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Flavobacterium columnare genomovar influences mortality in channel catfish (*Ictalurus punctatus*)

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Abstract

Flavobacterium columnare, causal agent of columnaris disease, is pathogenic to many species of freshwater fish throughout the world. The United States channel catfish (*Ictalurus punctatus*) aquaculture industry is severely impacted by columnaris disease. The majority of the *F. columnare* isolates recovered from diseased channel catfish belonged to either genomovars I or II. The objective of the present study was to determine if differences existed in the ability of these genomovars to induce mortality in channel catfish. Single strand conformation polymorphism analysis (SSCP) was used to ascribe the isolates used in this study to the appropriate genomovar. Immersion challenge experiments (15 min immersion exposure to $\sim 5 \times 10^5$ to 1×10^6 CFU/mL) were carried out to assess virulence of genomovar I and II isolates to channel catfish. The results demonstrated that genomovar II ($n = 4$) isolates were significantly ($P < 0.05$) more virulent to channel catfish fry (92–100% mortality) than genomovar I ($n = 3$) isolates (0–46% mortality). *In vivo* adhesion of the genetically characterized *F. columnare* also correlated ($r^2 = 0.73$) to increased mortality in the challenged fry. In fingerling channel catfish, significantly higher mortality ($P < 0.05$) resulted with genomovar II isolates ALM-05-182 and ALG-00-530 as compared to all the genomovar I isolates ($n = 3$). Mortality of genomovar II isolate BGFS-27 with similar to genomovar II isolate (ALG-00-530) and two genomovar I isolates (ALM-05-53 and 140). The results suggest that although both genomovars are present in the aquatic environment, genomovar II appears to be more pathogenic for channel catfish.
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Keywords: *Flavobacterium columnare*; Channel catfish; Genomovar; Virulence

1. Introduction

Flavobacterium columnare is a pathogen of worldwide importance in freshwater fish. In the United States channel catfish (*Ictalurus punctatus*) industry, *F. columnare* is responsible for significant economic losses each year (Wagner et al., 2002).

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Many environmental factors have been suggested to influence the virulence of *F. columnare* to fish, such as water salinity, temperature, host stress, etc., (Chowdhury and Wakabayashi, 1990; Altinok and Grizzle, 2001; Shoemaker et al., 2003; Suomalainen et al., 2006a). Decostere et al. (1999a,b,c) suggested that virulence of isolates was dependent on the ability to adhere to gill tissue of fish. Thomas-Jinu and Goodwin (2004) demonstrated that differences in virulence existed between *F. columnare* isolates from different genomic groups defined by random amplified polymorphism DNA (RAPD). However, no clear association was made to virulence with respect to RAPD group (Thomas-Jinu and Goodwin, 2004). Arias et al. (2004) assigned 30 *F. columnare* isolates recovered from farm raised fish from different geographic locations to the already established genomovars I and II by 16S rDNA-restriction fragment analysis (RFLP) (Triyanto and Wakabayashi, 1999). Subgroups within those genomovars were established by sequencing the internal spacer region (IRS) and by amplified fragment length polymorphism (AFLP®[1]) analysis. The genetic diversity of *F. columnare* was confirmed by recent studies (Schneck and Caslake, 2006; Suomalainen et al., 2006b). The objective of this study was to determine if differences existed in the ability of *F. columnare* genomovar I and II isolates to induce mortality in channel catfish.

2. Materials and methods

2.1. Fish and experimental conditions

Channel catfish (*Ictalurus punctatus*) (NWAC-103 strain) fry that ranged from 0.3 to 0.6 g in weight were used in experiment 1. Prior to experimentation 15 randomly-selected fry were microbiologically examined and found to be culture negative for *F. columnare* (Shoemaker et al., 2004). Fifteen fry were stocked into 1 L beakers (duplicate beakers for each isolate) supplied with de-chlorinated water and aeration through air stones. The water temperature was 28 ± 2 °C and the fry were allowed to acclimate for 24 h (fish were not fed until after challenge). Following challenge, fry were fed with AQUAMAX Fry Starter 100 (Purina Mills Inc., St. Louis, MO) at 2% body weight daily. One-half the volume (500 mL) of water was replaced daily. Dissolved oxygen ranged from 4.8 to 5.3 mg/L and ammonia ranged from 1.5 to 2 mg/L prior to water exchanges. After water exchange, ammonia levels were <1 mg/L.

Four hundred and fifty channel catfish fingerlings (average weight = 2.76 g/fish) were stocked at 25 fish into each 57 L aquarium for experiment 2. Fifteen randomly-selected fingerling channel catfish were microbiologically examined and found to be culture negative for *F. columnare* prior to the experiment (Shoemaker et al., 2004). Aquaria were supplied with 26 ± 2 °C flow-through, dechlorinated water at a rate of 0.5 L per min. Fish were fed daily to apparent satiation with AQUAMAX Grower 400 (Purina Mills, Inc., St. Louis, MO). Fingerling catfish in each of three aquaria per *F. columnare* isolate were challenged by bath immersion for 15 min exposure (see Section 2.2). Mortalities were recorded daily for 14 days and used to calculate cumulative percent mortality. Fresh dead fish were cultured to confirm *F. columnare* presence and to determine the genomovar of the isolate used to challenge the fish was the same genomovar recovered. Fish care and use was performed in accordance with the requirements of the Aquatic Animal Health Research Unit, Institutional Animal Care and Use committee.

