Aquaculture 448 (2015) 142–150

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Concurrent infections of *Flavobacterium columnare* and *Edwardsiella ictaluri* in striped catfish, *Pangasianodon hypophthalmus* in Thailand☆

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ARTICLE INFO

Article history:
Received 14 April 2015
Received in revised form 28 May 2015
Accepted 31 May 2015
Available online 2 June 2015

Keywords:
Edwardsiella ictaluri
Flavobacterium columnare
Striped catfish
Pangasianodon hypophthalmus

ABSTRACT

Flavobacterium columnare and *Edwardsiella ictaluri* are two major bacterial pathogens threatening catfish aquaculture globally. Earlier studies have focused on the characterization of single bacterial infection. In reality, multiple bacterial pathogens are present in aquaculture systems and are probably responsible for disease outbreaks and considerably outweigh single infection. The objectives of this study, therefore, were to investigate whether single or concurrent bacterial pathogens were involved in naturally diseased striped catfish (*Pangasianodon hypophthalmus*) and subsequently investigate the pathogenicity of single- and dual-infection through experimental challenges. The investigation revealed coinfections of *F. columnare* and *E. ictaluri* found in naturally diseased Thai striped catfish exhibiting columnaris and edwardsiellosis diseases. Bacterial identification was confirmed by phenotypic tests, species-specific PCR and 16S rDNA sequence analysis. Molecular data analysis also identified that the infected fish species was *P. hypophthalmus*. Experimental challenges of striped catfish juveniles with single and dual bacterial species using both immersion (i.m) and injection (i.p) approaches were performed. Injection of two different doses of combined bacteria caused markedly high mortality of 86.7–100%, indicating high virulence of the bacterial isolates. Immersion (i.m.) coinfection of *E. ictaluri* (2.6×10^6 CFU mL^{-1}) and *F. columnare* (1.0×10^4 CFU mL^{-1}) caused significantly high cumulative mortality ($96.7 \pm 5.8\%$) compared to i.m. of single infection of *E. ictaluri* ($80.0 \pm 20\%$) or *F. columnare* ($3.3 \pm 5.7\%$) with the same dose of bacteria. Both coinfection challenge routes i.p. and i.m. successfully mimicked typical signs and histopathological manifestations of natural coinfection. This study had fulfilled Koch's postulates through single- or dual-challenged tests to mimic the natural disease case in striped catfish.

Statement of relevance:

The authors strongly believe that our manuscript would provide significant knowledge to fish aquaculture especially to that of the striped catfish *P. hypophthalmus*.

1. Introduction

Striped catfish (*Pangasianodon hypophthalmus*) is native to the Mekong, Chaophraya, and Maeklong basins of Southeast Asia, including Cambodia, Laos, Viet Nam and Thailand (Poulsen et al., 2004; Robert and Vidthayanon, 1991). It has been introduced to other Asian countries such as Bangladesh, China, Indonesia, Malaysia, Myanmar, and India for aquaculture purposes (FAO, 2010–2011). The recent boom in striped catfish culture and significant export levels in Vietnam has brought global attention to the Asian catfish culture industry (Nguyen and Dang, 2009). The high stock density of intensively cultured farms, however, faces devastation through infectious pathogens such as channel catfish virus (Siti-Zahrah et al., in press), parasitic monogenea *Thaparocleidus caecus* and *Thaparocleidus siamensis* (Šimková et al., 2013; Tripathi et al., 2014) or important bacteria *Edwardsiella ictaluri*, *Edwardsiella tarda*, *Flavobacterium columnare*, and

☆ The GenBank accession numbers for 22 sequences reported in this paper are KR080244 to KR080265.

