Zebrafish as a model for zoonotic aquatic pathogens

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Abstract

Aquatic habitats harbor a multitude of bacterial species. Many of these bacteria can act as pathogens to aquatic species and/or non-aquatic organisms, including humans, that come into contact with contaminated water sources or colonized aquatic organisms. In many instances, the bacteria are not pathogenic to the aquatic species they colonize and are only considered pathogens when they come into contact with humans. There is a general lack of knowledge about how the environmental lifestyle of these pathogens allows them to persist, replicate and produce the necessary pathogenic mechanisms to successfully transmit to the human host and cause disease. Recently, the zebrafish infectious disease model has emerged as an ideal system for examining aquatic pathogens, both in the aquatic environment and during infection of the human host. This review will focus on how the zebrafish has been used successfully to analyze the pathogenesis of aquatic bacterial pathogens.

Keywords

Zebrafish; aquatic pathogens; environmental reservoir; zoonotic; aquaculture; bacteria; infectious disease

1. Introduction

Aquatic pathogens are found in all water environments including marine, freshwater, brackish water, sewage, ground and even drinking water. Seawater alone harbors about 100 million bacteria per liter (Finkelstein and Oren, 2011). Humans can be exposed to all of these aquatic sources through various activities, ranging from consumption of contaminated seafood, ingestion of contaminated drinking water, recreational activities such as swimming, surfing, boating or fishing, exposure of open wounds to contaminated water sources or through handling of contaminated fish.

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A significant source of human pathogens from aquatic habitats is the many fish species that are used for food consumption. Fish are an important part of the human diet worldwide and can therefore serve as vectors of human disease. Extensive growth in the aquaculture industry in the last 30 years has extended from an increased desire for lean protein and healthy fats, as well as depletion of fish species in natural waters from overfishing. In addition, restrictive quotas have been imposed for many wild caught fish in certain geographical areas, leading to further decline in fish availability in the food supply. To increase overall production to meet the rising demand, fish farms often have overcrowded ponds that can serve as hot transmission sites for fish disease and serve as bacterial reservoirs that can be passed on to humans during food preparation. In many parts of the world, fish that can harbor potential bacterial pathogens live in human water supplies, creating a reservoir that can be passed on to humans in drinking water. Incidences have been reported in which the source of a bacterial disease outbreak in wild fish came from a nearby contaminated aquaculture facility (Zlotkin et al., 1998). Therefore, one of our major sources of protein can be the origin for potential human pathogens and also be a major contributor to contamination of essential water supplies (Table 1).

For many years, zebrafish (*Danio rerio*) have served as an important tool of biomedical research, providing great advances in our understanding of vertebrate development and disease modeling. Importantly, in recent years, zebrafish have been used to effectively model human infectious disease (Allen and Neely, 2010; Davis et al., 2002; Meijer and Spaink, 2011; Neely et al., 2002; Phelps, 2005; Sullivan and Kim, 2008; van der Sar et al., 2004). Zebrafish pathogenesis closely reflects pathogenesis observed in diseased wild and farmed fish and clinical symptoms observed during human infections (Davis et al., 2002; Neely et al., 2002; Weinstein et al., 1997). Zebrafish can also become colonized by some aquatic pathogens without succumbing to disease, providing an excellent model for the role of fish as an "environmental reservoir" for aquatic pathogens (Runft et al., 2014). "Environmental reservoirs" describes locations that allow bacterial persistence and replication in the environment while favoring pathogen transmission to susceptible hosts (Vezzulli et al., 2010). This review will focus on recent research on zebrafish as experimental hosts of zoonotic aquatic bacterial pathogens. Using zebrafish as an aquatic colonization model of human pathogens can provide information on environmental reservoirs of pathogens and how they contribute to transmission of human disease. Of all the infectious diseases modeled using the zebrafish host, those diseases caused by aquatic organisms would appear to have the greatest potential to add to our knowledge of pathogenesis because of the environmental niche they share.

2. Utilizing zebrafish as an infectious disease model

Natural pathogens of fish, such as *Mycobacterium marinum* and *Streptococcus iniae*, have been utilized in zebrafish to provide excellent models for understanding the pathogenesis of human tuberculosis infections caused by *Mycobacterium tuberculosis* (see (Deng et al., 2011b; Krishnan et al., 2010; Lesley and Ramakrishnan, 2008; Meijer and Spaink, 2011; Pozos and Ramakrishnan, 2004; Ramakrishnan, 2013; Tobin and Ramakrishnan, 2008) for review and citations within) and human streptococcal systemic infections (Allen and Neely, 2011; Harvie et al., 2013; Locke et al., 2008; Lowe et al., 2007; Miller and Neely, 2005;

Neely et al., 2002; Phelps, 2005). Using a model host allows comparison in side-by-side infections of bacterial mutants and wild type bacteria to examine the role of virulence genes in aquatic and human disease. Information gained from these analyses allows development of methods to better target infection either in the vector (fish) itself or in the human or animal host. One advantage of using the zebrafish as an infectious disease model is the ability to knockdown zebrafish genes during early life stages using morpholino technology. This method allows the researcher to suppress expression of a particular immune gene to determine its role in fighting disease. Furthermore, vaccine and treatment strategies studied in the zebrafish have the potential to be translated to aquaculture and to human clinics. Consequently, the use of the zebrafish for analysis of aquatic pathogens makes it an ideal infectious disease model.

Zebrafish disease models have utilized multiple life stages of the host, from embryos to adults (Meijer and Spaink, 2011). Because the innate immune system is active within 24 hours post fertilization (hpf) in zebrafish, but the adaptive immune system is not completely developed until 3-4 weeks later, important differences in required immune responses for fighting specific infections can be analyzed using the different stages of development (Clay et al., 2007; Clay et al., 2008; Davis et al., 2002; Lam et al., 2004; Meeker and Trede, 2008; Sullivan and Kim, 2008; Trede et al., 2004).

Multiple inoculation methods have been used to infect zebrafish and the success of a particular inoculation method depends on the ability of the pathogen to breach natural defense barriers. The outer mucus layer of the zebrafish provides a natural barrier to infection, much as the mucous membranes do in human tissues (Chang and Hwang, 2011). The epidermis and scales of the zebrafish also act as a protective layer, just as the epidermis and dead skin cell layer in humans act as a barrier to pathogens (Chang and Hwang, 2011). Natural routes of infection in the model system include simply adding bacteria to the tank water; bath immersion, in which the zebrafish are placed for a limited period of time in a high concentration of bacteria and then placed into fresh tank water; and dermal abrasion prior to bath immersion (Harriff et al., 2007; Neely et al., 2002; Phelan et al., 2005; Pressley et al., 2005; van Soest et al., 2011). Fish can also be infected or colonized by the oro-gastric route, by being exposed to inoculated tank water or allowed to feed on inoculated food sources (Kanther and Rawls, 2010; O'Toole and Kolter, 1998; Rawls et al., 2004; Runft et al., 2014; Szabady et al., 2009). All of these test the ability of the bacteria to breach natural barriers, or mimic inoculation conditions that would be encountered in the natural aquatic environment. However, they do not allow for an accurate measurement of the amount of bacteria delivered to the host.

Other methods of inoculation include oral gavage, in which bacteria are inserted into the esophagus through a small tube attached to a syringe (Cocchiaro and Rawls, 2013; Collymore et al., 2013). This allows inoculation into the gastrointestinal tract and is particularly important for analysis of pathogens that have a GI tropism. Although this method does provide more precision in determining the amount of bacteria inoculated than the natural routes discussed above, some of the inoculum may escape through the gills or be lost through the mouth. Therefore, subsequent bacterial counts taken shortly after inoculation can be inconsistent.

