Evaluation of environmental samples as a sampling method for detecting pathogens in zebrafish

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Abstract

Zebrafish are becoming increasingly popular to use in different kinds of research projects as research animals, replacing rodents in many fields. When using animals for research, it is important to keep track of the animal health in order to get reliable results. The purpose of the study was to investigate whether these pathogens could be found analyzing environmental materials with real-time PCR instead of euthanizing fish and submitting them for histopathology. Also, to see if any material differentiated from the rest regarding accessibility to work with in a routine diagnostic laboratory.

This study was performed on environmental samples, such as filters, swabs, detritus and water, from a recirculating water system holding zebrafish. The pathogens analyzed were Mycobacterium chelonae, M. haemophilum, M. abscessus, M. marinum, M. fortuitum and Pseudoloma neurophilia, all common pathogens that can affect zebrafish.

All materials tested gave at least one positive result for most of the pathogens tested. Two pathogens were not detected, M. marinum and M. abscessus. Due to poorly working PCR-system for M. fortuitum, the results for that bacteria were deemed inconclusive. The filter materials and the swabs of the filter materials gave the best results in this small study, although all materials gave satisfactory results.

In conclusion this study shows that environmental samples can be used to detect pathogens in zebrafish, but larger studies should be performed to better evaluate which material is the best one to use.

Keywords

e-DNA; Mycobacterium haemophilum; Mycobacterium spp; Pseudoloma neurophilia; Danio reiro
Introduction

Zebrafish (*Danio rerio*) are becoming increasingly common as experimental animals in various types of studies (Whipps et al., 2012), ranging from cancer (Manni et al., 2019) to neurological studies (Ijaz & Hoffman, 2016) as they have similar cellular and neurological functions as humans.

The fish are often kept in facilities with recirculating water systems, that is, most of the water is filtered and purified in different steps and then circulated back into the aquariums. The filtration of water varies between different systems. In the research facility were samples were gathered for this study, the water was first filtered with a coarse filter pad to sort out fish and larger debris, to be followed by a biological filter, a fine filter, a carbon filter and finally a UV filter.

There are many advantages in keeping fish in recirculating systems; it is primarily easier to keep water parameters, such as nitrate, pH, temperature and ammonium at a good level with large amounts of water. The disadvantage of a recirculating water system is if pathogens enter the system, there is a risk that many fish will be infected. If fish with different immune systems are kept in the same system, infected fish with better immune defense and do not show any symptoms, can be brought into the system and infect the weaker fish (Murray et al., 2011).

Some of the more common pathogens in zebrafish in research facilities are *Mycobacterium* spp. and *Pseudoloma neurophilia* (Kent et al., 2012). Common symptoms in fish suffering from mycobacteriosis are skin lesions, raised scales and erratic or lethargic behavior (Whipps et al., 2012). However, the majority of mycobacteria infections in zebrafish show subclinical symptoms and are therefore difficult to detect. For fish that have skin lesions, bacteria may shed and spread to the rest of the fish in the tank. Mycobacteria have the ability to multiply.
both inside and outside the host and are often found in biofilm on aquarium materials such as filters, making them ideal for use as environmental samples.

Many common pathogens that can affect zebrafish produce subclinical symptoms, which can lead to research being conducted on sick or subclinically infected fish. This can partly be harmful for the animals but can also give uncertain results as it is not possible to determine whether observations are due to the fish being sick or immunocompromised or to the research done (Collymore et al., 2016).

In order to ensure that reliable results are reported in research studies, it is important that facilities that house zebrafish perform regular health check-ups. One common way to control the health of a system is by using sentinel fish, which make it possible to investigate health in a system without sacrificing valuable research fish. The fish used as sentinel fish may be old breeding fish, or fish with specific genetic sets, and are usually subjected to the recirculating water for a fixed period of time before examination. The time between examination varies between facilities and their individual health protocols, but three months is a common time span for regular health check-ups. In cases of suspected infections, these intervals might be shorter. During health check-ups, several sentinel fish are euthanized for examination by use of histopathology and PCR for the presence of bacteria and parasites. However, these methods have been shown to have a draw-back, as pathogens may occur in the system even if it is not found in the sentinel fish (Murray et al., 2016).

