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Attenuated *Mycobacterium marinum* protects zebrafish against mycobacteriosis

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Fish mycobacteriosis is a common bacterial disease in many species of freshwater and marine fish (Decostere, Hermans & Haesebrouck 2004). Infections are common in wild fish, aquaculture and ornamental fish (Jacobs, Stine, Baya & Kent 2009). This chronic progressive disease is commonly caused by aquatic *Mycobacterium* spp. such as *M. marinum*, *M. fortuitum* and *M. chelonae* (Decostere *et al.* 2004), although some previously recognized human mycobacteria such as *M. haemophilum* and *M. peregrinum* have also been found in zebrafish during mycobacteriosis outbreaks (Kent, Whipps, Matthews, Florio, Watral, Bishop-Stewart, Poort & Bermudez 2004). Fish mycobacteriosis usually leads to systemic infections, which have reportedly caused massive mortalities among fish grown in intensive aquaculture systems (Bruno, Griffiths, Mitchell, Wood, Fletcher, Drobniewski & Hastings 1998; Diamant, Banet, Ucko, Colorni, Knibb & Kvitt 2000). With the remarkable increase in fin fish culture, there has been a concurrent increased concern about mycobacteriosis in cultured species (Ostland, Watral, Whipps, Austin, St-Hilaire, Westerman & Kent 2008). In addition to acute mortalities, chronic mycobacterium infections may result not only in low level mortality but also in reduced growth or marketability of fish due to macroscopic lesions. In addition, *Mycobacterium* spp. infecting fish are all potentially zoonotic (Tchornobay, Claudy, Perrot, Levigne & Denis 1992; Vazquez & Sobel 1992; Parent, Salam, Appelbaum & Dossett 1995; Lehane & Rawlin 2000). There are a few reports of treatment of infections with antibiotics (Santacana, Conroy, Mujica, Marin & Lopez 1982; Lawhavinit, Hatai, Kubota, Toda & Suzuki 1988; Conroy & Conroy 1999), but these have been limited and mostly at the experimental level. Presently, there are no effective drugs for treating food fish on a commercial scale.

Vaccination for mycobacteria has limited success in mammalian systems. The BCG vaccine prepared from *Mycobacterium bovis* bacillus Calmette-Guerin is the most widely used vaccine worldwide for human and bovine tuberculosis. It is a live attenuated vaccine, which is effective against severe forms of childhood tuberculosis, but appears to have limited effects against adult pulmonary disease (Martin 2006; Liu, Tran, Leung, Alexander & Zhu

2009). Many alternative vaccine candidates against tuberculosis have been developed and actively evaluated in clinical trials, including plasmid DNA, subunit and alternative live attenuated vaccines (Hernandez Pando, Aguilar, Infante, Cataldi, Bigi, Martin & Gicquel 2006; Huygen 2006; Martin 2006; Gupta, Katoch & McMurray 2007; Liu *et al.* 2009). Vaccine candidates based on rational attenuated mutants of *Mycobacterium tuberculosis* seem very promising (Hernandez Pando *et al.* 2006; Liu *et al.* 2009). While there are no reports of live vaccines for mycobacteriosis in fish, a DNA vaccine encoding a major secreted fibronectin-binding protein of aquatic *Mycobacterium* spp. was shown to provide protection to hybrid-striped bass against an *M. marinum* challenge (Pasnik & Smith 2005). However, it is unclear whether such a DNA vaccine will become commercially viable for fish immunization because it is based on a single antigen. In addition, DNA vaccines in general are still in the experimental stage despite of years of intensive research (Cui 2005). Similarly, killed mycobacteria and the extracellular products of *Mycobacterium* spp. were shown to induce cell-mediated responses and antibody responses, respectively, when injected into rainbow trout, but there are no data showing whether the immune responses afforded the trout protection against a mycobacterial challenge (Bartos & Sommer 1981; Chen, Yoshida, Adams, Thompson & Richards 1996). Therefore, there continues to be a need to develop an efficacious vaccine against fish mycobacteriosis.

In the present study, we preliminarily evaluated the feasibility of prophylaxis against fish mycobacteriosis by immunizing zebrafish, *Danio rerio* (Hamilton), with a candidate vaccine based on a live *M. marinum* mutant (L1D) that had impaired ability to replicate in macrophages (Ramakrishnan, Federspiel & Falkow 2000). As controls, fish were also immunized with the extracellular culture filtrate proteins (CFPs) from *M. marinum* culture or a heat-killed *M. marinum* strain, both adjuvanted with polyinosinic-polycytidylic acid [(poly(I:C)], a synthetic double-stranded RNA (dsRNA). Poly(I:C) is a ligand of Toll-like receptor 3 (TLR3) (Alexopoulou, Holt, Medzhitov & Flavell 2001) and has been confirmed to be present in fish (Bilodeau & Waldbieser 2005; Phelan, Mellon & Kim 2005; Novoa, Romero, Mulero, Rodriguez, Fernandez & Figueras 2006). The interaction between dsRNA and TLR3 promotes hosts to initiate innate immune responses and orchestrates the transition from innate to adaptive immune responses (van Duin, Medzhitov & Shaw 2006). The poly(I:C) was used in this study as an adjuvant to enhance the immune responses induced by the CFPs and heat-killed *M. marinum* strain. The commercial applicability of the poly(I:C) for massive vaccinations is likely to be limited due to its potential toxicity (Giantonio, Hochster, Blum, Wiernik, Hudes, Kirkwood, Trump & Oken 2001). Highly virulent *M. marinum* OSU-214 was cultured in Middlebrook broth (7H9 with ADC) (Becton-Dickson) in a shaker incubator at room temperature for 5 days. To prepare the CFPs, the culture was centrifuged (3500 g for 20 min at 4 °C) (Wedlock, Denis, Skinner, Koach, De Lisle, Vordermeier, Hewinson, Van Drunen Littel-Van Den Hurk, Babiuk, Hecker & Buddle 2005). The supernatant was sterile-filtered twice through a 200 nm filter and dialysed against distilled water using a 5-kDa molecular weight dialysis tube (Spectrum Chemicals and Laboratory Products). The CFPs were lyophilized and stored at -80 °C. The protein content in the CFP preparation was determined using a Bio-Rad Quick Start Bradford Protein Assay Kit (Hercules). To prepare the killed OSU-214 bacteria, the pellets after centrifugation were re-suspended in distilled water, dialysed overnight against water and incubated in a 90 °C oven for 1 h. The killed bacteria were then freeze-dried and stored at -80 °C. Again, the total protein content in the killed bacterial preparation was quantified. Both the CFP and the killed bacterial preparations were confirmed to be free of live bacteria when cultured on 7H9 agar plates. The *M. marinum* mutant L1D was grown in 7H9 broth similarly (Ramakrishnan *et al.* 2000). The bacteria were harvested by centrifugation and washed with sterile phosphate buffered saline (PBS, 10 mm, pH 7.4) three times, re-suspended in PBS, and used immediately.

