Vibrio anguillarum and *V. ordalii* Disinfection for Aquaculture Facilities

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Keywords: *Vibrio anguillarum*, *Vibrio ordalii*, disinfection, aquaculture, marine

ABSTRACT

One of the major limitations to intensive aquaculture is disease. Diseases spread rapidly in an aquatic environment and pose a major threat to development and utilization of all species in aquaculture. Bacteria of the genus *Vibrio* play a major role in the diseases of cultured species of marine fish. The goal of reducing the incidence of disease in a population is either to eliminate potential pathogens or to increase the resistance of the host. To reach that goal, a disinfection assay to test the effectiveness of nine common aquaculture chemical compounds was evaluated against two marine bacterial pathogens (*Vibrio anguillarum* and *V. ordalii*). Both bacterial species were susceptible to a variety of common disinfecting compounds including Chloramine-T®, chlorine, ethanol, iodine, Lysol®, Roccal®, and Virkon-S®.

International Journal of Recirculating Aquaculture 9 (2008) 43-51.

INTRODUCTION

Vibriosis, a disease caused by numerous species of *Vibrio*, is a primary disease of fish in marine and brackish waters. Vibriosis has been reported in over 50 species of marine fishes, and is a major obstacle for marine salmonid culture (Woo and Bruno 1999). In intensive culture, disease outbreaks often occur in late summer, when water temperatures increase.

Vibrio (Listonella) anguillarum is a halophilic Gram negative, curved rod with polar flagella. Vibriosis caused by this bacterial species has been identified in many finfish species including turbot (*Scophthalmus maximus*), eels (*Anguilla anguilla*) and salmonids (*Oncorhynchus nerka)* (Austin and Austin 1987, Tiecco *et al*. 1988, Antipa *et al*. 1980). High mortalities are often observed, with 100% morbidity (Reed and Francis-Floyd 2002) and mortality commonly over 80% in cultured cobia, *Rachycentron canadum* (Liu *et al*. 2004). Fish less than 4 months old (< 500g) appear to be the most susceptible, with the highest mortalities recorded for this bacterial pathogen (Lin *et al*. 2006). Clinical signs may present as hemorrhagic septicemia, skin discoloration, red necrotic lesions in the abdominal muscle, abdominal distension, exopthalmia, and erythema at the base of the fins, vent and in the mouth (Austin and Austin 1987).

Vibrio ordalii, formerly referred to as *Vibrio anguillarum* biotype 2, has been reclassified as a distinct species (Schiewe *et al*. 1981). *Vibrio ordalii* is another causative agent for vibriosis in fish. It can be distinguished from *Vibrio anguillarum* by culture and biochemical characteristics, as well as DNA sequence relatedness (Schiewe *et al*. 1981). Though the type strain (LMG 13544) of *V. ordalii* was initially isolated from coho salmon (*Oncorhynchus rhoddiurus*), *V. ordalii* has been reported from numerous marine species (Thompson *et al*. 2004). Clinical signs are similar to *V. anguillarum* with differences including microcolony formation on skeletal and heart muscle, gills and gastrointestinal tract, a slower progression of bacteremia, and marked leucopenia (Austin and Austin 1987).

Disinfection is the process whereby an antimicrobial agent is applied to a non-living object or surface to reduce or eliminate microorganisms. A variety of disinfection procedures are applicable to aquaculture situations including ozonation, ultraviolet exposure (UV), and chemical disinfection. Ozone and UV are commonly used to disinfect raw seawater to prevent

the introduction of pathogens into fish culture systems, or to disinfect recirculated water in a closed aquaculture system. In addition, a variety of chemical disinfectants are currently utilized in aquaculture, with concentration and time of exposure playing an important role in the efficacy of the given disinfectant.

