edwardseilla ictaluri (CFU/mL)	Negative Control	10^2	10^3	10^4	10^5	10^6	10^7
1L – Test 1	-	-	-	-	+	+	+
1L – Test 2		-	-	-	-	+	+
1L – Test 3		-	-	+	+	+	+
2L – Test 1	-	-	-	+	+	+	+
2L – Test 2		-	-	-	+	+	+
2L – Test 3		-	-	+	+	+	+
3L – Test 1	-	-	-	+	+	+	+
3L – Test 2		-	-	-	+	+	+
3L – Test 3		-	+	+	+	+	+

- Negative   + Positive

Figure 1. Results obtained from submitting filters for PCR testing with known concentrations of *E. ictaluri* in 1L, 2L, and 3L samples.

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A.hydrophila (CFU/mL)	Negative Control	10^4	10^5	10^6	10^7	10^8
1L – Test 1	-	-	-	-	+	+
1L – Test 2		-	-	+	+	+
1L – Test 3		+	-	-	+	+
2L – Test 1	-	-	+	+	+	+
2L – Test 2		-	-	+	+	+
2L – Test 3		-	-	-	+	+
3L – Test 1	-	-	+	+	+	+
3L – Test 2		+	-	+	+	+
3L – Test 3		-	-	+	+	+

- Negative + Positive

Figure 2. Results obtained from submitting filters for PCR testing with known concentrations of *A. hydrophila* in 1L, 2L, and 3L samples.

Mycobacterium marinum	Negative Control	10^4	10^5	10^6	10^7	10^8
1L – Test 1	-	-	-	-	+	+
1L – Test 2		-	-	+	+	+
1L – Test 3		-	-	-	+	+
2L – Test 1	-	-	+	-	+	+
2L – Test 2		-	-	+	+	+
2L – Test 3		-	-	-	+	+
3L – Test 1	-	-	+	+	+	+
3L – Test 2		-	-	+	+	+
3L – Test 3		-	-	+	+	+

- Negative + Positive

Figure 3. Results obtained from submitting filters for PCR testing with known concentrations of *M. marinum* in 1L, 2L, and 3L samples.

Results

Cultures were diluted to obtain 1L, 2L, and 3L samples of bacteria. All sample concentrations were run in triplicate for each volume ($n = 3$ at 1L, $n = 3$ at 2L, and $n = 3$ at 3L) except for the negative controls. The negative controls (100 µL media in RO water with no bacteria) were tested once at each volume (1L, 2L, and 3L). Samples were subjected to vacuum filtration and the filters sent to a commercial diagnostic lab for PCR analysis (see Methods). Results from the diagnostic lab were reported as either positive or negative.

For *E. ictaluri*, 1, 2, and 3 L samples were tested between 10^2 CFU/mL and 10^7 CFU/mL. Almost all samples at 10^5 CFU/mL and higher resulted in positive PCR results, while lower concentrations were not reliably detected. These results are presented in Figure 1.

For *A. hydrophila* samples were run between 10^4 CFU/mL and 10^8 CFU/mL. Samples with 10^7 CFU/mL concentrations and higher and 3L samples at 10^6 CFU/mL returned positive PCR test results. These results are presented in Figure 2.

Cultures of *M. marinum* were tested at concentrations between 10^4 CFU/mL and 10^8 CFU/mL. All samples at 10^7 CFU/mL and higher and 3L samples at 10^6 CFU/mL returned positive PCR test results. These results are presented in Figure 3.

To test whether the volume of water or the concentration of bacteria predictably increased the probability of obtaining positive PCR results, the data obtained were fitted to a logistic regression model.

To illustrate the fitted model, we let

$$\eta = b0 + b1\ X1 + b2 \log (X2 + 1),$$

so that

$$P(Y = 1) = \exp(\eta) / (1 + \exp(\eta)).$$

Figure 4 shows the fitted model using the outcome of *E. ictaluri* with η (x-axis) plotted against the probability of a positive result (y-axis).

The fitted coefficients are b_–13.4189 intercept, 1.3582 volume, and 1.1786 for *E. ictaluri* concentration, b_–7.3394 intercept, 0.2298 volume, and 0.5609 for *A. hydrophila* concentration, and b_ –20.0398 intercept, 1.4931 volume, and 1.2948 *M. marinum* concentration. Further, our results show that the volume of water and the concentration of bacteria are positively associated with a positive outcome and that this association is statistically significant for changes in the concentration of bacteria (P value less than 0.01) and changes in the volume of system water tested (P value less than 0.05). Specifically, a one-unit increase in the concentration of bacteria increased the likelihood of a positive result by 3.25 times for *E. ictaluri*, 1.72 times for *A. hydrophila*, and 3.65 times for *M. marinum*.