Zebrafish were from the Zebrafish International Resource Center (ZIRC) (Eugene, OR) and maintained in a BSL-2 fish facility in the Microbiology Department in Oregon State University. Fish were randomly assigned into four groups. They were injected intraperitoneally with sterile PBS, the mixture of CFPs and poly(I:C) dissolved into PBS, killed OSU-214 suspended together with poly(I:C) into PBS, or the live L1D *M. marinum* mutant (3.5×10^4 per fish). The dose of poly(I:C) was 0.5 lg per fish. The zebrafish were around 0.5 g. Fish were immunized on days 0 and 10. Water temperature in tanks was maintained at 27–28 °C. Ammonia and nitrite levels were monitored daily using test kits and water changes were performed periodically. In addition, box-type aquarium filters with porous lava rock were placed into each tank for biological filtration. Fish were fed twice daily (Zeigler adult zebrafish diet, Zeigler Bros Inc.). About 10 fish from each group were euthanized and bled on day 25. On day 26, the remaining fish were intraperitoneally injected with 5×10^4 of live *M. marinum* OSU-214 and monitored for 62 more days. Dead fish were carefully examined, and their liver homogenate was occasionally cultured to confirm the presence of *M. marinum*. Specific antibodies (Abs) against the CFPs or the killed OSU-214 bacteria were determined using ELISA as described previously (Sloat & Cui 2006), except that the plates were coated with either the CFPs or the killed OSU-214 (100 ng per well), and that the secondary Ab was replaced with a protein A-peroxidase conjugate (1:500-fold dilution, Sigma-Aldrich).

As shown in Fig. 1, strong Ab responses against both the CFPs and the killed OSU-214 bacteria were induced by all three vaccine candidates, suggesting that there were antigens that were presented in both the CFPs and the bacteria. However, our preliminary data showed that only those fish that were immunized with the live attenuated L1D *M. marinum* mutant were protected against a challenge using the virulent *M. marinum* OSU-214 strain (Fig. 2, $P = 0.003$, Live L1D vs. PBS). Apparently, the strength of the Ab response against mycobacterial CFPs and the killed bacteria was not correlated with the protection against *M. marinum* infection. This was probably because mycobacteria reside intracellularly in macrophages and cellular immune responses were required to control them. In fact, efforts on identifying correlates to protective immunity against mycobacteria are mainly focused on cellular immune responses, with whole blood interferon-gamma being the best available correlate (Ellner, Hirsch & Whalen 2000; Buddle, Wedlock, Denis & Skinner 2005; Maglione & Chan 2009). Alternatively, it may be because fish produce only IgM-like antibodies, which are of intrinsic low avidity and lack affinity maturation (Bengtén, Clem, Miller, Warr & Wilson 2006). Fish circulating antibodies are often non-protective and even high levels of specific antibodies often do not correlate with protection or disease status (Alvarez-Pellitero 2008). Finally, it should be noted that the L1D *M. marinum* strain has a kanamycin insertion mutation in its mag 24–1 gene (macrophage activated gene) (Ramakrishnan *et al.* 2000). It is possible that the mutant strain will reacquire virulence once introduced back into a live host. The re-acquisition of virulence is a recurrent phenomenon that has often prevented the application of vaccines based on attenuated pathogens (Hofmann-Lehmann, Vlasak, Williams, Chenine, McClure, Anderson, O'Neil & Ruprecht 2003). Thus, an alternative, more stable mag 24–1 mutant strain may need to be created in the future.

In conclusion, our preliminary data showed that immunization of zebrafish with an attenuated live *M. marinum* vaccine reduced mycobacteriosis. Further studies with other fish species are needed to determine whether this phenomenon can be extended to commercially important aquaculture species.

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Figure 1.

(a) Anti-CFP Abs and (b) anti-killed bacterial Abs induced in zebrafish by all three vaccine candidates, CFPs/poly(I:C), killed OSU214/poly(I:C) and live L1D. Blood samples from 10 fish in every group were pooled and assayed three times. Data shown are the mean $\pm$ SD.

Figure 2.

Only the live attenuated L1D vaccine candidate significantly protected zebrafish from a challenge using the virulent *Mycobacterium marinum* OSU-214 strain. The initial number of fish in each group was around 20. Log Rank analysis of Kaplan–Meier survival curves indicated that the Live L1D curve was significantly different from that of the other groups (Live L1D vs. PBS, $P = 0.003$; L1D vs. CFP, $P = 0.001$; L1D vs. killed OSU-214, $P = 0.0004$).