Common disinfectants used in aquaculture include halogens such as chlorine and iodine, quaternary ammonia compounds, alcohols such as isopropanol and ethanol, phenolic compounds such as cresol, benzyl-4-chlorophenol-phenylphenol (used in Lysol®), and alkylating agents such as formalin, glutaraldehyde and ethylene oxide (Ellis 1988). Most disinfectants are toxic to animals as well as dangerous to the people using them. Therefore, animals may have to be removed from the facility prior to disinfection, and proper personal protection is required for all individuals during the disinfection process. Thus, the list of possible disinfectants is reduced by what is appropriate for use in the aquaculture industry and those that are relatively non-toxic to both animals and humans.

The goal of this study was to examine the efficacy of common aquaculture disinfecting compounds against two *Vibrio* species to provide a recommendation of the most effective compound(s) for the prevention of vibriosis in an aquaculture setting.

MATERIALS AND METHODS

Cultures of *Vibrio (Listonella) anguillarum* (NFHRL #5) and *Vibrio ordalii* (NFHRL #57) were obtained from the National Fish Health Research Laboratory in Kernersville, WV (USA). Cultures were inoculated on brain heart infusion agar (Fisher Chemicals, Fair Lawn, NJ, USA) with 1% NaCl (Fisher Chemicals, Fair Lawn, NJ, USA) (BHIA + 1% NaCl), and grown for 24 hours at 25ºC. Ten ml brain heart infusion broth with 1% NaCl (BHI + 1% NaCl) was inoculated from the plate and grown for 24 hours at 25ºC.

Bacteria were harvested by centrifugation at 1900 x g for 10 minutes at room temperature (22°C). Bacteria were washed twice in 10 ml sterile phosphate buffered saline (PBS, Sigma, St. Louis, MO, USA), and the final pellet resuspended in 5 ml sterile PBS (stock solution). One ml of stock solution was added to 6 ml of sterile PBS (working solution).

At this point, 100 µl of working solution was added to each of three labeled, sterile 1.5 ml microcentrifuge tubes (A, B, and C). For the control, Tube A, 900 µl sterile PBS was added. Next, 100 µl from Tube A was taken and added to 9.9 ml sterile PBS and 10 x serial dilutions were made to 10^{-5}. Serial dilutions were made with 100 µl of the previous concentration, in 900 µl of sterile PBS. Dilutions were plated with a multi-channel pipette in 10 µl drops, with four dilutions and five rows to a plate. For the replicates, Tube B and Tube C, 900 µl of individual disinfectant was added. The disinfectants used were Chloramine-T® (H&S Chemical, Covington, KY, USA), Clorox® regular bleach (The Clorox Company, Oakland, CA, USA), ethanol (AAPER Alcohol and Chemical Company, Shelbyville, KY. USA), formalin (Fisher Chemicals, Fair Lawn, NJ, USA), iodine (P.V.P, Western Chemical Inc., Ferndale, WA, USA), Lysol® (Reckitt Benckiser North America Inc., Parsippany, NJ, USA), Roccal-D Plus® (Pharmacia and Upjohn Company, Kalamazoo, MI, USA), sterile autoclaved tap water (Municipal Blacksburg, VA, USA), and Virkon S® (Pharmacal Research Laboratories, Waterbury, CT, USA) (Table 1). Samples were diluted and plated as with the control (Tube A) at 1, 5, 10, 20, 30, and 60 minutes exposure time. After the 60 minute samples were made, another dilution was taken of the control, Tube A, and plated. Colonies were counted after 24 and 48 hours incubation for separate trials of *V. (L.) anguillarum* and *V. ordalii*, respectively, and the number of colony forming units (CFUs) per ml was calculated.

RESULTS

The results of the disinfection assay (Table 1) demonstrated that Chloramine-T®, Clorox®, ethanol, iodine, Lysol®, Roccal®, and Virkon-S® eliminated all growth of both species of bacteria at exposure times of 1 minute and longer. Formalin reduced bacterial growth only after 60 minutes, and was not effective in elimination of either of the species of bacteria within 60 minutes. Autoclaved tap water demonstrated bacterial growth for only 10 minutes with *V. anguillarum* and for 5 minutes for *V. ordalii*, with no growth of either bacteria after those times. Control plates (PBS only) showed no significant change in CFU count over 60 minutes in any of the trials.

