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Comparative analysis of *Edwardsiella* isolates from fish in the eastern United States identifies two distinct genetic taxa amongst organisms phenotypically classified as *E. tarda*

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ABSTRACT

Edwardsiella tarda, a Gram-negative member of the family Enterobacteriaceae, has been implicated in significant losses in aquaculture facilities worldwide. Here, we assessed the intra-specific variability of *E. tarda* isolates from 4 different fish species in the eastern United States. Repetitive sequence mediated PCR (rep-PCR) using 4 different primer sets (ERIC I & II, ERIC II, BOX, and GTG_5) and multi-locus sequence analysis of *16S SSU rDNA, groEl, gyrA, gyrB, pho, pgi, pgm, and rpoA* gene fragments identified two distinct genotypes of *E. tarda* (DNA group I; DNA group II). Isolates that fell into DNA group II demonstrated more similarity to *E. ictaluri* than DNA group I, which contained the reference *E. tarda* strain (ATCC #15947). Conventional PCR analysis using published *E. tarda*-specific primer sets yielded variable results, with several primer sets producing no observable amplification of target DNA from some isolates. Fluorometric determination of G + C content demonstrated 56.4% G + C content for DNA group I, 60.2% for DNA group II, and 58.4% for *E. ictaluri*. Surprisingly, these isolates were indistinguishable using conventional biochemical techniques, with all isolates demonstrating phenotypic characteristics consistent with *E. tarda*. Analysis using two commercial test kits identified multiple phenotypes, although no single metabolic characteristic could reliably discriminate between genetic groups. Additionally, anti-microbial susceptibility and fatty acid profiles did not demonstrate remarkable differences between groups. The significant genetic variation (<90% similarity at *gyrA*, *gyrB*, *pho*, *phi* and *pgm*; <40% similarity by rep-PCR) between these groups suggests organisms from DNA group II may represent an unrecognized, genetically distinct taxa of *Edwardsiella* that is phenotypically indistinguishable from *E. tarda*.

1. Introduction

Edwardsiella tarda, a Gram-negative, motile, rod-shaped bacterium, is the causative agent of edwardsiellosis in a wide variety of cultured fish and has been implicated in significant losses in aquaculture worldwide (Mohanty and Sahoo, 2007). First described from humans (Ewing et al.,

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1965), it is the most widespread member of the *Edwardsiella* genera, having been reported from over 20 species of freshwater and marine fish from 25 countries in the Americas, Europe, Asia, Australia, Africa and the Middle East (Hawke and Khoo, 2004). In channel catfish, *E. tarda* is the causative agent of emphesematous putrefactive disease of catfish and was the first member of the genus described as a pathogen in channel catfish (Meyer and Bullock, 1973). Although traditionally considered less important than the closely related *E. ictaluri*, case submissions to the Aquatic Diagnostic Laboratory of the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS (http://tcnwac.msstate.edu/publications.htm) suggest *E. tarda* is a potential emerging disease in catfish aquaculture in the southeastern United States.

Several studies have demonstrated a wide degree of intraspecific diversity for *E. tarda* from different geographic regions and host species, making the development of broad-spectrum molecular based diagnostic tools difficult (Castro et al., 2006; Panangala et al., 2006, Acharya et al., 2007; Maiti et al., 2008; Maiti et al., 2009; Castro et al., 2011; Wang et al., 2011; Yang et al., 2012). As a result, several researchers have reported the development of *E. tarda*-specific PCR assays with varying levels of success (Chen and Lai, 1998; Sakai et al., 2007; Lan et al., 2008; Sakai et al., 2009). The purpose of this study was to determine if *E. tarda* isolates from fish in the eastern United States demonstrate the same level of intraspecific variability seen in other geographic regions, in turn providing baseline information for the development of more reliable molecular diagnostic tools.

