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Virulence and molecular variation of *Flavobacterium columnare* affecting rainbow trout in Idaho, USA

Jason P. Evenhuis [a,*], Haitham Mohammed [b], Scott E. LaPatra [c], Timothy J. Welch [a], Covadonga R. Arias [d]

[a] USDA-ARS, National Center for Cool and Cold Water Aquaculture, 11861 Leetown Rd, Kearneysville, WV 25430, USA
[b] Department of Animal Medicine, Faculty of Veterinary Medicine, Assiut University, Assiut 71526, Egypt
[c] Clear Springs Foods, Inc., Research Division, Buhl, ID 83316, USA
[d] School of Fisheries, Aquaculture, and Aquatic Sciences, 203 Swingle Hall, Auburn University, Auburn, AL 36849, USA

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ABSTRACT

Columnaris disease, caused by *Flavobacterium columnare*, is an emerging problem in the rainbow trout (*Oncorhynchus mykiss*) aquaculture industry of Idaho. All *F. columnare* isolates taken from disease outbreaks in the rainbow trout producing region of southern Idaho, and for rainbow trout, are all genomovar I. Virulence phenotyping of 11 genomovar I and 1 genomovar I/II isolates, taken from 6 different farms, suggests significant variation in virulence toward rainbow trout with the most virulent strains having an LD50 of 1×10^6 CFU/ml^{-1} when using a waterborne challenge model. The least virulent strain required 8×10^6 CFU/ml^{-1} to reach the LD50. AFLP fingerprinting of these strains and the resulting phylogenetic tree show that all of the strains tested had a percent similarity of 75% or higher, save one, the MS-FC-4 strain had <60% identity to all of the other strains tested. These results suggest strains of *F. columnare* isolated from disease outbreaks in rainbow trout production have a higher degree of diversity than previously thought.

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1. Introduction

Flavobacterium columnare was first described by Davis (1922), later isolated in 1944 (Ordal and Rucker, 1944) and is the etiological agent for columnaris disease. *F. columnare* is found in aquatic environments worldwide (Decostere et al., 1998; Austin and Austin, 1999), and columnaris disease is commonly associated with external lesions on the skin and gills (Decostere et al., 1999; Noga, 2000; Thune, 1993) of finfish in aquaculture production but can also be isolated from internal tissues (Hawke and Thune, 1992). Columnaris was estimated to cause up to 60% of bacterial disease loses in the catfish aquaculture industry (Olivares-Fuster et al., 2007a; Decostere et al., 1997, 1998; Thomas-Jinu and Goodwin, 2004) and results in millions of dollars of losses. Recently, columnaris disease has been characterized as an emerging problem for early-life stage rainbow trout (LaFrentz et al., 2012; Evenhuis et al., 2014).

F. columnare harbors a significant intraspecies diversity and it has been divided into three genomovar (designated I, II and III) based on DNA:DNA hybridization studies (Triyanto and Wakabayashi, 1999). For routine typing, genomovar ascription can be carried out by restriction fragment length polymorphism (RFLP) of the 16S rDNA gene (Triyanto and Wakabayashi, 1999). Recent studies by LaFrentz et al. (2012) and Olivares-Fuster et al. (2007a) have increased the number of subdivisions to include I-B, II-B and I/II genomovars by improving on the original RFLP assay.

A study by Olivares-Fuster et al. (2007b) suggested an association between host species and *F. columnare* genomovar where genomovar I isolates were commonly found in threadfin shad (*Dorosoma petenense*) and genomovar II isolates were found in channel catfish and fresh water drum. In that study, all fish species sampled shared the same ecosystem but there was a significant correlation between fish species and which genomovar they harbor. To date, all *F. columnare* isolates taken from rainbow trout outbreaks have been classified as genomovar I, except for one genomovar III isolate from Georgia GA-02-14 (Welker et al., 2005). A study describing the emergence of early life stage columnaris disease outbreaks in Idaho (Evenhuis et al., 2014) suggested the expansion of high virulent genomovar I strains in trout aquaculture. Over 70 isolates were tested by 16S rDNA RFLP, multilocus sequence typing and SDS-PAGE analysis. Only one banding pattern difference was observed by SDS-PAGE. In this study we take a closer look at the molecular make up and virulence phenotype of 11 additional *F. columnare* isolates taken from 5 different rainbow trout farms in the Idaho region, including the farm previously described by Evenhuis et al. (2014), as well as the alternative genomovar I/II strain isolated from yellow perch in Indiana. Herein we describe difference in virulence phenotypes, variations in genome fingerprints and differentiation of a targeted gene. This was done to define genetic and virulence variations amongst the *F.*