The sentinels can be placed in strategic places such as before or after filtration to make sure they are exposed to as many pathogens present in the system as possible. In the system analyzed in this study, the sentinel fish were zebrafish held in the same conditions as the other zebrafish in the system, a 5 L tank with approximately 5 fish/L. The tanks were placed randomly in the system, that is, neither before or after filtration in any specific order.
The purpose of this study was to investigate whether environmental samples could be used to detect common pathogens in zebrafish. Samples were taken from system filters, water samples, detritus and swabs of both the system filters but also the aquarium walls and the filter holders. The pathogens examined were *Mycobacterium chelonae*, *M. marinum*, *M. abscessus*, *M. haemophilum*, *M. fortuitum* and *P. neurophilia*. As PCR is routinely used in diagnostics, including at the National Veterinary Institute (Uppsala, Sweden), were the study was carried out, this method was chosen to analyze the samples. The PCR-system detecting *P. neurophilia* was from a previously developed local protocol at the National Veterinary Institute, while the remaining PCR systems came from previously published systems (Meritet et al., 2017; Rocchetti et al., 2017; Sanders & Kent, 2011). The sequences used in the study can be seen in Chart 1 below.

The study was conducted on environmental material from five systems at Science for Life Laboratory (SciLifeLab, Evolutionary Biology Center, Uppsala University, Uppsala, Sweden), where the regular health monitoring consisted of daily check-ups of the tanks during feeding to detect sick or dead fish, and a more thorough check-up once a week outside the feeding schedule. In this specific facility, *M. chelonae*, *M. fortuitum* and *P. neurophilia* had been previously detected in fish by using histopathology and PCR, which was the reason for including them in this study.

Of the bacteria included in this study, *Mycobacterium haemophilum* causes most problems in fish facilities, partly because of increased mortality (Whipps et al., 2012). *Pseudoloma neurophilia* is a parasite that primarily affects the nervous system in the fish, which is why the presence of this parasite in zebrafish in neurological studies may give misleading results. The main symptoms caused by this parasite, aside from the impact on the nervous system, are emaciation, spinal deformity and morbidity (Ramsay et al., 2010). *M. marinum* causes zoonotic infections and, if present in a facility, might infect the staff handling the fish causing
painful skin lesions and even deep infections in some cases (Aubrey et al., 2002). It is also known to cause high mortality in zebrafish facilities. *M. cheloneae, M. fortuitum* and *M. abscessus* are associated with low mortality and chronic disease in zebrafish (Watral & Kent, 2007; Whipps et al., 2012).

For zebrafish to continue to be used in research and provide reliable results, there must be ways to guarantee their health. This without the need for valuable research fish to be euthanized, and in a way that can guarantee reliable results with as few false negative results as possible. With current methods; histopathology of fixed tissues and PCR of euthanized fish, there is a risk that pathogens go undetected. This can lead to unknowingly using sick fish in research projects and the publishing of uncertain results.

This study aims to investigate whether environmental samples from zebrafish tanks and environment can contribute to more reliable results on a population's health, but also to evaluate different forms of sampling methods depending on accessibility and ease to examine.
Materials and Methods

Study materials

For the collection of environmental samples from the zebrafish tanks, various amounts of sampling materials were used; cotton and nylon swabs, 2 mL detritus, 150 mL water from each sentinel tank, 150 mL outlet water and 50-micron filters (Pentair). Filter pieces of 5x5 mm were cut out from each filter and analyzed individually or in groups of 5 pieces from the same filter in the same tube. The tank samples were collected from sentinel tanks from five different systems with recirculating water. The filters and the outlet water samples were collected from each of the five systems. One fish that had been euthanized due to illness from one of the sentinel tanks was also analyzed using the same methods.