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http://dx.doi.org/10.1016/j.aquaculture.2015.05.046
0044-8486/

Aeromonas hydrophila (Crumlish et al., 2010; Panangala et al., 2007; Shetty et al., 2014). Among bacterial pathogens, *E. ictaluri* and *F. columnare* are recognized as the most highly pathogenic bacteria that cause enteric septicemia of catfish (ESC) and columnaris disease in freshwater fish respectively (Declercq et al., 2013; Hawke et al., 1981). *E. ictaluri* was reported in channel catfish (*Ictalurus punctatus*) in the United States (Hawke et al. 1981), walking catfish (*Clarias batrachus*) and hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) in Thailand (Boonyaratpalin and Kasornchan, 1986; Kasornchandra et al., 1987; Suanyuk et al., 2014), striped catfish in Vietnam and Indonesia (Crumlish et al., 2002; Ferguson et al., 2001; Yuasa et al., 2003), wild ayu (*Plecoglossus altivelis*) in Japan (Nagai et al., 2008), yellow catfish (*Pelteobagrus fulvidraco*) in China (Ye et al., 2009), as well as cultured Nile tilapia (*Oreochromis niloticus*) (Soto et al., 2012). *F. columnare* is one of the oldest known bacterial pathogens, having affected the global population of aquaculture freshwater fish species since the beginning of the last century (Bernardet, 1989; Bernardet and Bowman, 2006; Declercq et al., 2013). A wide range of fish hosts have been reported, 37 fish species were addressed by Anderson and Conroy (1969), and many economic aquaculture fish recently such as Nile tilapia (Figueiredo et al., 2005), red tilapia (*Oreochromis* sp.) (Dong et al., in press—a,b), Indian carp (*Catla catla*) (Vermaand and Rathore, 2013), striped catfish (Tien et al., 2012). It is noticeable that *F. columnare* was considered as the second most important bacterial pathogen threatening the U.S. catfish culture industry after *E. ictaluri* (Shoemaker et al., 2007). Earlier studies have focused on characterization of single pathogen infection. Most likely, the reality of the disease manifestation in cultured fish farms frequently occurred as the result of dual or multiple infections. Coinfection between bacteria and parasites has been primarily described in channel catfish and tilapia (Xu et al., 2007, 2012a,b). Understanding the coinfection of important bacterial pathogens, however, remains undetermined, especially in striped catfish. This study aims 1) to characterize a natural coinfection of *E. ictaluri* and *F. columnare* in cultured striped catfish and 2) to experimentally investigate pathogenicity of single and dual infection.

2. Materials and methods

2.1. Infected fish samples and experimental fish

Infected juvenile striped catfish *P. hypophthalmus* ($n = 20$) were obtained from two different hatcheries in Ratchaburi, a central province of Thailand through a commercial fish store in Bangkok in June, 2014. Affected fish exhibited typical clinical signs of both columnaris and edwardsiellosis diseases (see details in Section 3.1). For experimental challenge tests, healthy striped catfish juveniles (mean weight, 26.7 ± 8.7 g; mean length, 5.4 ± 0.7 in.) were kindly provided by Charoen Pokphand Company (Ayutthaya province, Thailand) and were allowed to acclimate in the aquarium for 11 days prior to infection. Prior to challenge tests, a subset of five fish were randomly subjected for bacterial isolation and found to be free of *F. columnare* and *E. ictaluri*. Remaining fish were observed by the naked eye and none of them had any clinical signs or any abnormalities.