The most common route of inoculation is injection of bacteria directly into the host, which gives reliable reproducible infection results, but is not a natural route of infection. In adult zebrafish this includes intramuscular injection into the dorsal muscle or intraperitoneal injection (Neely et al., 2002; Phelps, 2005). Microinjection is also used for larval infections where bacteria are inoculated into the otic vesicle, the yolk sac, tail muscle, the tail vein, the caudal vein, or the Duct of Cuvier (Cosma et al., 2006; Li and Hu, 2012; Meijer and Spaink, 2011). All of these can be used to mimic a certain type of infection and to ask a specific question about the ability of the pathogen to survive in a particular tissue or to disseminate to other tissue environments.

3. Aquatic bacterial pathogens modeled in the Zebrafish

While mammalian models of human disease have proven invaluable in providing information on pathogenesis, they do not always accurately mimic disease symptoms that occur during human infections. Furthermore, some human pathogens do not readily infect mice, rats or rabbits. In the case of zoonotic infections, information on transmission from the vector to the human host is completely lacking. Many questions remain about the ability of the pathogen to persist during the environmental stage or in an alternate host, often in a non-pathogenic state, before being transferred to humans. Using the zebrafish to address these types of questions with aquatic pathogens provides an opportunity to acquire new knowledge that could be used to develop strategies to eliminate the pathogen prior to human exposure.

3.1 *Aeromonas*

Aeromonas species have had a devastating effect on the aquaculture industry with reports of major losses in fish populations worldwide resulting in great financial loss (Monette et al., 2006). Disease states in fish from *Aeromonas hydrophila* infections range from hemorrhagic septicemia, red sore disease and ulcerative infections in multiple species of fish (Janda and Abbott, 2010). Although the Aeromonads are mostly considered associated with aquatic environments i.e., found in fresh water, seawater, drinking water and groundwater, they are also ubiquitous in the environment, and are associated with a number of recreational habitats, animals and consumable products that can lead to human exposure and potential infection (Janda and Abbott, 2010). There are a plethora of clinical syndromes, both intestinal and extraintestinal, that *Aeromonas* can cause in humans ranging from relatively mild gastroenteritis to infections with high mortality rates such as septicemia, necrotizing fasciitis, and myonecrosis ((Janda and Abbott, 2010) and the references within). Serious wound infections can occur from simple abrasions if the affected area is exposed to contaminated water sources, such as streams, lakes and recreational waters. Therefore, aquatic habitats can serve as an environmental reservoir for human *Aeromonas* infection, which is now considered an emerging pathogen for humans (Igbinosa et al., 2012).

A. hydrophila is also a natural pathogen of zebrafish, making zebrafish an excellent model for the study of pathogenesis. A recent outbreak of hemorrhagic disease in laboratory zebrafish was isolated and identified to be *A. hydrophila* (Rodriguez et al., 2008). Naïve fish challenged with this strain by either IP injection or a wound-immersion route quickly developed the same symptoms as fish in the original outbreak. Importantly, injection with

just the extracellular secreted products gave similar mortality, indicating that the major
virulence factor(s) is secreted. Live bacteria and their extracellular secreted products were
hemolytic against fish blood and cytotoxic to cultured zebrafish kidney cells. Pro-
inflammatory cytokines (TNFα, IL-1β and IFNγ) and iNOS were strongly induced by
infection with live bacteria as well as with extracellular secreted products alone. These
results strongly indicate that *A. hydrophila* causes disease by triggering massive
inflammation, mediated by the extracellular secreted products (Rodriguez et al., 2008). In
support of these results, when Li et al. (2011) focused on three known virulence factors of *A.
hydrophila*, the aerolysin, the cytotoxic enterotoxin and a serine protease, it was found that
these three factors act synergistically in causing disease (Li et al., 2011). Isolates encoding
none or only 1 or 2 of these virulence genes had similar LD_{50} when tested in the zebrafish
model, whereas isolates with all three virulence genes had a reduced LD_{50}. Isolates positive
for all three virulence genes were also more hemolytic, more cytotoxic towards Vero cells,
and had more proteolytic activity in their supernatants, suggesting that these virulence
factors cause disease by tissue destruction (Li et al., 2011). However, there were strain
specific differences, indicating that other virulence factors, beyond the three tested, also play
a role in disease.

Much work has been done on finding ways to combat *A. hydrophila* infection in aquaculture
to preserve fish populations as well as circumvent transmission to humans. Antibiotic
treatment is one of the major options for treating an outbreak. However, use of inappropriate
antibiotic treatment in the food supply has been highly controversial. One reason is that
inappropriate antibiotic use can lead to development of antibiotic resistance in pathogens
through selective mutations or transfer of antibiotic resistance genes between bacteria.
Cantas et al. (2012) recently utilized the zebrafish infectious disease model to examine the
role of antibiotic use in treating *A. hydrophila* infection (Cantas et al., 2012). Oral gavage
was used to inoculate adult zebrafish with *A. hydrophila* resulting in systemic disease.
Infected zebrafish were then treated with antibiotics that were either effective for the
infection or at sub-inhibitory levels leading to ineffective treatment. Expression of innate
immunity genes in the gut of the zebrafish was then measured under the different conditions.
In addition, expression of bacterial antibiotic resistance plasmid transfer genes was
examined to determine how the antibiotic treatments affected potential transfer of antibiotic
resistance. Infection alone increased expression of IL-1β and IL-8, but not TNFα or
complement factor C3. Infection plus effective antibiotic treatments increased all four
examined immune genes, as did ineffective treatment with trimethoprim, sulphonamide or
flumequine, but ineffective doses of tetracycline reduced expression of TNFα and C3 below
levels measured in untreated infected fish. In addition, ineffective antibiotic treatment can
also activate conjugative transfer genes of a bacterial antibiotic resistance plasmid, while
transfer genes were repressed in bacteria during effective antibiotic treatment. Taken
together, using zebrafish and *A. hydrophila* as a model, this study demonstrated the danger
in ineffective antibiotic treatment leading to activation of resistance plasmid transfer genes,
and in the case of tetracycline, repression of immune genes, whereas effective antibiotic
concentrations not only kill the pathogen, but block transfer of resistance plasmids and can
activate innate immunity genes to help the immune system clear the infection. This supports

observations that single high dose antibiotic therapy can clear infection (Cantas et al., 2012), but highlights the importance of using antibiotics appropriately.

The zebrafish model has also been used to explore alternatives to antibiotic treatments to *A. hydrophila* infection. Many bacteria use "quorum sensing", a signaling system based on population density, to communicate with each other. *A. hydrophila* use quorum sensing to regulate virulence gene expression using the signaling molecule acyl homoserine lactone (AHL) (Bi et al., 2007; Khajanchi et al., 2009; Vilches et al., 2009). One alternative to antibiotic use would be to block the quorum sensing signaling between bacteria using a treatment with an AHL degrading enzyme (lactonase), acting as a broad spectrum inhibitor of virulence. Zebrafish exposed to *A. hydrophila* infection by bath immersion that were subsequently fed with food supplemented with an AHL lactonase showed significantly reduced mortality compared to fish not treated with the enzyme providing a new potential therapy for infection (Cao et al., 2012).

Vaccination methods in fish populations are also being pursued as a mechanism for combating *A. hydrophila* infection. This reduces the dangers from broad scale antibiotic use in ponds, but is more labor-intensive as each fish needs to be treated. The zebrafish model has provided an excellent model for testing vaccination methods for many types of aquatic pathogens, including *Aeromonas*. One way in which to provide immunity to a population without having to vaccinate every fish is to use a vaccine that allows immunity to be transferred from mother to offspring. Wang et al. (2009) demonstrated that vaccination of breeding females with formalin-killed *A. hydrophila* provided significant protection to the embryos (Wang et al., 2009). This was not mediated by maternal transfer of antibody, but rather by maternal transfer of complement components C3 and Bf. Vaccination of breeding females caused a rise in C3 and Bf levels in both the whole body homogenate of the adult fish and in the extract of eggs produced by that fish. Treatment of the fish with an anti-C3 or anti-Bf antibody reversed these effects. Embryos from vaccinated and unvaccinated fish were challenged with live, fully virulent *A. hydrophila*. Embryos from vaccinated fish survived significantly more than those from unvaccinated fish. Embryos from vaccinated fish were able to kill the bacteria, providing evidence that the maternal derived complement components are able to lyse the pathogen (Wang et al., 2009).