2. Materials and methods

2.1. Isolation and identification of Edwardsiella tarda

A total of 47 *E. tarda* isolates were obtained from the archived collections of the Thad Cochran National Warmwater Aquaculture Center (NWAC), The Aquaculture/Fisheries Center of the University of Arkansas-Pine Bluff, The Department of Biological Sciences at Auburn University, The Aquatic Microbiology Laboratory at Auburn University and the Louisiana Aquatic Diagnostic Laboratory (LADL). All isolates were collected from diseased fish. Briefly, cryostocks were streaked for isolation on Mueller-Hinton agar plates supplemented with 5% sheep blood (Becton, Dickinson and Company, Sparks, MD) and grown overnight at 37 °C. Individual colonies were used for identification by the BBL™ Crystal™ Enteric/Nonfermenter ID kit (BD, Franklin Lakes, NJ, USA) following the manufacturer's suggested protocol. Isolates were grouped by biotype based on their identification code and two representatives from each biotype were chosen for further analysis with the exception of biotype 20, which had only 1 representative. An ATCC *E. tarda* isolate (#15947) as well as *Edwardsiella ictaluri* (S94-711; S97-773; S07-698) isolates obtained from diseased channel catfish were included in the analysis.

2.2. Conventional bacterial characterization

For phenotypic analysis, cryostocks were streaked for isolation on Mueller-Hinton agar plates supplemented with 5% sheep blood (Becton, Dickinson and Company, Sparks, MD) and grown overnight at 37 °C (28 °C for *E. ictaluri*). An individual colony from each isolate was then subcultured onto Trypticase Soy Agar (TSA; Difco, St Louis, MO) for 48 h at 37 °C and each isolate was then characterized by classical microbiological and biochemical tube tests and standardized procedures as described by MacFaddin (1981) and Lennette et al. (1985). All bacteriological media and supplies were prepared and sterilized according to manufacturer's recommendations. Based upon the results from individual tests, bacteria were classified according to referenced flow charts and identification schemes (Lennette et al., 1985; MacFaddin, 1981; Panangala et al., 2006).

2.3. DNA extraction

Individual colonies served to inoculate 5 ml of Brain Heart Infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD) overnight at 37 °C (*E. tarda*) or 28 °C (*E. ictaluri*) without shaking and cultures were pelleted by centrifugation. Genomic DNA from all isolates was extracted using the Puregene DNA Isolation Kit (Qiagen, Valencia, CA) following the manufacturer's suggested protocols for gram-negative bacteria and quantified spectrophotometrically (Nanodrop, Wilmington, DE, USA).

2.4. Edwardsiella tarda specific PCR

Genomic DNA from all isolates were analyzed using previously established protocols for PCR amplification of *Edwardsiella* spp., *E. ictaluri*, and *E. tarda* (Chen and Lai, 1998; Sakai et al., 2007; Lan et al., 2008; Sakai et al., 2009; Castro et al., 2011; Griffin et al., 2011) (Table 1). Briefly, the 25-μl PCRs consisted of EconoTaq PLUS GREEN 2X Master Mix (Lucigen Corporation, Middleton, WI, USA); 20 pmol of each primer, 5 ng of DNA template and nuclease-free H_2O to volume. Amplification cycles used for denaturation, primer annealing and extension were carried out according to the respective protocol. Aliquots of each amplification reaction (10 μl) were electrophoresed through a 1.25% (w/v) agarose gel, stained with ethidium bromide and visualized under ultraviolet light for the presence of the appropriate sized bands, determined by direct comparison with concurrently run DNA standards (Hyperladder II, Bioline USA inc., Taunton, MA, USA).

2.5. Repetitive sequence mediated PCR (rep-PCR)

Genetic fingerprinting for each isolate was carried out using modifications to existing protocols (Versalovic et al., 1991, 1994; Castro et al., 2011; Griffin et al., 2011) (Table 2). Three *E. ictaluri* isolates (S94-711; S97-773; S07-698) and an *Escherichia coli* (ATCC# 25952) were included in the analysis. Genomic DNA for these additional isolates was obtained as described above. Briefly, the analysis consisted of 25-μl reactions comprised of 13 μl of IQ Supermix (BioRad, Hercules, CA, USA), 20 (ERIC I and II) or 40 (BOX, ERIC II, GTG5) pmol of primer, 100 ng of DNA template and nuclease-free H_2O to volume. Amplifications were performed on a PTC 200 gradient cycler (MJ Research,