Using the fitted coefficients described above, we calculated the probability of obtaining a positive PCR ($P(Y = 1)$) with escalating concentrations of *E. ictaluri* (Figure 1), *A. hydrophila* (Figure 2), or *M. marinum* (Figure 3) if testing 1 L, 2 L, or 3 L of water. This represents the probability of a positive result if the experiment is repeated independently with the same . The y axis represents the probability that a single experiment will be positive and is not related to the number of experiments.

Simple line graphs for *E. ictaluri* show the probability of obtaining a positive PCR test result as a function of the CFU/mL for 1, 2, and 3 L of water (Figure 5). The sensitivity of the test increases with both increasing volumes of water tested (shift of the graph to the left) and increasing concentration of CFU (slope of the curve). The difference in the probability of obtaining a positive PCR test result as a function of the volume of water

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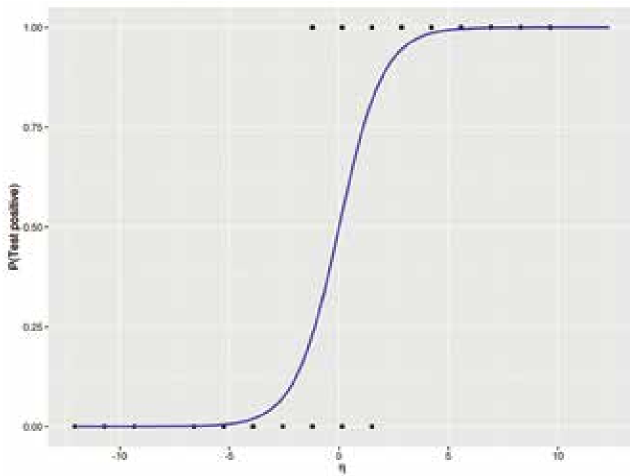

Figure 4. The fitted model using the outcome of *E. ictaluri* with η (x-axis) plotted against the probability of a positive result (y-axis).

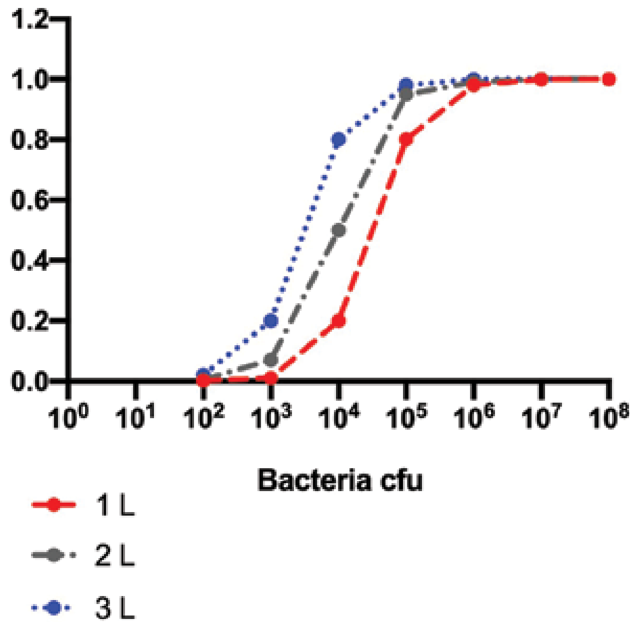

Figure 5. Simple line graphs for *E. ictaluri* (Figure 2), visually demonstrate the probability of obtaining a positive PCR test result as a function of the CFU/mL for 1L of water (red line), 2L of water (gray line), and 3 L of water (blue line).

filtered was most striking at the 10^4 CFU/mL concentration of *E. ictaluri*. At 10^4 CFU/mL, the probability of detecting the organism was 80% when 3 L of solution were filtered, compared with only 20% when 1 L of solution was filtered. However, as the concentration of the organism increased to 10^6 CFU/mL, the probability of obtaining a positive PCR test result after filtering 1 L of solution (98%) was close to the probability obtained after filtering 3 L of solution (99%).