In a follow-up study, vaccination of female zebrafish with the hapten-carrier trinitrophenylated bovine serum albumin TNP-BSA raised a specific antibody response in the fish (Wang et al., 2012a). These antibodies were transferred to the offspring of vaccinated females. Embryos from females vaccinated with TNP-BSA were less susceptible to *A. hydrophila* than embryos from unvaccinated females. This protection was IgM specific, as an anti-IgM treatment blocked protection. Embryos of vaccinated mothers were able to kill *A. hydrophila* suggesting that the protection is maternal antibody mediated bacterial lysis. These were not *A. hydrophila* specific antibodies, indicating that these maternal antibodies were able to act non-specifically in protecting the embryo from water-borne infection. This mechanism of specific maternal antibodies acting non-specifically against bacterial pathogens, in addition to maternally derived natural antibodies, may protect embryos from water-borne infection until development of the adaptive immune system (Wang et al., 2012a).

3.2 *Edwardsiella*

Edwardsiella tarda and *Edwarsiella ictaluri* can cause septicemia in fish populations from both freshwater and marine environments. While *E. ictaluri* only infects fish, *E. tarda* is a zoonotic pathogen that can infect both fish and humans (Janda and Abbott, 1993). Transmission to humans is through exposure to contaminated aquatic environments and infected fish or through ingestion of contaminated fish (Janda and Abbott, 1993). Gastroenteritis is the most common clinical syndrome observed in humans and can range from an asymptomatic carrier state to intestinal colitis (Schlenker and Surawicz, 2009). *E. tarda* can also cause extraintestinal infections including soft tissue infections, meningitis, peritonitis, septicemia, bacteremia, endocarditis and urosepsis (Wang et al., 2005). Although infections in humans are more rare than in fish, *E. tarda* septicemia infections occurring in individuals with impaired immune systems can be severe and result in high mortality rates up to 50% (Janda and Abbott, 1993; Tamada et al., 2009; Wang et al., 2005).

Along with the *Aeromonads*, the *Edwardsiella* bacterial species cause the most severe morbidity and mortality in aquaculture (Mohanty and Sahoo, 2007). Multiple species of fish are susceptible to edwardsiellosis including carp, tilapia, eel, catfish, mullet, salmon, trout and flounder. Conditions most favorable for *E. tarda* infection include high temperature, poor water quality and high organic content (Mohanty and Sahoo, 2007). These conditions can often occur in aquaculture farms without proper diligence to hygienic practices. The bacteria can survive in pond water and mud for long periods of time and can increase in concentration with increasing temperature and organic load (Mohanty and Sahoo, 2007). The clinical pathology of edwarsiellosis includes small cutaneous lesions that can develop into large necrotic abscesses, petechial hemorrhaging of the fin and skin, rectal hernia and swelling of the abdomen due to fluid accumulation (Park et al., 2012; Pressley et al., 2005). The ability of *E. tarda* to survive outside the host provides an environmental reservoir that can lead to outbreaks of infection in the fish population, and subsequently humans, when the conditions are favorable.

Experimental infection analyses have shown zebrafish to be an effective experimental host for *E. tarda* infection, exhibiting similar clinical pathology to that observed in naturally infected wild and farmed fish populations (Pressley et al., 2005). While larvae were susceptible to infection by bath immersion, adult zebrafish were only susceptible to infection if the dermis was abraded prior to bath immersion, or if the fish were injected with bacteria. Mortality was observed from all infection methods in both larvae and adult fish. Gross symptoms in adult fish included discoloration and petechial hemorrhages at the site of wounding, if infected via the abrasion-bath immersion method, and distended abdomen and characteristic peri-anal edema, if infected by IP injection (Pressley et al., 2005). The blood of infected fish contained inflammatory cells with intracellular bacteria in macrophages, similar to that shown in kidneys of experimentally infected rohu fish (Mohanty and Sahoo, 2007). Infected larvae showed systemic inflammation, leading to epithelial and muscle tissue destruction, and numerous inflammatory cells in the bloodstream, with intracellular bacteria in macrophages (Pressley et al., 2005).

To mimic the environment of a fish farm or site of high bacterial load in the natural environment, van Soest et al. (van Soest et al., 2011) used bath immersion as a route of infection. Their results indicated that infection levels, and especially immune responses, following immersion in *E. tarda* culture are highly variable from fish to fish. However, injection into the tail vein of the larva caused a more reproducible infectious dose and a more consistent induction of immune related genes. While bath immersion resulted in a range of 25-75% mortality, tail vein injection resulted in 100% mortality. Therefore, while the zebrafish can be used to mimic natural infection routes of *E. tarda* that may occur in the environment, using methods that produce 100% inoculation (such as injection) allows experimental analysis of virulence pathologies and host response by bypassing the first protective barriers to infection. Pro-inflammatory responses were measured at the single embryo level and showed a strong induction of the *il-1b* and *mmp9* genes (van Soest et al., 2011). Pressley et al. (2005) also showed a strong upregulaton of IL-1β in both *E. tarda* infected larvae and adult zebrafish, as well as an increase in TNFα (Pressley et al., 2005). The results of a microarray performed on RNA isolated from infected zebrafish larvae revealed an increased expression of genes involved in defense and regulation of the immune response (van Soest et al., 2011). Results from these types of analyses with zebrafish provide new information about host response to *E. tarda* infection allowing development of more effective treatments.

Using a responsive experimental host also allows for analysis and identification of major virulence genes in the pathogen. Very little was known about the pathogenic mechanisms of *E. tarda* until recently. Using the zebrafish infectious disease model, several groups have been able to identify key factors in *E. tarda* pathogenesis. Xiao et al. (2012) showed *E. tarda* encodes two catalases, enzymes that allow the bacteria to break down hydrogen peroxide. Hydrogen peroxide is often the first line of defense used by the innate immune system against invading pathogens. Comparison of zebrafish infections with an *E. tarda* wild type strain and a strain that had a knockout of one of the catalase genes showed partial attenuation, with an approximate 10-fold increase in LD_{50}. However, a strain with a deletion of both catalase genes showed a 100-fold increase in LD_{50} (Xiao et al., 2012). Similarly, Wang et al. (2012) showed that deletion of the *rpoN* gene, which encodes an alternative sigma factor implicated in multiple species as a transcriptional regulator of virulence genes, had an 8-fold increase in LD_{50} compared to infection with the wild type strain (Wang et al., 2012b). The *rpoN* strain also had reduced ability to survive peroxide, acid, osmotic, and starvation stresses and produced less biofilm and chondroitinase, all of which could be implicated in virulence (Wang et al., 2012b). The invasin protein of *E. tarda* was examined for its role in virulence during zebrafish infection. Both deletion and overexpression of the invasion gene was analyzed. While deletion showed decreased virulence, most likely through reduced hemolytic activity and biofilm formation, overexpression of the invasin protein demonstrated higher adherence and internalization levels in cultured zebrafish epithelial cells (Dong et al., 2013).

