The efficacy of a commonly used commercial hatchery disinfectant against epizootic epitheliotropic disease virus (EEDV)

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1. Abstract

Epizootic epitheliotropic disease virus (EEDV) causes substantial losses in hatchery-reared lake trout (*Salvelinus namaycush*) in the Great Lakes Basin. Due in part to the inability to culture EEDV *in vitro*, almost nothing is known about effective means for inactivating the virus using disinfectants that are approved for use in hatcheries/aquaculture facilities. One disinfectant that is widely used in hatcheries is Virkon® Aquatic (active ingredient, potassium peroxymonosulfate; Syndel, Ferndale, Washington), as previous experiments have found it to be efficacious against multiple microbial fish pathogens. Thus, our study aimed to investigate the efficacy of Virkon® Aquatic against EEDV. Positive control lake trout were challenged with EEDV by soaking a net in $1.77x10^5$ virus copies/mL of water and then transferring naïve fish into the EEDV-laden net. The Virkon® Aquatic treatment groups were EEDV challenged analogously, with the exception that prior to fish transfer, the virus laden net was soaked in a 1% Virkon® Aquatic suspension for 20 minutes (i.e, the manufacturer recommended disinfection concentration/duration). Beginning at 26 days post-infection (pi), characteristic signs of EED were observed in positive control fish but were never observed in the Virkon® Aquatic treatment groups throughout the 148 days challenge period. At the end of the study period, 80% mortality occurred in the positive control groups compared to 3.3% in the Virkon® Aquatic treatment group. Similarly, EEDV was detected in 90% of the positive control fish but was never detected in the Virkon® Aquatic treatment group. Results from this study suggest that Virkon® Aquatic is effective at preventing EEDV contagion on hatchery tools at the manufacturer recommended concentration/duration and highlights a promising tool for improving lake trout hatchery biosecurity.

2. Introduction

Lake trout (*Salvelinus namaycush*) are indigenous to the Laurentian Great Lakes and have been the focus of substantial hatchery conservation and rehabilitation efforts to address massive previous population declines (Lawrie and Rahrer, 1972). Like many other hatchery-based fishery conservation efforts, infectious diseases are an impediment to the rearing of lake trout in Great Lakes hatcheries. In particular, epizootic epitheliotropic disease virus (EEDV) has directly killed millions of juvenile lake trout in multiple Great Lakes hatcheries since the 1980's and led to further losses due to subsequent hatchery depopulation efforts to prevent virus perpetuation and spread (Bradley et al., 1989). To date, there are no means for treating EEDV-infected fish, limiting EED prevention and control to avoidance, culling, and/or depopulation. Biosecurity is a primary means of infectious disease prevention and control within hatchery environments, including the use of hatchery disinfectants to not only prevent the introduction of fish pathogens into hatchery systems via contaminated fomites, but also to control their spread within hatcheries themselves (reviewed in Tørgesen and Hårsein, 1995). Virkon® Aquatic (Syndel, Ferndale, Washington) is a potassium peroxymonosulfate (PPMS) based disinfectant that has viricidal and bactericidal effects (Hernández et al., 2000) and is commonly used commercially in aquaculture facilities to disinfect fish husbandry tools (Paetzold and Davidson, 2011). Additionally, Virkon® Aquatic is relatively safe for fish when used according to manufacturer protocols (Stockton-Fiti and Moffitt 2017).

PPMS-based disinfectants have proven to be efficacious at inactivating a range of viruses. For example, an investigation by Rohaim et al. (2015) showed that 1% Virkon® completely inactivated the Egyptian H5N1 avian influenza virus at a 5 to 30 minutes contact time. Another study reported that 1% Virkon® could inactivate the H7N2 strain of avian influenza virus as well (Bieker, 2006). The use of Virkon® Aquatic has also been reported to eliminate viruses by immersing common carp in 3 ppm of the soluble disinfectant for 2 hours before experimental treatment was conducted (Sunarto et al., 2012). In addition, PPMS inactivates some dsDNA enveloped viruses, as demonstrated with Virkon® No Foam against the hepatitis B virus (Scioli et al., 1997). Lastly, PPMS was experimentally studied to investigate its efficacy against a herpesvirus *in vitro*, which revealed that even at low temperatures, Virkon® inactivated equine herpesvirus-1 (EHV-1; Tsujimura et al. 2015).

Nevertheless, in herpesviruses that infect fish, especially EEDV, specific knowledge on the efficacy of Virkon® Aquatic has yet to be determined. Therefore, this study was designed to investigate the efficacy Virkon® Aquatic against EEDV, thereby providing hatchery managers with scientific evidence to hatchery managers with the goal of establishing proper biosecurity measures targeting EEDV.