Ethics considerations

The purpose of the study was to examine environmental samples from zebrafish tanks, not the zebrafish themselves. However, the opportunity was given to examine one zebrafish that had been euthanized by the animal husbandry staff at the facility due to illness, but no fish were euthanized for the sake of the trial. When fish aren’t killed for the sake of the trial, no ethical condition is required according to the Swedish Board of Agriculture [SJVFS 2017: 40].

DNA extraction

Sampling materials were put in 2 mL Eppendorf tubes (Eppendorf), each containing four 2 mm steel beads. Then 500 µL G2 buffer (provided with the DNA extraction kit from QiaGen) was immediately added and the samples homogenized with bead beating 2 x [6,5 m/s x 1 min] using FastPrep-24 (MPBio). To remove the bubbles formed after homogenization the samples were centrifuged at 6000 g for 5 min, 25 µL proteinase K (QiaGen) was added and the samples were frozen at -70°C for 10 min and thereafter put in a 56°C thermomixer for 30 min
at 600 rpm. The samples were then centrifuged at 13 000 g for 5 min and 200 µL of the supernatant was transferred into a new tube. The DNA extraction was made with an EZ1 DNA Tissue Kit (Qiagen) in a EZ1 Advanced XL machine (Qiagen), 100 µL was extracted. The water samples collected were individually filtered through a fine filter which was then submitted to the same method as the tank filters. The 2 mL of collected detritus samples were centrifuged at 6 000 g for 5 min to make a detritus pellet, from where DNA was extracted in the same way as described above.

A comparison was made between using one piece of filter material versus using five pieces of filter material in the tubes for extraction. The tubes with cotton swabs were put on a shaker (IKA VIBRAX VXR basic) at 1500 rpm for 10 min due to them being more fragile than the nylon swabs and broke when homogenized with the bead beating method. They were then boiled at 98-100°C for 10 min, centrifuged at 6 000 g for 5 minutes then Proteinase K was added. Thereafter, the standard method was followed.

One fish was mashed before being put in a tube with the steel beads. A comparison between the effects of DNA-extraction with 2 mm steel beads and 2 mm steel beads together with 0,5 mm zirconia beads (Biospec) was performed to evaluate whether the combination of the beads resulted in a better DNA-extraction.

PCR

Five different kinds of primer/probe mixes were made, one each for *M. marinum* (Meritet et al., 2017), *M. chelonae/M. abscessus* (MC/MAG) (Rocchetti et al., 2017), *M. haemophilum* (Meritet et al., 2017), *M. fortuitum* (Rocchetti et al., 2017) and *P. neurophilia* (Sanders & Kent, 2011) (see sequences in the chart below). These mixes contained TE-buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA) and the different primers and probes. Each mix contained forward and reverse primers and a probe, except for MC/MAG which contained two probes.
Chart 1. Nucleotide sequences for the primers and probes used in the study.