Yu et al. (2012) took a different approach by analyzing the role in virulence of an antibiotic resistance plasmid (Yu et al., 2012). Virulence genes are often encoded on these plasmids, leading to transfer of virulence genes between bacterial populations and increased survival

of bacteria in environments with high antimicrobial concentrations such as fish farms. An *E. tarda* strain cured of an antibiotic resistance plasmid showed attenuation in mortality in zebrafish as well as goldfish and Japanese flounder. Subsequent sequencing of the plasmid identified 84 open reading frames, revealing that multiple virulence genes other than the genes for antibiotic resistance are encoded on the plasmid harbored by *E. tarda* (Yu et al., 2012). All of these analyses provide new information on genes required for pathogenesis, identifying new targets for antimicrobial therapies.

A major reason for the success of the zebrafish infectious disease model is that the zebrafish and human immune systems are very similar, although there are some differences, including absence of lymph nodes and additional Toll-like receptors compared to their mammalian counterparts (Phelps and Neely, 2005; Trede et al., 2004) and the references within). This means that zebrafish can also be a good model for vaccine development and analysis of immunization against infections. In an effort to find a vaccine for *E. tarda* infections, Yang et al. (2013) examined the zebrafish immune response using a live attenuated *E. tarda* vaccine strain for immunization and a fully virulent *E. tarda* strain for the subsequent challenge. The vaccination led to 78% of fish surviving a challenge 4 weeks post-immunization whereas only 12% of naïve fish survived challenge. Immunity was mediated through TLR5 signaling and activated a CD8+ T-cell response through cross-presentation of extracellular antigen. CD8+ responses were induced to a higher level during the challenge phase than the immunization phase. Vaccination also induced specific antibody in the fish serum, indicating that vaccination activated both cell and humoral mediated immunity (Yang et al., 2013).

3.3 *Mycobacteria*

Mycobacterium marinum has been analyzed extensively in zebrafish as a model for human tuberculosis infections caused by *M. tuberculosis* (see (Deng et al., 2011b; Krishnan et al., 2010; Lesley and Ramakrishnan, 2008; Meijer and Spaink, 2011; Pozos and Ramakrishnan, 2004; Ramakrishnan, 2013; Tobin and Ramakrishnan, 2008) for review and citations within). However, *M. marinum* is also a human and fish pathogen in its own right. As a human pathogen, *M. marinum* most often causes a granulomatous skin and soft tissue infection in fish hobbyists named "fish tank granuloma". This normally results from superficial wounding during routine fish tank maintenance. Additionally, infections have been linked to swimming in contaminated aquatic environments (Bonamonte et al., 2013; Wu et al., 2012). These infections can become severe, requiring prolonged multi-antibiotic therapy and/or surgical interventions (Bonamonte et al., 2013; Huang et al., 2012; Wu et al., 2012). In patients being treated with immunosuppressive drugs, these infections can be more prevalent and severe (Ferreira et al., 2012). Superficial infection can rarely disseminate to bones and joints or cause a pulmonary infection (Bonamonte et al., 2013; Wu et al., 2012). *Mycobacteria* are also natural pathogens of marine and freshwater fish (see (Gauthier and Rhodes, 2009; Jacobs et al., 2009) for review and citations within).

Fish kept in aquaculture farms and zebrafish research colonies are susceptible to natural infection by several species of *Mycobacteria*, including *M. abscessus, M. chelonae, M. fortuitum, M. marinum, M. haemophilum* and *M. peregrinum.* Most species are opportunistic

pathogens but *M. marinum* and *M. haemophilum* are highly pathogenic. Bacteria are acquired from other infected fish or infected biofilm. (for more extensive discussion see (Whipps et al., 2012) and citations within).

To further examine the virulence of mycobacterial species isolated from research facilities, Watral and Kent (2007) experimentally infected zebrafish, by intraperitoneal injection, with several strains of *M. marinum, M. abscessus, M. peregrinum, and M. chelonae* (Watral and Kent, 2007). Only fish infected with *M. marinum* exhibited symptoms of disease and succumbed to infection. However, histopathologic examination of infected fish found granulomatous lesions in the peritoneum, kidneys and ovaries of fish infected with *M. marinum* and non-*marinum* species, indicating that these fish can be sub-clinically infected with *Mycobacteria*. Live bacteria were only consistently recovered from the fish infected with *M. marinum*, but some individual fish were asymptomatic carriers of other the other mycobacterial species. Symptoms of *M. marinum* disease in experimentally infected fish were skin erythema, raised scales, swollen abdomens and rarely, skin lesions (Watral and Kent, 2007).

To study possible natural transmission routes, Peterson et al. (2013) examined inoculation of *M. marinum* and *M. chelonae* by the oro-gastric route by using the single cell eukaryotic ciliate *Paramecium caudatum* as a vector (Peterson et al., 2013). Larval, juvenile and adult fish exhibited more extragastric (mainly kidney and spleen) granulomas containing *Mycobacteria* when fed paramecia infected with *M. marinum* or *M. chelonae* than when the bacteria were incorporated into a food gel matrix or free bacteria were added to the water. Gastrointestinal bacteria were observed for all infection routes, suggesting that bacteria are more virulent after passing through a ciliate host, and are better able to cause disseminated infection (Peterson et al., 2013).

Although the ESX-1 (RD1) transport system has been extensively studied for its role in virulence in mycobacteria (Abdallah et al., 2007; Champion and Cox, 2007), including in the zebrafish (Davis and Ramakrishnan, 2009; Volkman et al., 2010), the ESX-5 Type VII secretion system has more recently found to also play a role in virulence in many mycobacterial species. To examine the effect of the *M. marinum* type VII protein secretion system ESX-5 in establishment of granulomas in zebrafish, Weerdenburg et al. (2012) used the zebrafish model. Their analysis demonstrated that this protein secretion system serves a complex role in virulence (Weerdenburg et al., 2012). When 100 cfu of the ESX-5 mutant was microinjected into the caudal vein of zebrafish larvae 28 hours post fertilization, establishment of initial infection was mildly attenuated compared to injection with the wild type strain. Fewer bacteria were isolated from the infected larvae and granuloma formation was delayed in fish infected with the ESX-5 mutant than with wild type bacteria. However, adult fish infected with the ESX-5 mutant succumbed more rapidly to infection and had higher bacterial loads in the spleen and liver than fish infected with wild type bacteria. Since larvae do not yet have a functional adaptive immune system, the role of the adaptive immune system was examined to see if it was responsible for the discrepancy between attenuation in larvae and hypervirulence observed in adult fish. However, infection with the ESX-5 mutant into Rag1 deficient adult fish, which are deficient in an adaptive immune response, showed that the hypervirulent phenotype was not dependent on the adaptive

immune system. Histopathologically, fish infected with the ESX-5 mutant bacteria developed granulomas sooner and in higher numbers than fish infected with wild type bacteria. Additionally, pro-inflammatory cytokines (IL-1β, TNF-α and IFN-γ) were more highly expressed in ESX-5 granulomas. Co-infection of wild type and ESX-5 bacteria, to determine if the secreted factors from the wild type could complement the hypervirulence of the ESX-5 mutant, showed that the ESX-5 mutant outcompeted the wild type strain. These results suggest that the ESX-5 protein secretion system functions to modulate *M. marinum* virulence and potentially contribute to persistence (Weerdenburg et al., 2012).

In addition to a type VII secretion system, the SecA2 alternative protein translocation system is another putative virulence factor of *M. marinum*. SecA2 mutants were attenuated in a larval zebrafish model of *M. marinum* infection (van der Woude et al., 2013). Fish were infected by microinjection into the caudal vein 24 hours post fertilization. Five days post-infection, bacterial loads were five times higher in fish infected with wild type than *secA2* mutant bacteria. Mutant bacteria showed less aggregation in larvae and leukocytes formed smaller and less compact aggregates in fish infected with mutant bacteria. Complementation of the *secA2* mutation restored wild type levels of bacterial load and aggregation with leukocytes (van der Woude et al., 2013).