3. Materials and methods

3.1.Fish maintenance

Juvenile Lake Superior strain lake trout (*Salvelinus namaycush)* were obtained from Marquette State Fish Hatchery (MSFH; Marquette, Michigan) at 4 months of age while still raised on a closed (i.e., deep well) water source and were maintained at the Michigan State University – University Research Containment Facility (URCF) until experimental challenge (at 22 months of age) and for the duration of the study. Prior to EEDV-challenge, fish were housed in a 680-L flow-through fiberglass tank supplied with dechlorinated water (temperature at 14 °C) and fed AquaMax® Fingerling Starter 300 (Purina®, Gray Summit, Missouri) *ad libitum* with

detritus/feces siphoned and removed once per day. A subset of lake trout was examined for the presence of EEDV via qPCR (section 2.6 below) to ensure freedom from infection. All fish handling and maintenance during the study period (148 days) was in accordance with Institutional Animal Care and Use Committee (IACUC) standards.

3.2.Disinfectant solution preparation

One hour prior to the infection challenge, a 1% Virkon® Aquatic solution was prepared following the manufacturer's protocol. In brief, 8.5 grams Virkon® Aquatic was dissolved into 946 mL clean water. Then, 600 mL of this solution was transferred into an 11.4-L glass aquariums. These steps were repeated for each replicate.

3.3.Infection challenges

Because EEDV has yet to be successfully cultured *in vitro*, infectious inoculum was prepared from EEDV-infected lake trout skin collected from a natural EED epizootic as previously described (Shavalier, 2017). The skin was homogenized and trimmed to ~ 1-2 mm in diameter, to which sterile sample diluent (pH 7.525 ± 0.025) containing 458 mL Minimal Essential Medium (MEM; Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts), 7 mL of 1 M tris buffer, 1 mL gentamycin sulfate (Sigma-Aldrich, St. Louis, Missouri), 5 mL penicillin/streptomycin (Invitrogen), and 5 mL Amphotericin B (Thermo Fisher Scientific) was added at a 1:3 (w/v) ratio. Afterwards, the suspension was homogenized (Seward Stomacher 80, Biomaster Lab System) for 120 seconds at high speed, centrifuged at 368 x g (20 minutes; 4 °C), and then frozen at -80 °C for use in infection challenges.

Prior to EEDV challenge, fish were randomly divided into five flow-through 42-L fiberglass cylindrical tanks filled with 18.9 L chilled water and categorized into three treatment groups: one tank for the negative control (NC) group that consisted of 20 fish, three replicate tanks for the Virkon® Aquatic group consisting of 20 fish each, and one tank for the positive control (PC) group that consisted of 20 fish. All fish were acclimatized for 15 days in the water at a temperature of 10 ± 1.0 °C, and all tanks were equipped with air-lines and air-stones in a flow-through water system. On day-0 of the infection challenge, three treatments were conducted as follows.

1. Negative control (NC) treatment group. A clean and disinfected net (net-A) was soaked for 5 minutes in 600 mL water containing 7 mL sterile sample diluent (section 2.3). A second clean net (net-B) was used to transfer all 20 fish from their holding tank into net-A, where they were held for 20 seconds, and then transferred back into the NC tank.
2. The Virkon® Aquatic treatment group. A clean and disinfected net (net-A) was soaked in 600 mL water mixed with 7 mL of the EEDV solution, making the final EEDV concentration $2.25x10^5$ virus copies/mL of the water EEDV suspension (Table 1), for 5 minutes. Net-A was then transferred into 600 mL of the 1% Virkon® Aquatic solution for 20 minutes (manufacturer recommended duration). A second clean net (net-B) was used to transfer all 20 fish from their holding tank into net-A, where they were held for 20 seconds and then returned to their respective aquaria. This protocol was repeated for each of the three replicates and each replicate used two different nets.
3. Positive control (PC) treatment group. Fish in the positive control treatment were treated identically as the Virkon® Aquatic groups, with the exception that net-A was soaked in a 1% sample diluent water suspension for 20 minutes instead of a 1% Virkon® Aquatic solution.