<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Nucleotide sequence</th>
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<tbody>
<tr>
<td><em>M. marinum</em> Forward</td>
<td>5’ CAA CCC GCT CGG TCT GAA 3’</td>
</tr>
<tr>
<td><em>M. marinum</em> Reverse</td>
<td>5’ CG ACCT CTT TGG CCG ACT T 3’</td>
</tr>
<tr>
<td><em>M. marinum</em> Probe</td>
<td>5’ TCA CCG AGA CCT TGC 3’</td>
</tr>
<tr>
<td><em>MC/MAG</em> Forward</td>
<td>5’ CAC GGG GTG GAC AGG ATT TA 3’</td>
</tr>
<tr>
<td><em>MC/MAG</em> Reverse</td>
<td>5’ TAA GGA GCA CCA TTT CCC AG 3’</td>
</tr>
<tr>
<td><em>MC</em> Probe</td>
<td>5’ ATT CAC CAA GCG AGT AAC CA 3’</td>
</tr>
<tr>
<td><em>MAG</em> Probe</td>
<td>5’ TCA CCA AGT AGA TAY? CAA CTA CAG A 3’</td>
</tr>
<tr>
<td><em>M. haemophilum</em> Forward</td>
<td>5’ GTT AAG GTG GCG TTG GAA GCT 3’</td>
</tr>
<tr>
<td><em>M. haemophilum</em> Reverse</td>
<td>5’ TCC AGC CCG GAG TTG AAG 3’</td>
</tr>
<tr>
<td><em>M. haemophilum</em> Probe</td>
<td>5’ CGC TGA AGC AGA TCG 3’</td>
</tr>
<tr>
<td><em>M. fortuitum</em> Forward 1</td>
<td>5’ ACC TGA CCT GCA CAA AGT 3’</td>
</tr>
<tr>
<td><em>M. fortuitum</em> Forward 2</td>
<td>5’ TCA CCT GAT CTG CAC ATA ATG T 3’</td>
</tr>
<tr>
<td><em>M. fortuitum</em> Reverse</td>
<td>5’ AGC ACC TCA TGC GAC TT 3’</td>
</tr>
<tr>
<td><em>P. neurophilia</em> Forward</td>
<td>5’ GTA ATC GCG GGC TCA CTA AG 3’</td>
</tr>
<tr>
<td><em>P. neurophilia</em> Reverse</td>
<td>5’ GCT CGC TCA GCC AAA TAA AC 3’</td>
</tr>
<tr>
<td><em>P. neurophilia</em> Probe</td>
<td>5’ – ACA CCG CCC GTC GTT ATC GAA GAC GA 3’</td>
</tr>
</tbody>
</table>

The different master-mixes contained PerfeCTa qPCR ToughMix with Low ROX (VWR Cat# 733-2097), the corresponding 20X primer-probe mix and nuclease-free water (Sigma).

The total reaction volume was 15 µL, 13 µL master mix and 2 µL sample.
The PCR analysis was performed on ABI 7500 Fast (Thermo Fisher) and the temperature profile used was: 95°C for 3 min followed by 45 cycles of 95°C for 3 sec and 60°C for 30 sec.
Results

A wide variety of environmental materials were analyzed during this study for the presence of *Mycobacterium marinum, Mycobacterium chelonae, Mycobacterium abscessus*, *Mycobacterium fortuitum, Mycobacterium haemophilum* and *Pseudoloma neurophilia*, all common pathogens in zebrafish at research facilities. The study was conducted on samples from Science for Life Laboratory (SciLifeLab) at Uppsala University (Uppsala, Sweden) where *M. chelonae, M. fortuitum* and *P. neurophilia* previously had been detected and were a known issue to the facility.

*Mycobacterium marinum and Mycobacterium abscessus*

All samples analyzed for *M. marinum* and *M. abscessus* showed negative results.

*Mycobacterium haemophilum*

All samples tested for *M. haemophilum* showed positive results (see Figure 1) and a few samples were sent to IDEXX Reference Laboratories for confirmation, given the fact the bacteria had not been detected previously in the facility and the severity of an outbreak of *M. haemophilum*. The results from IDEXX showed *M. haemophilum* was not present in the samples, meaning the PCR-system was not specific for *M. haemophilum*. 
Figure 1. A summary of results of the samples tested for *Mycobacterium haemophilum*. Positive results in green. Each category was analyzed once in each system, giving five results per material excluding “Fish”, “Cotton swab tank”, “Nylon swab2 tank” and the beads since only a few systems were sampled for those categories. The numbers 1 and 2 refer to different sampling opportunities, the materials were the same both times.