Simple line graphs for *A. hydrophila* show the probability of obtaining a positive PCR test result as a function of the CFU/mL for 1, 2, and 3 L of water (Figure 6). The calculated statistical probability of obtaining a positive result with increases in the volume of water tested (shift of the graph to the left) and the concentration of CFU (slope of the curve). However, for *A. hydrophila*, increasing the volume of water filtered resulted in a less marked changed in the calculated statistical probability

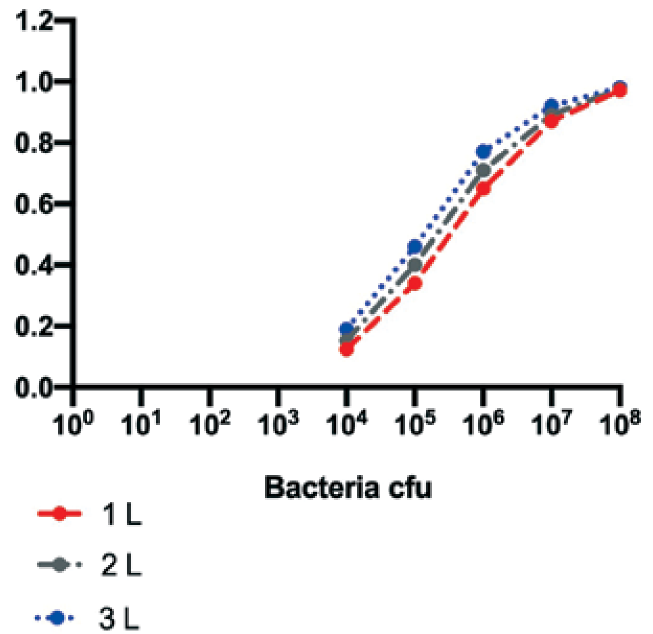

Figure 6. Simple line graphs for *A. hydrophila* (Figure 3) visually demonstrate the probability of obtaining a positive PCR test result as a function of the CFU/mL for 1L of water (red line), 2L of water (gray line), and 3 L of water (blue line).

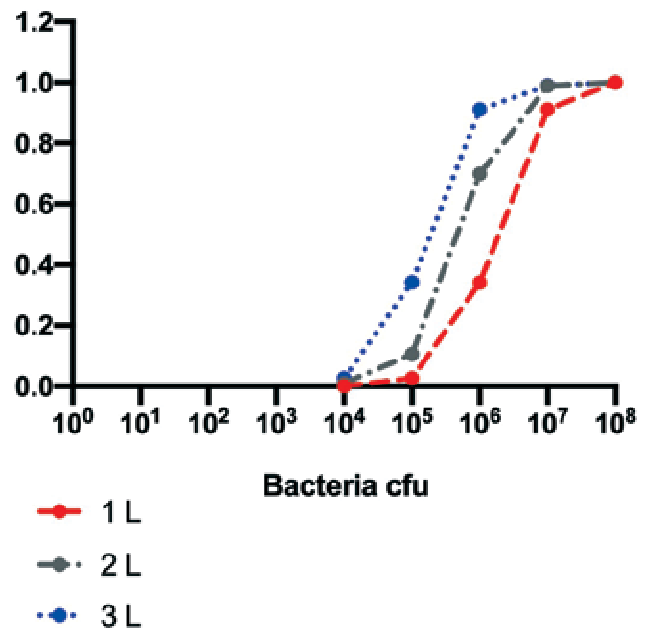

Figure 7. Simple line graphs for *M. marinum* (Figure 4) visually demonstrate the probability of obtaining a positive PCR test result as a function of the CFU/mL for 1L of solution (red line), 2L of water (gray line), and 3 L of water (blue line).

of obtaining a positive PCR test as for *E. ictaluri*. For example, at a concentration of 10^6 CFU/mL, the statistical probability of obtaining a positive test is 65% at when 1 L of water was filtered and 75% when 3 L of water was tested. At a concentration of 10^7 CFU/mL, the statistical probability of obtaining a positive test when 1 L of solution was filtered was 87% and 91% when 3L of solution was filtered.

Simple line graphs for *M. marinum* show the probability of obtaining a positive PCR test result as a function of the CFU/mL for 1, 2, and 3 L of water (Figure 7). The probability of ob-

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taining a positive result both with increases in volume of water tested (shift of the graph to the left) and with the concentration of CFU (slope of the curve). With *M. marinum*, increasing the volume of solution filtered for testing from 1L to 3L resulted in a change from 2% probability of detection to 38% at 10^5 CFU/mL, respectively, and a change from 34% to 99%, respectively, at 10^6 CFU/mL. However, as the concentration of the organism increased to 10^7 CFU/mL, the probability of obtaining a positive PCR test result after filtering 1 L of solution (91%) was close to the probability obtained after filtering 3 L of solution (95%).