One of the distinctive differences in Mycobacteria compared to other bacterial pathogens is the unique lipid components of the Mycobacterial cell wall. Recently, a study examining one of the proteins required for cell wall structure in *M. marinum*, MptC, encoding an α(1→2) mannosyltransferase, demonstrated that MptC also plays a role in virulence (Stoop et al., 2013). By examining both zebrafish adults and larvae, Stoop, et al (Stoop et al., 2013) took advantage of the ability to study the innate immune response to Mycobacterial infection in isolation from the adaptive immune response because of the later development of the adaptive immune system in zebrafish larvae. Examination of larval infection with an *mptC* mutant showed smaller bacterial aggregates, and *mptC* infected larvae had lower bacterial loads. Complementation with either the *M. marinum* or *M. tuberculosis mptC* gene restored wild type levels of bacterial growth and aggregation *in vivo*, indicating the *M. marinum mptC* gene serves the same function as in the human pathogen *M. tuberculosis.* However, virulence attenuation was greatly reduced when the same strains were used in an adult zebrafish intraperitoneal infection as both strains produced granulomas, although the *mptC* mutant produced fewer and showed less tissue destruction than fish infected with the wild type bacteria. Therefore, the larger magnitude of attenuation in larvae versus adult zebrafish indicates that MptC serves a role in virulence, but mainly in defense from innate immune mechanisms (Stoop et al., 2013).

In an effort to develop a vaccine to control *M. marinum* infection, Cui et al. (2012) examined the effectiveness of an attenuated strain of *M. marinum* with reduced replication in macrophages. In addition, heat killed *M. marinum* and extracellular secreted products were tested. Results showed that while all three vaccination methods led to the production of antibodies, only the live attenuated strain protected zebrafish from challenge with virulent *M. marinum* (Cui et al., 2010).

More recently, an important study examining the utility of zebrafish as a model for vaccination against mycobacterial disease was recently reported by Oksanen et al (Oksanen et al., 2013). They initially demonstrated that zebrafish could successfully be immunized against *M. marinum* by the BCG vaccine currently in clinical use to prevent human mycobacterial infection. Zebrafish were also shown to be able to stably express exogenous antigens following DNA vaccination, with expression lasting up to thirty days post vaccination. By vaccinating with three immunodominant antigens of *M. marinum*: Ag85B, CFP-10 and ESAT-6, individually and in combination, they were able to protect adult zebrafish from both slow and rapidly progressing *M. marinum* infection. Combination vaccination with all three antigens was able to induce a significantly higher IFN-γ response fourteen days post low dose *M. marinum* infection in vaccinated fish versus unvaccinated or fish vaccinated with a control plasmid. Following combination vaccination and a low dose challenge with *M. marinum*, vaccinated fish had fewer granulomas, and fewer affected organs. While combination vaccination and a high dose challenge of *M. marinum*, produced lower mortality and lower bacterial load in vaccinated fish. Therefore, this study demonstrates the effectiveness of pre-clinical vaccine research using the zebrafish infectious disease model (Oksanen et al., 2013).

3.4 *Streptococcus*

Streptococcosis in fish populations was first confirmed in Japan in the late 1950s (Hoshina et al., 1958). Since that time, multiple species of streptococci have been found to infect fish populations, but the major cause of economic losses from streptococcosis in aquaculture and wild fish populations are *Streptococcus iniae* and *Streptococcus agalactiae*. Both of these pathogens can also cause disease in humans and the aquatic habitat can act as a reservoir for human infection. Here we will discuss reports of the zebrafish infectious disease model to experimentally determine pathogenic mechanisms in both human and aquatic isolates of streptococci.

3.4.1 Streptococcus iniae—The first report of *Streptococcus iniae* infection was in 1976 in a captive Amazon freshwater river dolphin in which subcutaneous lesions called "golf ball disease" were described (Pier and Madin, 1976). However, in the last 35 years, *S. iniae* has emerged as a major finfish pathogen with a host range of over 27 species of fish from both freshwater and marine environments (Agnew and Barnes, 2007). While wild fish populations are just as susceptible to *S. iniae* infections, the most devastating effects of this pathogen have been observed in aquaculture with an estimated economic loss of over 100 million US dollars worldwide (Shoemaker et al., 2001). Although the type of disease presentation varies with fish species, most often a systemic infection occurs with accompanying meningitis and panophthalmitis (Bromage and Owens, 2002). In almost all cases, infection results in high morbidity and mortality.

S. iniae is also a zoonotic pathogen with the first reports of human infection occurring in 1995 after the handling of fresh fish from aquaculture (Weinstein et al., 1996a; Weinstein et al., 1996b). Infection in humans is usually a result of handling of contaminated fish and sustaining an injury, allowing access of the pathogen to the underlying dermis layer, most often of the hand. The resulting infection manifests as bacteremic cellulitis with the rare

complications of endocarditis, meningitis, arthritis, sepsis and toxic shock in immunocompromised individuals (Hansen and Strassburger, 2000; Koh et al., 2009; Lau et al., 2003; Sun et al., 2007; Weinstein et al., 1996a; Weinstein et al., 1996b). Although there have only been about 30 reports of *S. iniae* infections in humans, it has been suggested that infections are most likely underreported because clinical identification relies on biochemical testing and *S. iniae* is currently not listed in commercial or clinical databases (Lau et al., 2003; Weinstein et al., 1997).

The first publication using zebrafish as an infectious disease model for streptococcosis demonstrated that the histopathology as well as the clinical symptoms of *S. iniae* disease mimicked that which was observed in farmed fish populations (Neely et al., 2002). Zebrafish succumbed to a systemic infection within 4 days post intramuscular injection with an LD_{50} of 5×10^3 cfu. *S. iniae* was isolated from the skin, heart, gall bladder and brain (Neely et al., 2002). Subsequently, a large-scale screen investigating virulence factors in *S. iniae* highlighted the advantage of using the zebrafish infectious disease model (Miller and Neely, 2005). A signature-tagged mutagenesis screen was performed to identify mutants that had not survived in the heart or the brain of the zebrafish at 24 hours post-infection. The screen identified 41 mutants that were attenuated in the zebrafish host, 50% of which had homology to virulence genes from other streptococcal pathogens. These results confirmed the effectiveness of the zebrafish disease model for identifying virulence genes required for infection in the host. Importantly, 10 of the transposon insertions were found to be in the capsule operon of *S. iniae*, confirming the importance of capsule for systemic infections (Miller and Neely, 2005). By comparing the sequence of the capsule operon of the 9117 virulent *S. iniae* strain to a commensal strain of *S. iniae* isolated from healthy fish, Lowe et al. (2007) were able to show that the commensal strain had a large deletion in the capsule operon encompassing several important capsule synthesis genes (Lowe et al., 2007). In addition, the ability of various capsule mutants to survive in the presence of the wild type strain (competitive indices) or the ability to disseminate and survive in different tissue environments was examined using the adult zebrafish, providing new information on regions of the capsule operon required for virulence (Lowe et al., 2007).

To better determine the role of the innate immune system in protection from *S. iniae* infection, a zebrafish larvae model was employed to visualize host-pathogen interactions in real time. An *S. iniae* wild type strain and a *cpsA* capsule mutant strain were stained with the CellTracker red CMPTX dye prior to injection. Three zebrafish larvae strains were used as hosts: the Tg(*mpeg1:dendra2*) (Harvie et al., 2013) zebrafish line in which the macrophages are expressing EGFP; the Tg(*mpx-dendra2*) (Yoo and Huttenlocher, 2011) zebrafish line in which the neutrophils are expressing EGFP; and the Tg(*mpx:mCherry-2A-rac2-d57n*) zebrafish line in which neutrophils are expressing the inhibitory Rac2(D57N) mutation, making them unable to respond to inflammatory stimuli, including otic infection (Deng et al., 2011a). Injections were into the otic vesicle so as to observe recruitment of macrophages and neutrophils to the site of infection, as the otic cavity is usually free of leukocytes (Levraud et al., 2008). Injections of wild type *S. iniae* into larvae were lethal within 24 hours, while over 90% of larvae injected with *cpsA S. iniae* survived. Both neutrophils and macrophages were visibly recruited to the site of infection and were

observed to phagocytose bacteria. While depletion of both macrophages and neutrophils using Pu.1 morpholino injection led to an increased survival of the larvae, using a strain with impaired neutrophil function, but normal macrophage function, revealed that neutrophils are essential for control of *S. iniae* wild type infection (Harvie et al., 2013).

