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Transparent Tiger barb *Puntius tetrazona*, a fish model for *in vivo* analysis of nocardial infection

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ABSTRACT

Nocardiosis afflicts multiple species of cultured fish, resulting in substantial economic losses to the aquaculture industry, however, lack of detailed knowledge on disease pathogenesis has hampered the development of effective prevention and control strategies. In this study, we injected a green fluorescent protein (GFP)-labeled *Nocardia seriolae* strain into a transparent mutant strain of Tiger barb (*Puntius tetrazona*) to monitor tissue pathogen accumulation and tissue damage *in vivo*, and to clarify the relationship between pathogenic processes and overt symptoms. GFP-labeled bacteria were phagocytized by leukocytes and could proliferate within these cells, which in turn led to leukocyte aggregation, leukocyte death, and granuloma formation. In addition, intracellular bacteria could permanently colonize various tissues *via* leukocyte circulation, causing multi-organ infection as revealed by changes of tissue transparency. Histology revealed granulomatous lesions in organs such as muscle, kidney, and spleen that was corresponded to the tissue opacities *in vivo*. Confocal microscopy confirmed massive accumulations of GFP-labeled bacteria within these granulomas, which often contained a necrotic core. Tiger barb transparency allows for real-time observation of *in vivo* pathological changes within the same animal, and the pathogenic process can be evaluated based on the shape and size of body opacities. Thus, transparent Tiger barb is a promising model to study the pathogenesis of nocardiosis.

1. Introduction

Fish nocardiosis is a chronic infectious disease that is generally not accompanied by acute symptoms during the early stage, but gradual tissue invasion eventually forms nodular lesions in internal organs such as kidney, liver, and spleen. Due to the absence of early observable symptoms and prolonged disease course, nocardiosis in aquaculture is usually not detected until the midanaphase of breeding, resulting in substantial economic losses to breeders because most setup costs and labor have been invested and drug efficacy is relatively poor (Chen et al., 2000; Wang et al., 2007, 2009; Elkesh et al., 2013). The causative pathogen *Nocardia* has a wide epidemic area all over the world and a very broad host range from cold-water fish such as Atlantic salmon (*Salmo salar*) to tropical fish such as African catfish (*Clarias gariepinus*) and Snubnose pompano (*Trachinotus blochii*) (Bransden et al., 2000; Vu-Khac et al., 2016). In China, *Nocardia seriolae* has become a major pathogen in the aquaculture of fish such as Large yellow croaker (*Larimichthys crocea*) and Largemouth bass (*Micropterus salmoides*) (Chen et al., 2000; Wang et al., 2005).

Nocardia spp. are opportunistic pathogenic bacteria that primarily infect immunocompromised or injured fish. With increased intensification of aquaculture, fish are more prone to weakened constitutions and physical injury, thus enhancing infection risk. Indeed, nocardiosis in cultured fish are on the rise, but pathogenesis and spread are poorly understood. Since the first report of fish nocardiosis in Argentina in 1963, research on this disease has focused mainly on epidemiology (Itano et al., 2006; Shimahara et al., 2009), and there are currently no broadly effective methods for early detection or control within infected populations.

Fishes are usually transparent during the early stage of development, and this characteristic has been exploited for real-time studies. With chromatophore development, however, internal structures become more opaque, so continued real-time analysis requires anatomic sampling or advanced imaging methods (Zacharakis et al., 2005; Kelsh et al., 2009; Levraud et al., 2009). In a previous study, a Tiger barb (*Puntius tetrazona*) mutant population with prolonged body transparency was established due to a lack of iridophores. This breed was used to study inflammatory responses and pathological changes in real time following *in vivo* infection by *Pleistophora* (Li et al., 2012). The current study demonstrates that transparent Tiger barb is also a sensitive host

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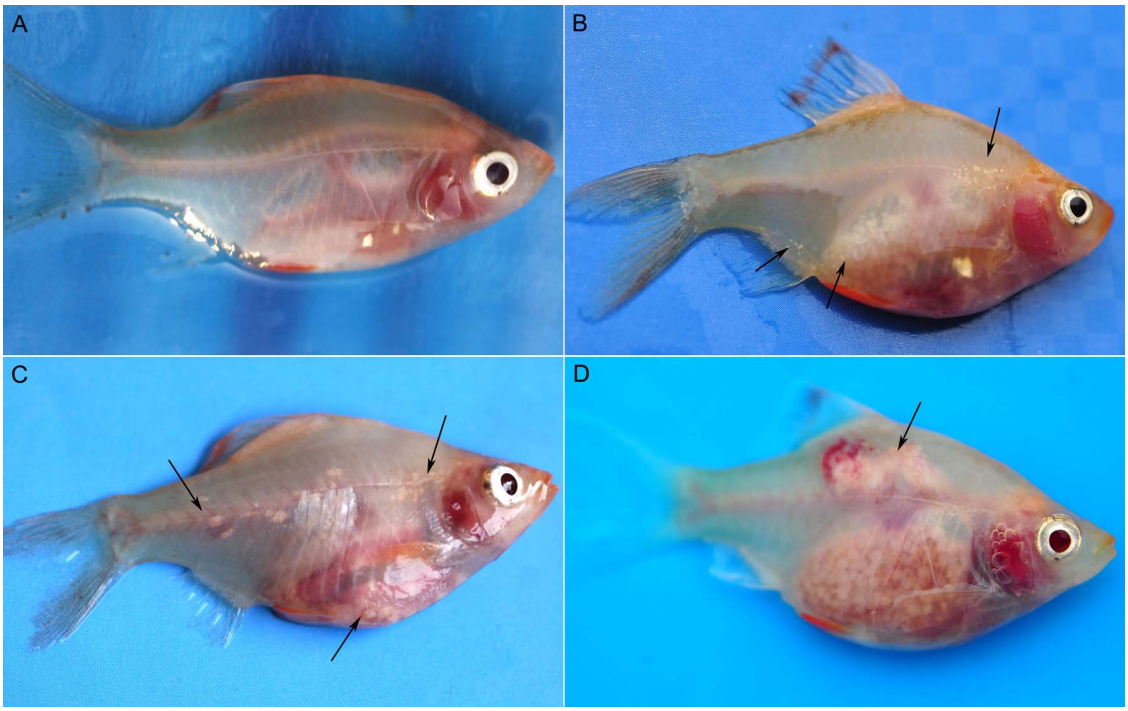