Discussion

Animal health status is a crucial factor in conducting reproducible research. Both opportunistic and primary pathogens can alter animal behavior, modulate physiologic processes, and increase morbidity and mortality.[3,9,10] The use of zebrafish as a model organism has risen dramatically in the past decade, leading to an increased demand for data-based information on how to maintain healthy colonies. Investigators have used a variety of methods, such as testing fish in the colony, maintaining sentinel fish in the sump, testing swabs or sludge from the sump,[1,3,13] or testing system water containing detritis.[11]

The experiments described in the present manuscript were designed to build on a previously published report demonstrating that system water could be filtered and the filtrate tested by PCR for microorganisms,[4] much as filtered exhaust air samples from individually ventilated rodent caging systems are tested by PCR. We tested whether increasing the volume of water filtered or the concentration of organism present altered the probability of obtaining a positive result as calculated by fitting the data to logistic regression model. In this study, the probability of detecting 2 of the 3 test organisms was affected by the volume of water assessed. For example, filtering 1L of water resulted in the reliable detection of *E. ictaluri* at 10^5 CFU/mL and of *M. marinum* at 10^7 CFU/mL. However, filtering 3L of water allowed the reliable detection of *E. ictaluri* at 10^4 CFU/mL and *M. marinum* at 10^6 CFU/mL. In contrast, the probability of obtaining a positive result was less affected by testing larger volumes of water were tested when *A. hydrophila* was the organism of interest.

As in a previous study,[4] we submitted our samples to one of 2 commercial diagnostic laboratories (IDEXX Bioanalytics, Columbia, MO. for *E. ictaluri* and *M. marinum*, and Charles Rivers Laboratories, Wilmington, MA. for *A. hydrophila*) for PCR testing using proprietary tests. Commercial diagnostic laboratories are also commonly used to test for pathogens in other species.

In general, our results support testing relatively large volumes of water (≥3L). This contrasts the previous results,[4] in which 100% of filter-membrane samples (144 total) were positive for *M. chelonae* when as little as 150 mL of system water was tested. The same study also found positive results when testing for *M. fortuitum*.[4] Several possible explanations could underlie this disparity. First, the previous study presents no information on the concentration of the organisms in the water tested.[4] Second, the 2 studies used different organisms. The previous study tested for *Mycobacterium chelonae*, *M. fortuitum*, *M. peregrinum*, *Pseudocapillaria tomentosa,* and *Pseudoloma neurophilia* while we tested for *M. marinum*, *E. ictaluri*, and *A. hydromonas*. Third, when obtaining samples from the aquatic system, the concentration of bacteria at the testing location could be positively affected by turbulence as water flowed over detritus or potentially biofilms. Our system was a clean and completely defined system. A fourth possible explanation is that system water was used in the previous study, while we used known quantities of bacteria suspended in reverse osmosis purified water. System water could contain fragments of DNA from dead microbes. Finally, the filter samples submitted to the testing lab differed in size. In the previous study, the filters were cut in half and only half of each filter was submitted for testing. We detached our filters by cutting along the perimeter of the filtration device and submitted almost the entire filter. This larger physical piece of filter could make the extraction of DNA more difficult.

Known infectious concentrations of *E. ictaluri* range from 10^2 to 107 CFU/mL.[6] *A. hydrophila* is lethal to larvae when present in system water at 10^8 CFU/mL and when delivered via intraperitoneal injection to adult fish at 10^5 CFU/mL.[11,18] Our results raise the question of whether the water filtration assay would detect these organisms before they reached an infectious concentration. However, if a facility uses a water filtration assay for either of these 2 organisms, we recommend filtering a large volume of water (>3L). Mycobacteria are ubiquitous in aquatic environments, and can grow to substantial concentration in sump biofilms; this helps explain why 40% of the clinical cases from zebrafish facilities submitted to the Zebrafish International Resource Center's (ZIRC) diagnostic lab are positive for mycobacteria.[2,14] A previous paper demonstrated disparate results between sentinel testing and testing samples from the dirty sump,[21] reporting that the mature biofilm in the sump routinely yielded a high concentration of mycobacteria. In contrast, the incidence of this pathogen in sentinel fish maintained in the dirty sump was only 1% over a 3-y period.[21]