The *cpsY* gene, which is directly upstream of the capsule operon of *S. iniae*, was originally thought to be involved with capsule expression because of its homology to the LysR transcriptional regulator family (Koskiniemi et al., 1998). A *cpsY* deletion strain was also highly attenuated in the zebrafish infectious disease model and showed an inability to disseminate to the brain, a key factor in *S. iniae* pathogenesis in fish (Lowe et al., 2007). However, it was determined that CpsY was not regulating capsule in *S. iniae* (Lowe et al., 2007) or *S. agalactiae* (Shelver et al., 2003) and was instead involved in methionine biosynthesis and uptake (Bryan et al., 2008). To elucidate the role of CpsY in pathogenesis, Allen and Neely (2011) combined in vitro whole blood and human neutrophil assays along with in vivo zebrafish infections to determine that CpsY is required for survival within neutrophils (Allen and Neely, 2011). Subsequent research confirmed that in addition to methionine biosynthesis and uptake, CpsY stimulates cell wall stabilization through peptidoglycan O-acetylation and repression of autolysins (Allen and Neely, 2012). Therefore, as was shown by the larval infection model detailed above (Harvie et al., 2013), neutrophils play a key role in controlling infection of *S. iniae*; however, the pathogen has evolved specific mechanisms that allow survival in that environment.

In an effort to learn more about key virulence genes required for pathogenesis of *S. iniae*, Locke et al. (2008) performed pyrosequencing of an *S. iniae* strain isolated from the brain of a diseased hybrid striped bass (K288) (Locke et al., 2008). Their analysis revealed an M-like protein (called SiM), which had high homology to the well-studied surface-anchored M protein from *Streptococcus pyogenes* that is required for virulence (Fischetti, 1989; Phillips et al., 1981). In addition, they identified a gene that encodes a protein homologous to the C5a peptidases found in both *S. pyogenes* and *S. agalactiae* that cleaves the chemoattractant C5a of the complement system (Cleary et al., 1992). In vivo analyses in both zebrafish and hybrid striped bass of *S. iniae* mutants in the *scpL* and *simA* genes showed that the SiM protein plays a role in virulence, while the C5a peptidase did not. They further tested the SiM deletion strain as a vaccine candidate in the hybrid striped bass model and found it to be highly protective from a lethal dose of the wild type strain 90 days after vaccination (Locke et al., 2008).

López-Muñoz utilized the zebrafish-*S. iniae* disease model to determine the role of zebrafish IFNs during infections (López-Muñoz et al., 2009). Their results demonstrated that while both the group I and group II zfIFNs could protect against viral infection, only the group I zfIFN was able to protect zebrafish from *S. iniae* infection through the induction of proinflammatory genes. Interestingly, they also found that unlike with mammalian IFN-γ, zfIFN-γ was unable to produce the proinflammatory response to infections (López-Muñoz et al., 2009).

3.4.2 Streptococcus agalactiae—*Streptococcus agalactiae*, better known as Group B *Streptococcus*, (GBS) is a Gram-positive pathogen responsible for human and agricultural

disease. Of all the streptococcal species, *S. agalactiae* appears to have the broadest host range for pathogenesis, having been isolated from both cold-blooded aquatic organisms and warm-blooded terrestrial animals. In humans, GBS is the leading cause of early onset neonatal sepsis and meningitis in the developed world (Edmond et al., 2012). GBS can also cause infections in non-pregnant adults and is considered as an emerging pathogen in immunocompromised and older individuals (Skoff et al., 2009). Outside of human infections, GBS is a major contributor to disease in agriculture, particularly bovine mastitis and fish sepsis.

Massive outbreaks of streptococcosis caused by GBS have been observed in both farmed and wild fish populations worldwide (Evans et al., 2002). There are also many reports examining the serotypes of strains isolated from different hosts to determine if certain serotypes are species specific (Evans et al., 2008; Pereira et al., 2010). There are only 9 serotypes of GBS and those that have been found to cause the most invasive disease in humans have also been isolated from aquatic mammals and fish (Delannoy et al., 2013). One study identified eating colonized fish as a risk factor for human colonization by GBS (Foxman et al., 2007).

Patterson et al. (2012) used the streptococcal-zebrafish model (Neely et al., 2002) to investigate disease characteristics of GBS (Patterson et al., 2012). Zebrafish infected with high doses of GBS by either the intramuscular or intraperitoneal route succumb to infection, whereas fish infected with a low dose can clear the infection. Bacteria are found within the bloodstream immediately after IP injection and dissemination to the brain was observed within 2 hours post injection. Inflammation within the brain was observed grossly as cerebral edema with higher expression of inflammatory cytokines IL-1β and IL-6 within the brain as compared to non-infected zebrafish (Patterson et al., 2012). This study accurately modeled the symptoms and response to meningitis observed in human GBS patients. Mutations in virulence genes of GBS led to attenuation in the zebrafish model, validating that factors important for human disease are also important for fish disease. Deletion of the transcriptional activator of capsule production *cpsA* leads to increased survival of fish infected by the IM route (Hanson et al., 2012) and *cpsA* mutants are attenuated in the ability to disseminate to the spleen, heart and brain (Fig 1, unpublished data from HMR). GBS strains carrying mutations in the hemolysin, *cylE*, a capsule transglycosylase, *cpsD*, or a two-component system responsible for virulence regulation, *covRS*, were attenuated in their ability to cause meningitis in the zebrafish. In contrast, the COH1 strain, which is hypervirulent in humans, caused increased concentration of GBS in the zebrafish blood and brain, resulting in increased lethality in zebrafish (Patterson et al., 2012).

3.5 *Vibrios*

Of the human pathogens that can be found in seawater environments, *Vibrios* are the most predominant species (Auerbach et al., 1987). Infection in humans is most commonly acquired by wound contamination from exposure to contaminated water or aquatic organisms, the ingestion of contaminated water or consumption of raw or undercooked seafood (Finkelstein and Oren, 2011). The major clinical syndromes associated with *Vibrio* infection from aquatic environments are gastroenteritis, soft tissue infection and primary

bacteremia (Austin, 2010; Blake et al., 1980). Soft tissue infections from wounds usually occur from recreational water activities such as swimming, surfing or boating, followed by fishing injuries and handling of seafood (Dechet et al., 2008). Although food borne infection is the most commonly recognized clinical syndrome from *Vibrio* aquatic exposure, soft tissue infections can result in high morbidity and mortality, leading the Centers of Disease Control and Prevention to recently add non-cholera vibriosis as a national notifiable disease (Dechet et al., 2008).

For non-food borne *Vibrio* infections, *Vibrio vulnificus* is the most common aquatic-associated infections in humans, followed by *Vibrio alginolyticus* and *Vibrio parahemolyticus* (Austin, 2010; Dechet et al., 2008). All three of these organisms have been modeled in the zebrafish (He et al., 2011; Pan et al., 2011; Paranjpye et al., 2013; Wang et al., 2008).

