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Zebrafish as a useful model for zoonotic *Vibrio parahaemolyticus* pathogenicity in fish and human

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ABSTRACT

Vibrio parahaemolyticus is an important aquatic zoonotic pathogen worldwide that causes vibriosis in many marine fish, and sepsis, gastroenteritis and wound infection in humans. However, the pathogenesis of different sources of *V. parahaemolyticus* is not fully understood. Here, we examined the pathogenicity and histopathology of fish (*V. parahaemolyticus* 1.2164) and human (*V. parahaemolyticus* 17) strains in a zebrafish (*Danio rerio*). We found that different infection routes resulted in different mortality in zebrafish. Moreover, death due to *V. parahaemolyticus* 1.2164 infection occurred quicker than that caused by *V. parahaemolyticus* 17 infection. Hematoxylin-eosin staining of liver, kidney and intestine sections showed histological lesions in all three organs after infection with either strain. *V. parahaemolyticus* 1.2164 caused more severe damage than *V. parahaemolyticus* 17. In particular, *V. parahaemolyticus* 1.2164 treatment induced more serious hydropic degeneration and venous sinus necrosis in the liver than *V. parahaemolyticus* 17 treatment. The expression levels of three proinflammatory cytokines, interleukin 1β (*il1β*), interferon phi 1 (*ifnϕ1*) and tumor necrosis factor α (*tnfα*), as determined by quantitative real-time PCR, were upregulated in all examined tissues of infected fish. Notably, the peak levels of *tnfα* were significantly higher than those of *il1β* and *ifnϕ1*, suggesting, together with pathological results, that *tnfα* and *il1β* play an important role in acute sepsis. High amounts of *tnfα* may be related to acute liver necrosis, while *ifnϕ1* may respond to *V. parahaemolyticus* and play an antibacterial role for chronically infected adult zebrafish. Taken together, our results suggest that the zebrafish model of *V. parahaemolyticus* infection is useful for studying strain differences in *V. parahaemolyticus* pathogenesis.

1. Introduction

The zebrafish (*Danio rerio*), with a complete (innate and adaptive) immune system, could be an efficient animal model for immunity and infectious disease because it is convenient to obtain specific mutant zebrafish for elucidation of pathogenicity (Lieschke and Currie, 2007; H Meijer and Spaink, 2011; Haenen et al., 2013; Yang et al., 2014). In comparison to other established vertebrate infection models such as mice and rats, the advantages of the zebrafish model include small size, rapid growth, relatively short life cycle, ease of breeding, and a transparent body in early life stages, allowing efficient genetic screens and real-time visualization (Kanther and Rawls, 2010; Goldsmith and Jobin, 2012; Kanwal et al., 2014; Rowe et al., 2014; Runft et al., 2014). Recently, zebrafish have been used for investigating *in vivo* host–pathogen interactions (van der Sar et al., 2004; Allen and Neely, 2010; Kanther and Rawls, 2010; Kanwal et al., 2014). This animal hosts Gram-positive and Gram-negative bacteria, fungi, mycobacteria, protozoa and viruses (Sullivan and Kim, 2008; Goody et al., 2014; Gratacap and Wheeler, 2014). Indeed, the zebrafish infectious disease model has emerged as an effective system for examining aquatic pathogens, both in the aquatic environment and in infection of humans (Rowe et al., 2014).

Abbreviations: TCBS, thiosulfate citrate bile salts sucrose; CFU, colony-forming unit; i.m. injection, intramuscular injection; i.p. injection, intraperitoneal injection; hpi, hour post infection; *tlh*, thermostable hemolysin gene; *il1β*, interleukin 1β; *ifnϕ1*, interferon phi 1; *tnfα*, tumor necrosis factor α.

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The *Vibrio parahaemolyticus*, a Gram-negative bacterium and an important zoonotic pathogen, was first isolated by Fujino et al. in Japan in 1950 from explosive food poisoning (Broberg et al., 2011). It is widely distributed in marine–estuarine environments, and causes vibriosis of fish, shellfish and other aquatic animals. In humans, waterborne *V. parahaemolyticus* can cause mild to severe infections, including wound infections, gastroenteritis, and septicemia (Hlavsa et al., 2011). Since its discovery, pathogenesis of *V. parahaemolyticus* has been examined in several cell lines as well as mammalian animal challenge models (Calia and Johnson, 1975; Brown et al., 1977; Boutin et al., 1979; Hoashi et al., 1990; Takahashi et al., 2000; Park et al., 2004; Kodama et al., 2008; Vongxay et al., 2008; Hiyoshi et al., 2010; Pineyro et al., 2010). However, these systems have not been used to compare the pathogenesis of distinct *V. parahaemolyticus* strains from different sources. In recent years, zebrafish have been used to study the pathogenesis of several *Vibrio* species, such as *Vibrio vulnificus* (Pan et al., 2011) and *Vibrio cholerae* (Runft et al., 2014), but there has been no direct comparison of infectious effects caused by the same pathogen from different hosts. *V. parahaemolyticus* is a very diverse and complex species, and shows signs of significant strain-specific differences (Izutsu et al., 2008; Okada et al., 2009; Li et al., 2014). In order to determine its role in epidemiology, we tested for virulence of, and other innate immune response characteristics to, *V. parahaemolyticus* strains isolated from different clinical and diseased fish sources using the zebrafish model.