Following experimental infection, all fish were observed daily for the development of disease signs and daily fish care was performed. Fish were fed *ad libitum* twice a day using AquaMax® Fingerling Starter 300. Any moribund fish during the study period were removed from the tanks and euthanized using a lethal dose (0.25 mg/mL) of tricaine methanesulfonate (MS-222; Western Chemical Inc., Ferndale, Washington) buffered with 0.5 mg/mL of sodium bicarbonate (Church & Dwight Co., Inc., Ewing, New Jersey). A necropsy was performed, eye, gill, and skin tissues were collected and frozen at -20 °C. The same protocol was followed for any mortalities. At the end of the study (140 days post infection (pi) for PC group, 148 days pi for Virkon® Aquatic and NC groups), all surviving fish were euthanized using a lethal dose of MS-222 at a dose of 0.25 mg/mL, buffered with sodium bicarbonate (0.5 mg/mL). Then, Pooled samples were taken from the eye, gill, and skin and frozen at -20 °C.

3.4.Water and suspension sampling

To assess EEDV loads, a 1 mL sample was collected from: a) the water used to prepare the net solutions; b) the water/sample diluent suspension before net soaking (NC group); c) the water/sample diluent suspension after net soaking (NC group); d) the water/EEDV suspension before net soaking (1% Virkon® Aquatic treatment group); e) the 1% Virkon® Aquatic solution after EEDV-laden net immersion (1% Virkon® Aquatic treatment group); f) the water/EEDV suspension before net soaking (PC group); and g) the sample diluent/water suspension after EEDV-laden net immersion (PC group; Table 1).

3.5.DNA extraction

Skin (for the PC group) and pooled eye, gill, and skin tissues (for the NC and Virkon® Aquatic group) were thawed and a maximum of 10 mg of tissue was transferred into a sterile 1.5 mL tube for DNA extraction. All extractions were performed following the protocol outlined by Mag-Bind® Blood & Tissue DNA HDQ 96 Kit (OMEGA Bio-tek, Inc, Norcross, Georgia) as previously described (*Chapter 1*). The DNA extractions of water and solution samples were performed following the Alternative PowerSoil Protocol for Low Bacterial Biomass Fluids from the Qiagen DNeasy® PowerLyzer® PowerSoil® Kit (Qiagen, Hilden, Germany) as previously described (*Chapter 2*). Extracted DNA was then quantified using the Qubit™ fluorometer (Invitrogen, Eugene, Oregon), and samples were diluted with sterile DNase-free water to obtain a maximum of 12.5 ng/uL qPCR template DNA.

3.6.Molecular detection of EEDV

The SYBR Green qPCR assay of Glenney et al. (2016a) that targets the glycoprotein gene of EEDV and differentiates the virus from all other currently recognized salmonid herpesviruses was utilized in this experiment. All qPCR reactions were carried out in a Mastercycler ep *realplex*^{2} real-time PCR machine (Eppendorf, Hauppauge, New York) and were performed as previously described (Glenney et al., 2016a; *Chapter 1*). EEDV-positive tissue homogenate was used as a positive extraction control (PEC) and sample diluent was used as the negative extraction control (NEC). Further controls included EEDV-positive purified DNA and nuclease-free water, which served as the positive reaction control (PRC) and negative reaction control (NRC), respectively. Samples were considered EEDV positive if the fluorescence exceeded 10% of the maximum florescence within 35 amplification cycles (Shavalier, 2017). Viral loads (copies/mg and copies/mL) were calculated using the Mastercycler ep realplex2 S

accompanying software via comparison to a standard curve that was generated via 8 serial 10-fold dilutions of EEDV positive standards (Shavalier, 2017).

4. Results

4.1.Gross disease signs and cumulative mortality

At the time of necropsy, lake trout in the three treatments ranged from 10.2 - 29.5 cm (average 18.2 ± 2.98 cm) in length and 8.6 – 110.1 grams (average 54.9 ± 27.1 grams) in weight. Disease signs consistent with EED were observed in the positive control group beginning at day 26 pi. Initially, these disease signs included lethargy and exophthalmia/corneal opacity, which continued to progress and become more severe. In addition to increasing lesion severity, ocular hemorrhage, severe congestion at the base of the fins, and secondary water mold invasion of the fins, body, and eyes were observed as infections progressed. The first mortality in the PC group occurred on day 29 pi and continued on days 31 (n=3), 34 (n=4), 38 (n=1), 39 (n=1), 40 (n=1), 42 (n=1), 44 (n=1), 47 (n=1), 56 (n=1), and 63 pi (n=1) until stabilizing at 80% cumulative mortality (Figure 1). In contrast, no EED disease signs were observed in any of the Virkon® Aquatic treatment replicates. However, one fish in replicate 1 and one fish in replicate 2 died during the study period (Figure 1; overall mean cumulative percent mortality of 3.3%). No mortality, nor EED disease signs, were observed in any negative control fish throughout the course of this study (Figure 1).