3.5.1. Vibrio alginlyticus—*Vibrio alginolyticus* causes septicemia in fish, as well as gastrointestinal, wound and mucosal infections in humans. To determine the role in pathogenesis in vivo of the twin-arginine translocation system (Tat) used by *V. alginolyticus*, He et al. (2011) utilized the zebrafish infectious disease model (He et al., 2011). The Tat system is used by the bacterium for translocating folded proteins and secretion of specific virulence factors as well as having roles in growth and motility. Construction and analysis of a strain deleted for the genes in the twin-arginine translocation system impacted biofilm formation and swarming motility and decreased secretion of extracellular proteases. Loss of these functions resulted in a reduction in virulence in the zebrafish compared to infection with the wild type strain (He et al., 2011). Similarly, Wang et al. (2008) used the zebrafish model to analyze mutants in iron uptake systems by demonstrating that the TonB1 and TonB2 systems are also required for systemic virulence of *V. alginolyticus*. Deletion of one locus resulted in an approximately one-log increase in LD_{50} and a double deletion resulted in over a 25-fold increase in LD50 (Wang et al., 2008).

3.5.2 Vibrio vulnificus—*Vibrio vulnificus* causes the greatest majority of soft tissue infections in humans from marine microorganisms (Finkelstein and Oren, 2011). However, the most lethal infection caused by *V. vulnificus* is primary septicemia, with a greater than 50% mortality rate (Blake et al., 1980).

V. vulnificus infection of zebrafish is lethal and shows a specific increase of innate immunity genes following *V. vulnificus* infection (Pan et al., 2011). Treatment of zebrafish with the antimicrobial peptide epinecidin-1 from Grouper (*Epinephelus coioides*) is able to protect zebrafish from *V. vulnificus* infection. Co-injection of epinecidin-1 conferred the best protection, but pre-treatment (one hour prior to injection of bacteria) or post-treatment (one hour after bacterial injection) also conferred protection. Fish surviving infection with epinecidin co-treatment were challenged again with bacteria 30 days post infection and had reduced mortality compared to naïve fish, indicating that epinecidin co-treatment acts similarly to a live-attenuated vaccine. Microarray analysis for inflammatory genes indicated that infection, epinecidin and co-treatment led to distinct transcriptional changes. Epinecidin acts as both an antibacterial and immunomodulatory molecule to confer protection to *V. vulnificus* infection (Pan et al., 2011).

3.5.3 Vibrio cholerae—Another *Vibrio* species that is responsible for a great deal of morbidity and mortality worldwide from consumption of contaminated seafood or water is *Vibrio cholerae*. Disease from *V. cholerae* manifests mainly as gastrointestinal symptoms, causing the disease of cholera, which leads to massive watery diarrhea resulting in dehydration and loss of electrolytes. Cholera afflicts an estimated 5 million people annually worldwide, causing over 100,000 deaths. Without rehydration therapy, cholera is lethal in 50-60% of patients; however quick treatment results in less than 1% mortality (Sack et al., 2004).

Like other *Vibrio* species, *V. cholerae* can be found associated with aquatic organisms (shellfish, insect egg masses, and plankton) as well as free swimming in water (Halpern et al., 2004; Hood et al., 1981; Rawlings et al., 2007; Senderovich et al., 2010). Much research has been done on the human pathogenesis of *V. cholerae*, and recently there has been increased interest on the environmental lifestyle and aquatic reservoirs of the organism, with hope of determining key factors required for transmission to humans. Current animal models for *V. cholerae* include the infant mouse model (Klose, 2000), the rabbit intestinal ileal loop model and the RITARD model (Formal et al., 1961; Spira and Sack, 1982; Spira et al., 1981). Unfortunately, neither the mouse nor the rabbit are natural hosts for *V. cholerae* and therefore, do not mimic human disease. Senderovich recently identified non-O1 *V. cholerae* in the intestinal tracts of multiple species of wild-caught fish (Senderovich et al., 2010), suggesting that *V. cholerae* may use vertebrate fish as an environmental reservoir, allowing for bacterial replication and transmission to water sources and other aquatic organisms.

Not much is known about the environmental lifestyle of *V. cholerae* or the key factors required for the pathogen to survive for long periods in an aquatic environment. Since previous mammalian models were not successful in reproducing human cholera symptoms, recent work investigated the efficacy of using the zebrafish host model as a model for cholera disease, and/or model as an environmental reservoir for *V. cholerae* (Runft et al., 2014). Runft et al. (2014) found that the zebrafish gut could be highly colonized over long periods of time by pandemic O1 serogroup *V. cholerae* strains through simple addition of the bacteria to the water. Furthermore, no prior manipulation of the natural intestinal flora is needed as is required with the other *V. cholerae* animal models, which suggests that colonization of the intestine in natural fish populations could easily occur with *V. cholerae*. Histology of infected zebrafish intestines illustrated that *V. cholerae* was closely attached to the intestinal epithelium and formed many micro-colonies in this environment (Runft et al., 2014). In an effort to mimic uptake of bacteria from both the water and the natural environment, providing a delivery vehicle of a contaminated food source was explored. Zebrafish rapidly eat freshly hatched brine shrimp as a natural food source. *V. cholerae* express chitin binding proteins and were found to bind readily to brine shrimp after a 15 minute incubation (Fig 2A & 2B). Addition of contaminated brine shrimp to an experimental tank of zebrafish resulted in an initial significant ($p < 0.05$) increase in colonization by 2 logs at 2 hours post inoculation compared to water inoculated fish (Fig 2C). However, by 24 hours post infection, levels of *V. cholerae* in the intestine were about the same as the water-inoculated fish (Runft et al., 2014).

One of the most interesting findings from this study was the ability of infected fish to pass the infection to naïve fish. Zebrafish were placed in water contaminated with *V. cholerae* for 3 hours, followed by multiple washing steps. An exposed fish was then placed into an experimental tank with fresh water and multiple naïve fish. Importantly, previous work showed that *V. cholerae* cannot be isolated from the outer surface of the fish (Runft, D. L. nonpublished results). After 24 hours, the naïve fish population, exposed to the contaminated fish, was colonized with *V. cholerae*. This type of disease transmission cannot be analyzed using the mammalian host models. Moreover, this occurrence is very likely to happen in the natural environment and may be one way in which *V. cholerae* maintains its presence in an aquatic habitat, by transfer from host to host (Runft et al., 2014).

This new model for *V. cholerae* provides a more natural route of infection of any previous animal model. Furthermore, it goes beyond most animal models as it provides a method for exploring the environmental lifestyle of the organism and transmission characteristics of the pathogen from infected to non-infected host. Further study needs to be done to determine how colonization of the fish gut may prime the organism for infection of humans or allows the organism to become physiologically competent for transmission and subsequent infection of humans.

4. Summary

The zebrafish infectious disease model has emerged as a highly useful tool for analyzing pathogenesis caused by a number of organisms. More recently, analyses of aquatic pathogens in the zebrafish model have provided new information on how fish can act as environmental reservoirs for both human and aquatic infections. The zebrafish presents an ideal model in which to study these interactions because of its aquatic lifestyle as well as the ability accurately mimic disease characteristics observed during human infections. The ability of the aquatic pathogens to persist in the environment is a determining factor in the subsequent transmission to humans. Identifying the mechanisms required for successful pathogen colonization in the aquatic organisms most certainly will lead to future strategies to eliminate the pathogens from the aquatic environment in an effort to limit subsequent transmission to humans. In this way, instead of treating infections after they occur in the human host, we may be able to stop initial transmission. Here we focused on those pathogens that are found in the aquatic environment and serve as environmental reservoirs for infection of fish species and, importantly, as a mode of transmission to humans.