* Corresponding author.
E-mail address: jason.evenhuis@ars.usda.gov (J.P. Evenhuis).

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columnare isolates taken from the aquaculture region of Idaho, isolates that were thought to be the expansion of a closely related, highly successful group of *F. columnare*.

2. Method and materials

2.1 Fish husbandry

Rainbow trout specific-pathogen-free eyed eggs were acquired from Troutlodge Inc., Sumner, WA, USA, or from the even year breeding line at the national center for cool and cold water aquaculture (NCCCWA) (Leeds et al., submitted for publication). Hatched trout were hand fed daily to satiation using a commercially available trout feed (Ziegler Inc., PA) and maintained in flow through water at approximately 12 °C, till they were ~2 g in size (Evenhuis et al., 2014). All fish use was approved under institutional animal care and use committee review.

2.2 Bacterial isolates

All of the genomovar I isolates were obtained from diseased rainbow trout or water from raceways harboring diseased fish, while the genomovar I/II isolate (LaFrentz et al., 2014) was taken from yellow perch (Table 1). *Flavobacterium columnare* isolation was conducted according to a published protocol (Decostere et al., 1997), on tryptone yeast extract salts (TYES) agar (Holt et al., 1994) with or without 1 μg ml^{-1} tobramycin and incubated overnight at 30 °C. A total of 12 isolates were characterized by virulence phenotyping and molecular typing (Table 1) and stored at −80 °C as 20% glycerol stocks. For challenge, isolates were grown on TYES agar plates with no antibiotics overnight at 30 °C followed by selection of a single colony for inoculation of 10 ml of TYES broth. This culture was then incubated overnight at 30 °C with constant agitation at 200 rpm in an Innova 44r (New Brunswick Scientific, USA) incubator. A total of 5 μl was used to inoculate a 2800 ml Fernbach flask containing 1 l of TYES, without antibiotics and again grown overnight at 30 °C. Cultures were allowed to grow till an OD540 reading between 0.7 and 0.75 was reached. These cultures were then used for immersion challenges.

2.3 Fish challenge

All fish were challenged in triplicate 4 l tanks, containing 30 fish per tank, with ~15.8 °C water flowing at 200 ml/min and were allowed to acclimate for 7 d prior to challenge. The immersion challenge protocol used was modified from LaFrentz et al. (2012). The challenges were performed in 3 l of water for 1 h statically. The challenge was performed 3 times with 2 different rainbow trout populations (two challenges were done using the NCCCWA even year population and one using a Troutlodge population (Supplemental Table 1). Bacterial concentrations were estimated by direct plate counting of the challenge water to estimate total CFU the fish were exposed to. Mortalities were removed and recorded daily for 21 d. Gills were sampled from approximately 20% of the daily mortality and were tested for the presence of *F. columnare* by direct plating onto TYES agar plates. Cumulative percent mortality (CPM) data was generated by 1-way ANOVA with Tukey's test for pairwise comparisons and graphs were produced using GraphPad Prism v5 software.

LD50 determination was accomplished using the method of Reed and Muench (1938). Serial 2-fold dilutions of stock *F. columnare* were used to challenge triplicate tanks, and repeated 3 times. Challenge water was plated onto TYES agar plates and CFUs were visually enumerated. The highest bacterial concentration was 2.75×10^7 CFU/ml^{-1} to a low of 1×10^5 CFU/ml^{-1}.