On the basis that the sources of the two strains are different, we hypothesized that a *V. parahaemolyticus* fish pathogenic strain (*V. parahaemolyticus* 1.2164) and a human pathogenic strain (*V. parahaemolyticus* 17) would have different pathogenicity to zebrafish, including gross symptoms, histopathology and inflammatory cytokine levels. TNFα, known to kill cancer cells but which causes acute liver and nerve cell necrosis at high doses, is the first cytokine released in the inflammatory immune response and leads to the downstream expression of IL-1β and chemokines and mainly produced by macrophages, whereas TNFβ is mainly produced by T lymphocytes. Cytokine IL-1β is the key proinflammatory cytokine, one of the first cytokine genes discovered in fish, known to stimulate inflammation. So, we selected *il1β* and *tnfα* for our cytokine analyses. Zebrafish IFNs are classified into two subfamilies, type I and II, on the basis of the cognate receptors they interact with and the subsequent immune responses they initiate (Zou and Secombes, 2011). Zebrafish type I IFNs, named interferon-phi (IFNϕ) are classified into two groups: group I (comprising IFNϕ1 and IFNϕ4) and group II (IFNϕ2 and IFNϕ3) (Stein et al., 2007; Zou et al., 2007; Hamming et al., 2011), and only group I zfIFN was able to protect the fish against bacterial infection (Lopez-Munoz et al., 2009). Recently, more and more researchers have selected the isoform IFNϕ1 (alternative names IFN, IFN1, IFN-a1) to evaluate the inflammatory level induced by various pathogens (Xiong et al., 2012; Varela et al., 2014; Feng et al., 2015). Thus, we also chose to analyze *ifnϕ1* levels.

Here, we describe the first direct comparison in an adult zebrafish model of pathogenic features of fish and human *V. parahaemolyticus* strains at the individual, tissue and molecular levels. We demonstrate that zebrafish are susceptible to *V. parahaemolyticus* infection and can distinguish the virulence of different strains. We use the typical proinflammatory cytokines *il1β*, *tnfα* and *ifnϕ1* to investigate the immune response in zebrafish challenged by *V. parahaemolyticus*. We show that *tnfα* and *il1β* play a pivotal role in *V. parahaemolyticus* infection and are relevant to the process of acute sepsis. High doses of *tnfα* may be related to acute liver necrosis. We also find that *ifnϕ1* plays a key antibacterial role in chronic adult zebrafish infection. These results provide a proof of principle for understanding zoonotic *V. parahaemolyticus* pathogens in the zebrafish model.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The fish pathogenic bacterial strain *V. parahaemolyticus* 1.2164, purchased from the China General Microbiological Culture Collection Center (CGMCC), and the human pathogenic strain *V. parahaemolyticus* 17, kindly provided by Dr. Jian Wang, Shanghai Animal Disease Prevention and Control Center, were routinely grown overnight in thiosulfate citrate bile salts sucrose (TCBS) agar culture medium at 28 °C for *V. parahaemolyticus* 1.2164 and 37 °C for *V. parahaemolyticus* 17. Sucrose non-fermenting colonies were selected by streak plating on TCBS agar and inoculated into sterile nutrient broth supplemented with NaCl (3% w/v), then were grown overnight at 28 °C for *V. parahaemolyticus* 1.2164 and 37 °C for *V. parahaemolyticus* 17 with shaking at 150 rpm. Logarithmic phase cultures were obtained by dilution of the overnight culture with sterile nutrient broth supplemented with NaCl (3% w/v) at 1:10 and allowed growth for another 3 h at the appropriate temperature, with shaking. Cultures were harvested by centrifugation (2000 rpm), washed twice and resuspended in saline solution (0.85% NaCl). In the following procedures, bacterial suspensions were prepared with saline, and control groups were treated with saline in the same way as test groups. The concentrations of the two strains were determined by McFarland nephelometry and plate count methods (Aldridge and Schiro, 1994). Each treatment (as described below) was carried out in triplicate, with ten animals for each replicate. Saline was used as a vehicle treatment.

2.2. Zebrafish care and maintenance

Wild-type AB adult zebrafish (7–8 months old) used throughout this study were obtained from Shanghai Institute of Biochemistry and Cell Biology (SIBCB). Fish husbandry followed the methods of Westerfield (Westerfield, 2000) (also see http://zfin.org/zf_info/zfbook/zfbk.html). Zebrafish were transferred to a stand-alone unit with a separate flow-through system, and acclimated for 2 weeks before infection treatment. Zebrafish were handled according to the procedures of the Institutional Animal Care and Use Committee (IACUC) of Shanghai Ocean University, Shanghai, China. The proposed research methodology received clearance from the Shanghai Ocean University Experimentation Ethics Review Committee.

2.3. Adult zebrafish infections and bacterial quantification

2.3.1. Exposure by immersion only

The first method used to infect zebrafish was immersion only, in which zebrafish were exposed to 6.0×10^6, 6.0×10^7 or 6.0×10^8 CFU/mL (CFU, colony-forming unit) *V. parahaemolyticus* 1.2164 or *V. parahaemolyticus* 17, respectively, by static immersion for 5 h in a total volume of 600 mL of bacterial solution. Zebrafish were then moved to 3 L tanks and maintained for 96 h. Zebrafish were observed daily for mortality and signs of disease (Pressley et al., 2005).

2.3.2. Exposure by immersion following dermal abrasion

In the second method of immersion exposure, zebrafish were subjected to abrasion prior to immersion in *V. parahaemolyticus* 1.2164 or *V. parahaemolyticus* 17. Zebrafish were lightly anesthetized with 0.1% 3-aminobenzoic acid ethyl ester (tricaine). After the zebrafish had been sufficiently anesthetized (~4 min), they were lightly scraped along the lateral surface behind the pectoral fins with a sterile scalpel to remove several scales and scratch the underlying dermis before immersion, as described by Neely et al.