Fig. 1. Distribution and progression of lesions following *N. seriolae* injection. Control fish injected with saline remained transparent throughout, and internal organs could be directly observed (A). Following intraperitoneal injection, punctate regions of turbidness (arrows) appeared all over the body (B), particularly within the abdominal cavity. Symptoms worsened gradually with time (C). Intramuscular injection resulted in large turbid lesions (arrow) at the infection site (D).

for *N. seriolae*, and infection can be monitored in real time by observing changes in tissue opacity associated with granuloma formation. Thus, transparent Tiger barb may be a useful model for studies of nocardial transmission and pathogenesis.

2. Materials and methods

2.1. Experimental fish and bacterial isolation

The transparent mutant breed of Tiger barb was selected, bred, and preserved in our laboratory. The breed is iridophore- and melanophore-deficient, so internal organs remain transparent throughout development. Six-month-old individuals, with 4.74 ± 0.45 cm in length and 2.73 ± 0.31 g in mass, were selected for this study.

N. seriolae strain ZJLN4, was isolated from an infected hybrid snakehead (*Channa argus* × *C. maculate*) and preserved in our laboratory. The fluorescent plasmid pRUALPGEN was generously donated by Dr. Mario C Salinas-Carmona, Universidad Autonoma de Nuevo Leon. Electrotransformation and positive bacterial screening were performed with reference to the Salinas-Carmona and Rocha-Pizaña method (2011). The positive strain was rejuvenated *via* snakehead infection.

2.2. Injection of Nocardia seriolae

Bacterial suspensions at 1.0×10^6, 1.0×10^7, and 1.0×10^8 cfu/mL were prepared in normal saline. Tiger barbs were anaesthetized using 0.2 mg/mL tricaine methanesulfonate (MS-222) for artificial infection by intraperitoneal or intramuscular injection at these three concentrations. Each dose group consisted of 20 fish injected at 0.05 mL per animal. Control group fish were injected with equal-volume normal saline.

Control and infected fish were maintained in water at 27 ± 2 °C with an exchange rate of one quarter volume every 4 days. Fish were observed regularly for the appearance of white opacities. Bacteria were isolated from these white turbid sites of dying fish by BHI plate culture. Infected tissues were also collected and prepared as wet mounts for observation or histological sectioning.

2.3. In vivo tracing of labeled strains

Tiger barbs were infected with labeled bacteria at 1.0×10^8 cfu/mL by intraperitoneal injection. Twelve hours later, blood and peritoneal fluid were extracted, and labeled bacteria examined under a confocal microscope. Sampling was performed every 12 h to trace the distribution of labeled bacteria in all fish organs.

2.4. Histopathological observation

Tiger barb tissues were fixed in 4% (w/v) paraformaldehyde for 48 h, dehydrated in an ethanol gradient, made transparent using dimethyl benzene, and embedded in paraffin. A Leica RM-2145 rotary microtome was used to prepared 3 μm slices, which were stained with hematoxylin and eosin (H & E), and examined under light microscopy.

2.5. DAPI staining of infected tissues

Tissues with obvious infection symptoms such as muscle and intestines were harvested from dying Tiger barb, fixed in 4% paraformaldehyde, washed in PBS, dehydrated in cane sugar, embedded in optimum cutting temperature (O.C.T.) compound, and sectioned at 10 μm using a Leica freezing microtome. Sections were dried, immersed in DAPI staining solution (Beyotime) at room temperature for 3–5 min, washed two or three times with PBS or normal saline (3–5 min/wash), dried, mounted, sealed, and examined under a confocal microscope.

3. Results

3.1. Visible symptoms of Nocardia seriolae infection in tiger barb

As neither intramuscular injection nor intraperitoneal injection led to acute death, the *N. seriolae* infection in Tiger barb was relatively slow, consistent with the prolonged disease course observed in other