Acknowledgments

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Highlights

- Review of analyses of aquatic bacterial pathogens in the zebrafish model

- Fish can serve as environmental reservoirs for human disease

- Zebrafish accurately mimic the clinical pathologies caused by aquatic bacterial pathogens
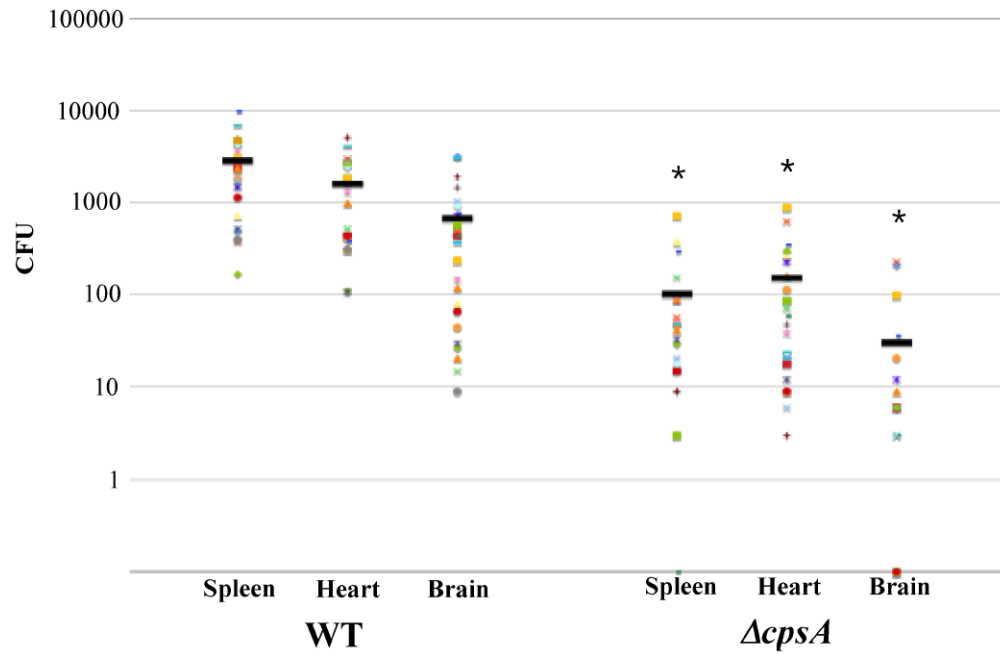
Figure 1. Dissemination of *S. agalactiae* during zebrafish infection
Adult zebrafish were injected intramuscularly with 10^6 CFU of *S. agalactiae* strain 515 or its isogenic ΔcpsA derivative. Four hours post injection, fish were euthanized and spleens, hearts and brains were isolated. Organs were homogenized and CFUs enumerated by viable plate count. Each dot represents 1 fish. (* p<0.01).
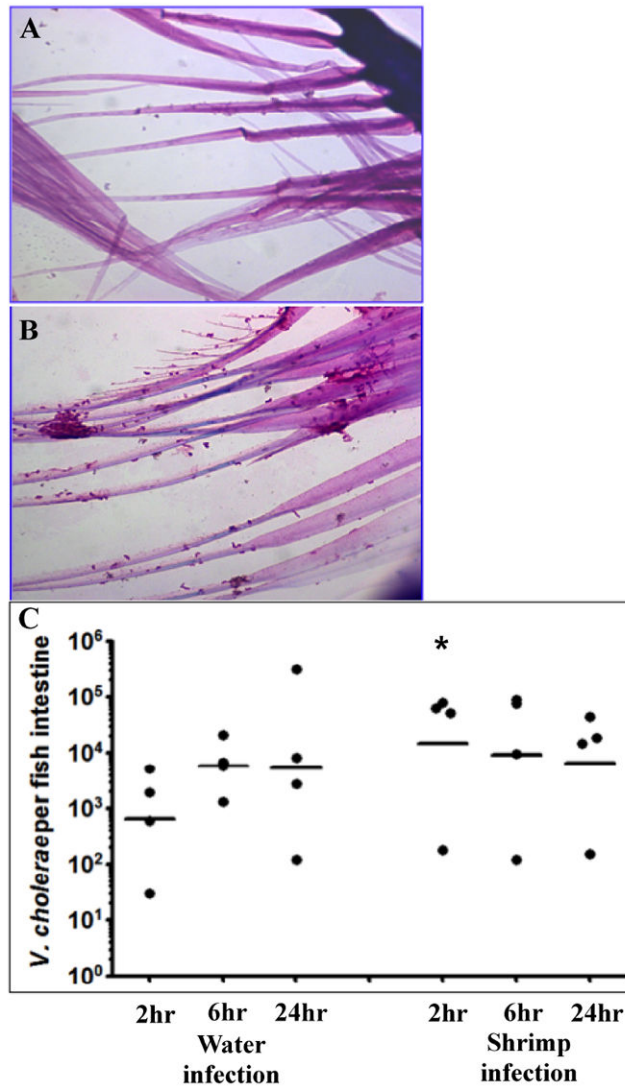
Figure 2. *Vibrio cholerae* infection of zebrafish using brine shrimp as a vehicle
Brine shrimp cultures are produced using brine shrimp eggs (Artemia cysts) and a brine shrimp hatchery (Aquatic Eco-systems). Freshly hatched brine shrimp (< 24 hours post hatch) were removed from the hatchery and washed 3X in tap water to remove excess salt. One hundred microliters of washed brine shrimp were added to 1 ml of 10^8 cfu of *V. cholerae* and mixed gently on a rotator for 15 minutes at room temperature. A). Hematoxylin and eosin (H & E) stained brine shrimp without *V. cholerae*. B). H & E stained brine shrimp after incubation with *V. cholerae*. C). Time course of colonization for fish infected after water exposure or using brine shrimp as a vehicle. Each dot represents the data from one fish and the horizontal bar indicates the mean bacterial load per fish. Total colonization per intestine was calculated after plating serial dilutions of intestinal homogenate 24 hr post infection. * $p < 0.05$.

Table 1

Aquatic bacterial pathogens that cause disease in fish and humans.

Organism	Symptoms in fish	Symptoms in humans	References
Aeromonas hydrophila	hemorrhagic septicemia, red sore disease and ulcerative infections	mild gastroenteritis, septicemia, necrotizing fasciitis and myonecrosis	(Janda and Abbott, 2010)
Edwardsiella tarda	septicemia, small cutaneous lesions, necrotic abscesses, petechial hemorrhaging of the fin and skin, rectal hernia, edema of the abdomen	Gastroenteritis, intestinal colitis, soft tissue infections, meningitis, peritonitis, septicemia, bacteremia, endocarditis and urosepsis	(Janda and Abbott, 2010; Mohanty and Sahoo, 2007; Schlenker and Surawicz, 2009; Tamada et al., 2009; Wang et al., 2005)
Mycobacterium marinum	skin erythema, raised scales, swollen abdomen, granulomatous lesions in the peritoneum, kidneys and ovaries	granulomatous skin and soft tissue infection	(Bonamonte et al., 2013; Gao et al., 2004; Huang et al., 2012; Jacobs et al., 2009; Wu et al., 2012)
Streptococcus agalactiae	sepsis and meningitis	early onset neonatal sepsis and meningitis	(Edmond et al., 2012; Evans et al., 2002; Skoff et al., 2009)
Streptococcus iniae	systemic infection with accompanying meningitis and panophthalmitis	bacteremic cellulitis with the rare complications of endocarditis, meningitis, arthritis, sepsis and toxic shock	(Agnew and Barnes, 2007; Bromage and Owens, 2002; Weinstein et al., 1996a; Weinstein et al., 1996b)
Vibrio alginolyticus	septicemia	gastrointestinal, wound and mucosal infections	(Blake et al., 1980; Dechet et al., 2008)
Vibrio cholerae	colonization of the intestinal track, mild edema, diarrhea	cholera disease causing dehydration and diarrhea	(Austin, 2010; Finkelstein and Oren, 2011; Runft et al., 2014; Sack et al., 2004)
Vibrio vulnificus	external haemorrhaging and ulcers, haemorrhaging of the gills, heart, liver and spleen	soft tissue infections, primary septicemia	(Blake et al., 1980; Dechet et al., 2008)