*Mycobacterium chelonae*

As seen in Figure 2 below, the results of *M. chelonae* came out a mix of positive and negative. Among the materials that did not pick it up as good as the others were; cotton and nylon swabs of the tank walls, the fish and the water samples, both from the outlet and the tanks.
Figure 2. A summary of results of the samples tested for *Mycobacterium chelonae*. Positive results in green and negative results in orange. Each category was analyzed once in each system, giving five results per material excluding “Fish”, “Cotton swab tank”, “Nylon swab2 tank” and the beads since only a few systems were sampled for those categories. The numbers 1 and 2 refer to different sampling opportunities, the materials were the same both times.

*Pseudoloma neurophilia*

Samples tested for *P. neurophilia* varied in outcome, with more negative results than positive, as can be seen in Figure 3 below. The materials with positive results for the parasite were the filters and the swabs from the filters, both cotton and nylon, the detritus, tank water and a nylon swab of the tank wall. One of the systems tested came back negative for the parasite with all the materials tested. Another system only showed two positive samples out of all the samples.
Figure 3. A summary of results of the samples tested for *Pseudoloma neurophilia*. Positive results in green and negative results in orange. Each category was analyzed once in each system, giving five results per material excluding “Fish”, “Cotton swab tank”, “Nylon swab2 tank” and the beads since only a few systems were sampled for those categories. The numbers 1 and 2 refer to different sampling opportunities, the materials were the same both times.

*Mycobacterium fortuitum*

A few samples were analyzed for *M. fortuitum* and all came out positive, as seen in Figure 4 below.
Figure 4. A summary of results of the samples tested for *Mycobacterium fortuitum*. Positive results in green and negative results in orange. Analyses were only made on one set of the different system filters and on the opportunistically collected fish.

**Materials**

The cotton swabs proved to be difficult to work with. During sampling of the tank walls the cotton swabs broke and had to be collected from the bottom of the tanks, while the nylon swabs stayed together. The same problem occurred during the swabs of the filter materials since the filter was coarse, which made the cotton swab break and leave small pieces of cotton on the filter.

The nylon swabs held together during all forms of sampling, both swabs of tank walls and coarse filters. Using the nylon swabs to swab the filters was easier cutting out pieces of the filters itself for analyzing. Swabbing the tank walls with the nylon swabs was easy, simply lifting the tank cap and swabbing the walls, eliminating the step of lifting down the heavy tanks from the systems.
The collection of the water samples required more work than the other materials. In order to collect the outlet water, a heavy part of the filter mechanism had to be moved. The tank water was easier to collect but did also require heavy lifting, the tanks had to be lifted down from the system in order to access 150 mL of water. That amount of water also required a lot of storage space. Another issue with the water samples was the extra filtration stage required.

To collect detritus the tanks had to be lifted off the systems to access the detritus that had accumulated near the outflow in the back of the tank. As similar the collection of the detritus and the tank water samples were, it was easier to store 2 mL of detritus than 150 mL of water.

The filters gave results for most of the pathogens tested, in many cases detecting pathogens were other materials failed. A comparison was made between using four 2 mm steel beads against using four 2 mm steel beads in combination with 0.5 mm zirconia balls. Both combinations gave similar results.
Discussion

Zebrafish are becoming increasingly popular as research animals, which has made the demand for evaluation of their health high. Currently the health of the zebrafish in the big zebrafish housing facilities has been determined by using histopathology on euthanized fish (Murray et al., 2016; Varga & Murray, 2016). Histopathology of euthanized fish has in some cases failed to detect pathogens in systems that were clearly infected. It is also time consuming to euthanize fish for examination with histopathology.

A faster and hopefully more reliable method for health data in fish could be to analyze environmental samples for pathogen-DNA using real-time PCR, which was performed in this study. A suitable method demands reliable results, time effective sampling methods, easy to replicate to be user friendly.