4.2.Molecular detection of EEDV

The estimated EEDV loads in the water/EED suspension prior to net soaking for the positive control and Virkon® Aquatic treatment groups ranged from $1.77x10^5$ - $2.25x10^5$ virus copies/mL and from $1.85x10^4$ - $7.29x10^4$ virus copies/mL after net soaking (Table 1). In contrast, EEDV was not detected in negative control water or sample diluents (Table 1), nor was it detected in lake trout prior to infection challenge. Likewise, EEDV was not detected in the pooled eye, gill, and skin samples of any NC fish, nor was it detected in any of the fish within the Virkon® Aquatic treatment (Table 1). In the positive control group, however, 18/20 fish were EEDV positive, whereby estimated virus loads exceeded the initial challenge concentration and ranged from $2.16x10^6$ - $3.64x10^{11}$ virus copies/mg skin tissue (Table 1). Among the EEDV positive individuals, 16 died and 2 were euthanized at 140 days pi.

5. Discussion

Contaminated equipment has been implicated in the transmission of numerous microbial fish pathogens (reviewed in Woo and Cipriano, 2017). In this context, disinfection is one of several tools that can be successfully used for preventing pathogen transmission into and within hatchery systems. Prior to this study, however, there was a complete lack of knowledge on the efficacy of hatchery disinfectants against EEDV, a matter hampering hatchery biosecurity efforts to prevent and control EEDV-associated losses. Herein, we provide evidence that under laboratory conditions, Virkon® Aquatic can prevent EEDV contagion to a highly susceptible host species/strain (i.e., Lake Superior strain lake trout) on contaminated fomites. Indeed, despite the development of severe and characteristic EED disease signs (Bradley et al. 1989), 80% cumulative mortality, and 90% EEDV infection prevalence with concomitantly high virus loads in lake trout that were netted with an EEDV-contaminated net, no signs of disease, EED-associated mortality, or the virus itself were detected in lake trout netted with an EEDV contaminated but then 1% Virkon® Aquatic treated net.

Although this study was the first to empirically assess EEDV susceptibility to Virkon® Aquatic, previous studies have examined the efficacy of Virkon® against other enveloped dsDNA viruses. For example, Gasparini et al. (1994) found that Virkon® damaged the surface antigen of the hepatitis B virus (Family Hepadnaviridae) and Scioli et al. (1997) similarly found that Virkon® modified the morphology of hepatitis B, thereby causing lost infectivity. Likewise, Bryan et al. (2009) found Virkon® was effective at inactivating a ranavirus (Family Iridoviridae) isolate, whereas McCormick and Maheshwari (2004) showed that Virkon® was also capable of inactivating adenovirus 5 and 6 (Family Adenoviridae). Specifically, against herpesviruses, Tsujimura et al. (2015) found that Virkon® effectively inactivated equine herpesvirus type 1 (Family Herpesviridae) and Hick et al. (2016) reported it inactivated ostreid herpesvirus-1 (Family Malacoherpesviridae). Results from the current study show that the use of Virkon® Aquatic holds promise against a fish-pathogenic herpesvirus belonging to the Family Alloherpesviridae, EEDV.

One of the limitations of the current study, primarily brought about by the inability to culture EEDV *in vitro*, was the relatively low concentration of EEDV used for experimental challenge of the fish. Although the utilized virus concentration in the net soak suspension (i.e., $1.77x10^5$ - $2.25x10^5$ virus copies/mL) exceeded the estimated median lethal dose for EEDV via immersion (i.e., $4.7x10^4$ virus copies/mL; Shavalier 2017), the exact concentration the fish were exposed to on the virus-contaminated net is unknown and may have been substantially lower than the suspension concentration. Nevertheless, EEDV loads in 90% of the positive control fish exceeded that of the virus suspension concentration, thereby showing EEDV infection and subsequent virus replication was achieved. Likewise, the current experimental challenge model led to initial mortality (day 29 pi) that was similar to what was observed via immersion challenge by Shavalier (2017; day 28 pi) and thus shows promise for future disinfection studies that mimic common hatchery practices.