2.4 Genomovar characterization

Genomovar classification was determined using a published protocol (Evenhuis et al., 2014). Briefly, total DNA was isolated for Gram-negative bacteria using the Qiagen DNeasy Blood and Tissue kit following the manufacturer's instructions, and concentrations were measured using a NanoDrop ND-1000 spectrophotometer. The 16S rDNA was amplified from total genomic DNA using primer mix (UN-20 (5′-AGAGTTTGATC(AC)TGGCTCAG-3′) and R1438 (GCCCTAGTTACCAGTTTTAC)) at a final concentration of 200 μM, 45 μl of Platinum PCR SuperMix High Fidelity polymerase master mix (Invitrogen) and 100 ng of DNA were combined, and the PCR reaction was run on a DNAengine thermocycler (Bio-Rad). The thermocycle program was as follows; 10 m initial 94 °C denaturing step, followed by 30 cycles at 94 °C, 55 °C annealing and 72 °C elongation. Each step was 1 m in length. The RFLP profile was generated by digestion of the 16S rDNA product with the *Hae*III or *Dnp*II restriction enzymes and by running the product on a tris-acetate-EDTA (TAE) + 1% (*w*/*v*) agarose gel cast with 1× SYBR Safe DNA gel stain (Invitrogen) and visualization by ultraviolet trans illumination.

2.5 Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) fingerprinting was performed as previously described (Arias et al., 2004; Olivares-Fuster et al., 2007a, 2007b). Briefly, 100 ng of genomic DNA was digested using 10 units of *Hind*III and *Taq*I (Promega, Madison, WI) restriction enzymes, in a final volume of 30 μl. Adaptors were ligated, following complete digestion, using the T4 DNA ligase to a final concentration of 0.04 μM for the *Hind*III adaptor and 0.4 μM for the *Taq*I adaptor. The two primers used for AFLP reactions and the PCR conditions are described elsewhere (Olivares-Fuster et al., 2007a, 2007b). The *Hind*III primer was labeled with an IR700 fluorochrome from LI-COR (LI-COR, Lincoln, NE). The final products were separated by electrophoresis on a NEN Global Edition IR2 DNA Analyzer (LI-COR) according to the manufacturer's instructions. Conversion, normalization, background subtraction with mathematical algorithms, and levels of similarity between fingerprints were calculated for AFLP analysis with the Pearson product moment correlation coefficient. Unique ALFP profiles were

Table 1
Isolate identification and description including the time, location, and tissue source of isolation. Also any descriptive notes and any previous citations.

ARS ID	Isolation date	Location	Tissue	Notes	Citation
CSF-298-10	10/13/2011	CSF-Box Canyon	Head kidney	Standard challenge strain	Evenhuis et al., 2014
FC-CSF-16	4/2/2012	CSF-Box Canyon	Spleen	Spleen	This paper
FC-CSF-20	4/2/2012	CSF-Box Canyon	Brain	Brain	This paper
FC-CSF-43	6/21/2012	CSF-Box Canyon	Water	Water	This paper
CSF-246-12	6/13/2012	CSF-Clear Lake	Gill	Alternative SDS PAGE pattern	This paper
CSF-253-12	6/13/2012	CSF-Briggs Creek	Gill	Briggs Creek	This paper
CSF-485-12-2	12/3/2012	CSF-Clear Lake	Gill	Alternative *Dnp*IIpattern	This paper
CSF-284-13-1	7/22/2013	CSF-Clear Lake	Gill	Clear Lake	This paper
CSF-301-13	8/13/2013	CSF-Box Canyon	Kidney	Kidney	This paper
MS-FC-4	7/18/2013	Magic Springs	Head kidney	Alternative SDS PAGE pattern	This paper
MS-FC-7	7/18/2013	Magic Springs	Gill	Magic Springs	This paper
F10-HK-A	6/6/2012	Indiana	Head kidney	Genomovar I/II, Perch	LaFrentz et al., 2014

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