Table 1. Results of disinfection assay examining various aquaculture compounds for efficacy against V. anguillarum, and V. ordalii. The results indicate the last time sample with the presence of growth.

	Bacteria Species	
Disinfectant (Concentration)	***Vibrio (L.) anguillarum***	***Vibrio ordalii***
Chloramine-T® (0.0015 g/100 ml)	no growth*	no growth
Clorox® (50 ppt)	no growth	no growth
Clorox® (200 ppm)	no growth	no growth
Clorox® (100 ppm)	no growth	no growth
Clorox® (50 ppm)	no growth	no growth
Ethanol (70%)	no growth	no growth
Ethanol (50%)	no growth	no growth
Ethanol (30%)	no growth	no growth
Formalin (250 ppm)	reduced growth at 60 min	reduced growth at 60 min
Iodine (50 ppm)	no growth	no growth
Lysol® (1%)	no growth	no growth
Roccal® [1:256 (3.9 ppt)]	no growth	no growth
Tap Water (autoclave sterilized)	growth at 10 min	growth at 5 min
Virkon-S® [1% (0.1 g/10 ml)]	no growth	no growth

** no growth – indicates no colonies present at any time periods and any concentrations.*

DISCUSSION

Both *V. anguillarum* and *V. ordalii* were susceptible to a number of common aquaculture chemicals, including the disinfectants and chemotherapeutics tested in this study. Chloramine-T®, Clorox®, ethanol, iodine, Lysol®, Roccal®, Virkon-S® were all effective at killing both species of *Vibrio* within 1 minute. Formalin and Chloramine-T® were also tested, as they have been commonly utilized as chemotherapeutics in the aquaculture industry as a disease treatment. Formalin is used to treat external protozoan parasitic infections as well as for prevention of fungal infection on fish and eggs, while Chloramine-T® has been used to treat external bacterial infections. Formalin was not effective at elimination of *Vibrio* spp. as it was being used at a concentration typical for treatment of living fish for external parasites.

It was observed that *Vibrio* spp. were susceptible to autoclave-sterilized municipal water. This effect was probably a result of osmotic imbalance, as *Vibrio* spp. used in this study were cultured in salt-enriched media, and washed in sterile PBS. It was also noted that washing of the bacteria in sterile de-ionized water also caused killing of the bacteria.

Each pathogen needs to be taken into consideration for disinfection. *Vibrio* spp. act differently than other bacterial species which may exhibit different levels of resistance to disinfection. For example, *Mycobacterium marinum* was resistant to many disinfectants and only susceptible to Lysol® and 50% ethanol with 1 minute contact time (Mainous and Smith 2005). In another study, *Edwardsiella* spp. was susceptible to most disinfectants, but not to Chloramine-T® and formalin (Mainous and Smith, accepted). *Aeromonas salmonicida* has also been shown to be susceptible to disinfection with iodophor (povidone iodine), which is used to reduce incidence of disease from contaminated salmon eggs (Cipriano *et al*. 2001).

Due to its high susceptibility to a variety of disinfectants, *V. anguillarum* and *V. ordalii* would most likely be eliminated by standard disinfection practices using these compounds at manufacturer's recommended dosages. Thus, the price of the disinfectant as well as discharge regulations would be the primary concerns for choosing a disinfectant for these species of *Vibrio.* Additional measures might need to be taken if other bacterial pathogens are suspected to be present, in order to

properly disinfect the facility. It is also important to address removal of organic matter and surface biofilms prior to disinfection to allow the disinfectant to work properly. This often poses some difficulty as tanks, filters and plumbing must be cleaned thoroughly to maximize disinfectant effectiveness.

Disinfection should be an essential part of standard biosecurity practices to prevent disease outbreaks. Proper disinfection can be expected to be less expensive than the economic cost of antimicrobial treatment of an infected population, or the loss of part or all of that population due to the disease outbreak.

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