A lot of different materials were collected and examined during this study. As seen in the results, all materials examined gave positive results for at least one pathogen. All materials tested for *M. haemophilum* gave positive results. Since the bacteria had not been reported in the zebrafish facility prior to this study, and since an outbreak of *M. haemophilum* is dealt with by wiping out all the fish and disinfecting the tanks (Francis-Floyd, 2011), some filter samples were sent to IDEXX Reference Laboratories for confirmation, showing negative results.

*M. chelonae* was already present in the systems analyzed prior to this study. The materials that did not detect it as good as the others were cotton swabs of the tank walls, the fish, the nylon swabs of the tank walls and the water samples, both tank and outlet water. *M. fortuitum* was already present in the systems and it was seen as an opportunity to gain data about the PCR-system. This system had previously been tried using control strains at the National Veterinary Institute, with poor results. But the real-time PCR curve had poor quality, as for the control strains, and the results were deemed inconclusive.
P. neurophilia was also a pathogen already present in the fish in this facility. The results from the materials varied more than for the bacteria. Some of the materials had at least a few positive results. These were the filters, the detritus and the swabs from the filters, both nylon and cotton, the tank water and nylon swab of the tank walls. None of the materials picked up the parasite in all of the materials, and at least one of the systems came back all negative for the parasite in this study. P. neurophilia differentiates from the bacteria, that grow on biofilms in the tanks, by only being able to reproduce inside the host (Murray et al., 2011). P. neurophilia can infect ovarian tissue in fish and therefore, eggs could be a potential carriers of the parasite (Kent et al., 2004). The parasite may also infect the gastrointestinal tract, spreading spores through feces into the tank, which could explain the higher presence in the filter materials and detritus rather than on the tank walls. The fish also tested negative for P. neurophilia, which could be due to that the fish contains inhibitors for the PCR-reaction.

Another theory is that the fish that was analyzed was not infected.

In 2017, Crim et al. performed a similar study were different kinds of environmental materials were analyzed for the presence of bacteria and parasites. They had similar results to this study regarding P. neurophilia, which was rarely detected in filtered water and detritus. For M. chelonae they listed their results for the same samples as “reliably detected”, and in this study it was detected in three out of five systems in outlet and tank water and four out of five times in detritus. In this study M. haemophilum was detected in all samples of all systems, and Crim et al. listed it as “reliably detected” in 150 mL water samples as well as detritus.

Since this study was a pilot study, the aim was to investigate a variety of materials to see if common pathogens could be detected. The results of this study showed that mycobacteria could be found in most materials, and that P. neurophilia could also be detected, mainly in filter material. It was when considering accessibility and ease of processing the materials prior
to analysis that the different materials differed most from each other. The cotton swabs required special treatment in order not to break, unlike other materials, which is not optimal for introducing it into routine diagnostics, but it was relatively easy to swab the tank walls and filters with them. The water samples from the tanks and outlets was more difficult to collect, especially the outlet water, and the water collected had to be filtered manually through special filter membranes prior to analyzing. Compared to the amount of water filtered through the system filters daily, the 150 mL collected was a relatively small volume to filter. The nylon filters were easier to used than the rest of the materials analyzed. Compared to the cotton swabs, they did not break during sampling, and gave better results. Comparing the nylon swabs to the filter pieces, the filter pieces had slightly better results. It was easier to collect samples with the nylon swabs rather than cutting out pieces from the system filters, which in a way makes up for the difference in results.

A comparison was made between using four 2 mm steel beads against using four 2 mm steel balls in combination with 0.5 mm zirconia balls. The results of these analyses showed no major difference in most cases, which resulted in only the 2 mm steel beads being used in the tubes for the DNA extraction.

In the future, larger studies are needed to test which of these materials are optimal for detecting as many pathogens as possible and which materials detect pathogens best. Properly health tested zebrafish before and during research projects, will increase the likelihood of research results being due to proper data from the study rather than to undiagnosed disease. In this study, the filters and related samples to them generally showed the best results, but further studies including more samples should be performed to verify the data.
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