2.2. Bacterial isolation and phenotypic assays

Infected fish ($n = 20$) were euthanized in ice-cold water before aseptically necropsied for bacterial isolation. Samples were collected by inserting a sterile metal bacteriology loop into the gills, skin lesion, kidney, and liver of each fish. Collected specimens were then streaked directly onto two different agar plates. Tryptic soy agar (TSA, Difco) plates supplemented with 5% bovine blood (Chulalongkorn University) were generally used for isolation of *Edwardsiella* sp. while Anacker and Ordal's medium (AO) supplemented with 1 μg mL^{-1} tobramycin (Sigma) was employed for culturing of *Flavobacterium* sp. Plates were incubated at 30 °C for 48 h (Dong et al., in press—a; Figueiredo et al., 2005; Hawke et al., 1981) and individual bacterial colonies were subcultured on respective agar plates to obtain pure isolates. Some conventional phenotypic assays including Gram staining, oxidase, catalase, oxidation/fermentation (O/F), and flexirubin pigment were performed as previously described by Bernardet (1989) and Crumlish et al. (2002). It was known later from the phenotypic assays and molecular data (see below) that the identified bacterial strains were *E. ictaluri* and *F. columnare*, respectively. Subsequently, six isolates of *E. ictaluri*, designated T1-1 to T1-3 and T2-1 and T2-3, and six individual colonies of *F. columnare*, named CF1 to CF6, were used in this study.

2.3. PCR amplification of bacterial DNA sequences

Universal primers targeting prokaryotic 16S rDNA (Weisburg et al., 1991) and species specific primers for *E. ictaluri* (Sakai et al., 2009) and *F. columnare* (Welker et al., 2005) used in this study are listed in Table 1. A PCR reaction volume of 20 μL contained a small amount of bacterial colony, 0.25 μM of each primer pair, 0.2 mM of dNTPs, 0.25 μM of $MgCl_2$, 1 unit of Taq polymerase (Invitrogen), and 1× reaction buffer. The PCR conditions were 94 °C for 5 min followed by 30 cycles of 94 °C for 40 s, annealing at 50 °C for 40 s and extension at 72 °C for 1 min/kb. PCR products were analyzed using 1.0% agarose gel electrophoresis.

2.4. PCR amplification of fish DNA sequences

Crude DNA extracts from fish samples were prepared according to a previous report (Kowasupat et al., 2014). Briefly, approximately 5 mg of fish muscle tissue was incubated with 180 μL of 50 mM NaOH at 95 °C for 10 min. The reaction was then neutralized by the addition of 20 μL of 1 M Tris–HCl (pH 8.0). DNA-containing supernatant was used for subsequent PCR reactions. Primers used for PCR amplification of fish DNA sequences listed in Table 1 included universal primers targeting eukaryotic 18S rDNA (Medlin et al., 1988), specific primers for fish COI (cytochrome c oxidase I), ITS (internal transcribed spacer), and RAG1 (recombinase activating gene 1). PCR reactions and thermocycling conditions were carried out using previously described protocols (Kowasupat et al., 2014).

2.5. DNA cloning and sequence analysis

Amplified DNA amplicons were gel purified using a Favogen Gel/PCR Purification Kit and cloned into pPrime cloning vector (5PRIME). Recombinant clones were verified by colony PCR (data not shown) prior to plasmid DNA purification using a FavoPrep Plasmid Extraction Mini Kit. DNA sequencing was performed by 1st BASE Pte Ltd. (Malaysia). A DNA sequence homology search was carried out using BLAST on the GenBank database. Multiple sequence alignments were performed by Clustal W (Thompson et al., 1994) and a Neighbor-Joining (NJ) tree and pairwise distance analysis were conducted using MEGA version 5 (Tamura et al., 2011) with 1000 replicates bootstrap values.

2.6. Challenge test by immersion

To minimize the effects from opportunistic pathogens, naïve striped catfish were treated with 1% NaCl for 20 min, kept in 0.1% NaCl for 1 day before being raised in pre-aerated freshwater. The treatment was repeated once after three days. Two bacterial isolates *E. ictaluri* T1-1 and *F. columnare* CF1 were used for challenged experiments. *F. columnare* CF1 and *E. ictaluri* T1-1 were cultured in AO broth and TSB, respectively at 30 °C with shaking (250 rpm) until reaching to an optical density of ~1.0 at 600 nm to get an expected density of ~10^8 CFU mL^{-1}. Conventional plate count method was then performed to determine the CFU mL^{-1}. For immersion challenge test, designed doses (see below) were prepared by diluting the cells in 50 L water. Fish were divided