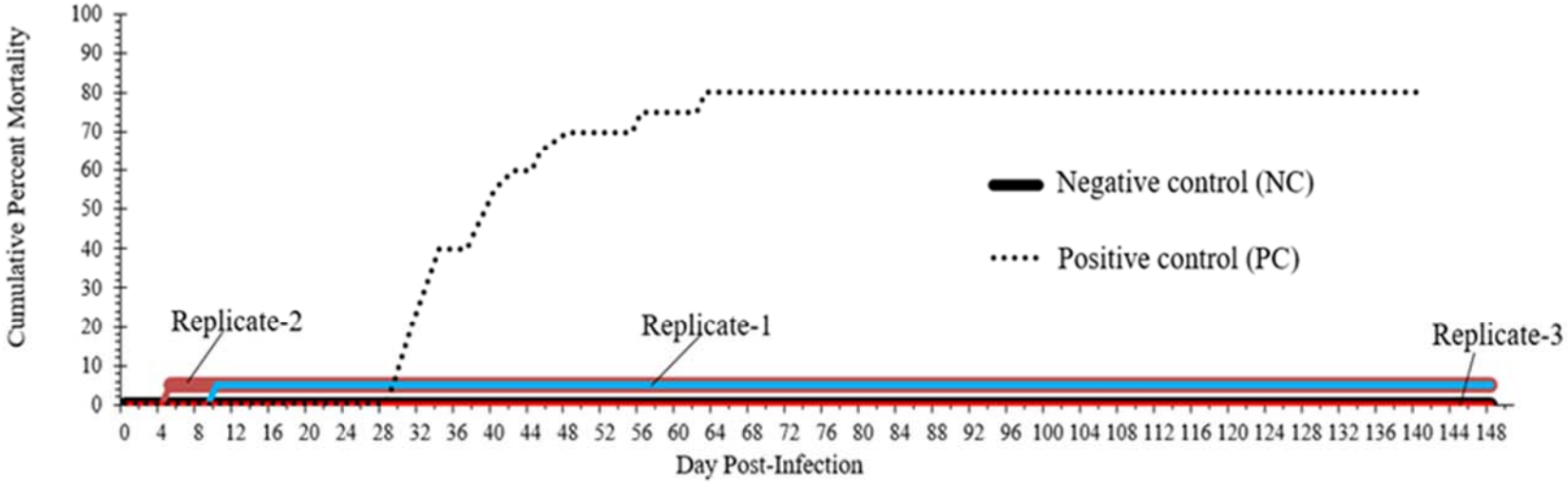
Although not the focus of this study, it is of interest that two of the four-lake trout that survived to 140-day pi still harbored relatively high EEDV loads ($2.16x10^6$ - $3.35x10^6$ virus copies/mg skin). In comparison, EEDV loads ranged from $1.33x10^4$ – $5.83x10^6$ virus copies/mg skin in Lake Superior strain lake trout at the end of the day 66 experimental challenge period (*Chapter 1*) and $1.59x10^7$ – $7.18x10^7$ virus copies/mg skin in Seneca strain lake trout at 100 days pi (*Chapter 1*). Even though virus delivery varied between this study and Chapter 1 (i.e., net exposure vs. intraperitoneal injection), both studies further demonstrate the extended periods that lake trout can harbor relatively high EEDV loads in their skin.

In conclusion, herein we provide the first evidence that Virkon® Aquatic is effective at reducing the risk of EEDV contagion on contaminated hatchery equipment. Although Virkon® Aquatic is marketed for effective disinfection in the presence of organic material, further studies should evaluate its capacity to prevent EEDV transmission on hatchery equipment under field conditions.

Table 1: The EEDV qPCR result of the samples of the negative control (NC), the Virkon® Aquatic, and the positive control (PC) group as well water and treated water.

Group	Number of samples	qPCR result	Virus load (virus copies/mg skin or mL water)
▪ Negative control (NC)	20	0/20	-
▪ Virkon® Aquatic			
Replicate-1	20	0/20	-
Replicate-2	20	0/20	-
Replicate-3	20	0/20	-
▪ Positive control (PC)	20	18/20	$2.16x10^{6}$ - $3.64x10^{11}$ /mg
▪ Sample of water that was used in this study	1	0/1	-
▪ Sample of water containing sample diluent before soaking it with net (NC)	1	0/1	-
▪ Sample of water containing sample diluent after soaking it with net (NC)	1	0/1	-
▪ Water sample collected from EEDV-laden water prior to soaking net in 1% Virkon® Aquatic	1	1/1	$2.25x10^{5}$ /mL
▪ Sample collected from 1% Virkon® Aquatic solution after EEDV-laden net immersion	1	1/1	$1.85x10^{4}$ /mL
▪ Water sample collected from EEDV-laden water prior to soaking net in the PC group	1	1/1	$1.77x10^{5}$ /mL
▪ Sample collected from sample diluent treated water after EEDV-laden net immersion	1	1/1	$7.29x10^{4}$ /mL

Figure 1: The cumulative mortalities that occurred in the positive control (PC), negative control (NC) and Virkon® Aquatic (Replicate-1, 2, and 3) group during the study period.



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