One group estimated that 259 out of 1000 fish would have to be tested to reliably detect a 1% infection rate in a system.[20] Fewer samples may be necessary if older fish are tested. However, in most colonies, testing the number of animals necessary to detect low-level infections is not feasible. One report supports taking samples of biofilm from the dirty sump as the most sensitive method of detecting mycobacteria.[20] Another report suggests that a filter water assay might be equally useful for detecting mycobacteria.[4] Our results indicate that the filter water assay might be useful only if large volumes of water were filtered. More study will be necessary to understand the sensitivity of this method to various species of pathogens and to determine whether changes in technique, such as optimizing the size of the filter submitted, may improve test results.

In our study, we used the 3 selected bacteria (*E. ictaluri*, *A. hydrophila*, and *M. marinum*) in part because the techniques needed to culture defined concentrations of these agents were readily available. In addition, each of these agents can be a significant pathogen in zebrafish, and 2 of them, *A. hydrophila* and *M. marinum*, have zoonotic potential.

E. ictaluri is considered a primary pathogen of zebrafish. Natural transmission is thought to occur through the shedding of bacteria from carriers or by cannibalism of dead fish. *E. ictaluri* can persist in the environment. Outbreaks of *E. ictaluri* are not common in zebrafish, but when they occur, they cause high morbidity and mortality.[5,9] One study found that zebrafish could show signs of infection (abnormal swimming) as soon as 12 d after exposure to a dose as low as 10^2 CFU/mL.[15] Another study found that bath exposure to 10^7 CFU/mL led to 100% mortality by 10 d after exposure.[6]

A. hydrophila is considered an opportunistic pathogen of zebrafish. These Gram-negative bacteria can infect both fish and humans. *A. hydrophila* is found worldwide, and can be part of the normal intestinal microflora of healthy fish. One group found that otherwise healthy larval zebrafish exposed to 10^8 CFU of *A. hydrophila* began to die beginning at 12 h after exposure, culminating in 33% mortality at 96 h after exposure.[16] *A. hydrophila* has been reported in swim bladder infections and hemorrhagic

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septicemia in zebrafish.[7,17] These infectious outbreaks may have been facilitated by overcrowding or other stressful conditions.[7,17] *A. hydrophila* is also considered zoonotic. Exposed humans can develop a range of illnesses, including gastroenteritis, endocarditis, osteomyelitis, and localized skin infections.[7]

Mycobacterium spp. are exceedingly common and mycobacteriosis is the second most common infectious disease in zebrafish colonies.[3,9,14] Mycobacteria are generally considered to be ubiquitous in aquatic systems. The organism readily survives in biofilms, is present in live feed, and has spores that persist in the environment. Multiple species of *Mycobacterium* can infect zebrafish, of which *M. chelonae*, *M. fortuitum*, and *M. marinum* are historically considered the most common.[3,9] Infection is characterized by granulomas in various tissues, including the kidney and in the coelomic cavity. *M. marinum* is of particular concern in the lab animal environment because of its zoonotic potential.[3,5,9,10] In humans, *M. marinum* is a nodular granulomatous disease that can spread along lymphatics. It usually presents as a single or limited number of lesions, but may progress to disseminated lesions in the skin, the lung, and viscera.[5,10]

Our study has some limitations. First, the need to empty the receptacle repeatedly increases the potential for operator error and contamination of the system. Additional studies should be explored to determine if filtration of larger volumes of water would to improve the sensitivity of PCR filter assays. However, filters with a larger total diameter than that used in this study (90 mm) should not be used without first consulting the diagnostic lab because the size of the filter submitted must be compatible with the technique the diagnostic lab uses to isolate DNA. Second, our study used solutions of clean RO water containing a known concentration of a pathogenic organism. We did not test system water. Future studies could quantify levels of microbes present when the PCR filter test yields positive results, but that goal was beyond the scope of our study.

In conclusion, PCR testing of zebrafish system water after vacuum filtration may augment colony health monitoring, but the sensitivity may be much lower than that reported for testing swabs of biofilm from the dirty sump. Our results suggest that if the water filtration assay is used, relatively large volumes of water (3L) should be filtered before the filter is sent for PCR testing. Finally, because of a disparity in our results compared with those of others,[4] additional work will be necessary to define technical standards that will achieve more uniform results.

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