2.2. Bacterial cultures and challenge method

Six genomovar I and six genomovar II isolates typed according to Arias et al. (2004) were used in the experiments (Table 1). Isolates (retrieved from archived stocks stored in Shieh broth with 5% glycerol at −80 °C) were grown in modified Shieh broth (Shoemaker et al., 2005) with shaking at 100 revolutions per minute (rpm) on an orbital shaker at 26 ± 2 °C for 24 h. Cultures were adjusted to an optical density (OD) of 0.7 at 540 nm by dilution with sterile modified Shieh broth. Fish were infected by adding 0.5 mL culture into 500 mL filter sterilized water for a 15 min immersion exposure. Duplicate beakers containing non-infected channel catfish (0.5 mL sterile modified Shieh broth was added to 500 mL water) were included as negative controls. Following the 15 min immersion exposure, two fish were removed from each replicate, rinsed three times with sterile distilled water and used to quantify

[1] Mention of trade or manufacturer names is solely for providing specific information and does not imply endorsement by the U.S. Department of Agriculture.

Table 1
Flavobacterium columnare isolates used in challenge experiments to determine virulence in channel catfish, *Ictalurus punctatus*

Isolate	Source	Date isolated	Genomovar
LSU	Channel catfish	1999	II
ALG-00-515	Channel catfish	2000	II
ALG-00-530	Channel catfish	2000	II
AL-02-36	Largemouth bass	2002	II
ALM-05-182[a]	Channel catfish	2005	II
BGFS-27[a]	Channel catfish	2005	II
ARS-1	Channel catfish	1996	I
HS	Channel catfish	2000	I
MS-02-463	Channel catfish	2002	I
ATCC-23463	Chinook salmon	Unknown	I
ALM-05-53[a]	Channel catfish	2005	I
ALM-05-140[a]	Channel catfish	2005	I

[a] Wild channel catfish from Mobile River basin.

the number of bacteria associated with the fry (see below). The remainder of the fry (13) were rinsed in sterile filtered water and replaced into the 1 L beaker. Plate counts were performed from the water to determine the actual colony forming units (CFU) per mL used in the bath immersion. Three genomovar I and four genomovar II isolates were used in the fry study. Fry were monitored daily and mortalities recorded. Dead fry were examined microscopically, and freshly dead fish were cultured to confirm *F. columnare* as the cause of mortality (Shoemaker et al., 2003). The fingerling channel catfish were challenged by adding 1 mL *F. columnare* culture per L of water using the same method used for fry. Three genomovar I and three genomovar II isolates were used in the fingerling experiment.

2.3. Quantification of adherence to fry

Adhesion was estimated based on viable cell counts of *F. columnare* attached to homogenized tissues using a method similar to Ofek and Doyle (1994) with modifications (Bader et al., 2005). The fry (2/replicate or 4/treatment) used to quantify the adhering bacteria were killed with a lethal dose of tricaine methanesulfonate (MS-222; 300 mg per L) and the gut was aseptically removed and discarded to minimize contaminating bacteria. The remainder of the fry was homogenized in 5 mL sterile modified Shieh medium supplemented with neomycin (Bullock et al., 1986). A 100 μL sample of non-diluted or a 1/10 dilution of the homogenate for each fish was spread onto a modified Shieh agar plate and the number of bacteria quantified. Counts were expressed as CFU/g tissue or log CFU/g tissue.

2.4. Re-isolation and genomovar characterization of isolates used in fingerling trial

Skin and anterior kidney samples were cultured from fresh dead fish, and the bacteria isolated were presumptively identified as *F. columnare* based on colony morphology. At 48 h post isolation, isolates were identified as *F. columnare* using whole-cell fatty acid profiles (Shoemaker et al., 2005). Recovered isolates were ascribed to genomovars according to Triyanto and Wakabayashi (1999). Strain characterization was performed by internal spacer region (ISR)-single strand conformation polymorphism (SSCP) analysis according to Olivares-Fuster et al. (2007a). Briefly, total DNA was extracted using the DNeasy Tissue kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. The 16S-23S rRNA gene ISR sequence was PCR amplified using the universal primers 16S-14F (5′-CTTGTACACACCGCCCGTC-3′ (positions 1389–1407 *E. coli* numbering) and 23S-1R (5′-GGGTTTCCCCATTCGGAAATC-3′) positions 124–110 *E. coli* numbering) (Zavaleta et al., 1996; Welker et al., 2005). Amplified ISR fragments were digested using the restriction endonuclease TaqI (New England Biolabs, Beverly, MA). Following digestion, 1 μL of the restriction product was mixed with 5 μL of denaturing-loading solution (95% formamide, 0.025% bromphenol blue), heated for 5 min at 98 °C, and cooled on ice. The denatured product was then separated by electrophoresis on GeneGel SSCP non-denaturing polyacrilamide gels (Amersham Biosciences, Piscataway, NJ, USA). A GenePhore electrophoresis unit (Amerham Biosciences) was used with the following run conditions: 5 °C constant temperature, 25 min at 90 V, 6 mA, 5 W plus 60 min at 500 V, 14 mA, 10 W. Gels were silver stained with the DNA silver staining kit (Amersham Biosciences) and digitally photographed.

2.5. Statistical analysis

Fry mortality and adherence to fry data were analyzed by one-way analysis of variance (ANOVA)

Table 2
Cumulative percent mortality* and colony forming units (CFU)/g tissue** (mean ± S.E.) from channel catfish fry challenged with different *Flavobacterium columnare*

Isolate	Genomovar	Challenge dose (CFU/mL)	Mean cumulative percent mortality	CFU/g
LSU	II	7.7×10^5	100.0 (±0.0)[a]	130.7 (±83.9)[a]
ALG-00-515	II	3.3×10^5	100.0 (±0.0)[a]	2227.0 (±1045.4)[b]
ALG-00-530	II	3.8×10^5	96.2 (±3.9)[a]	2702.7 (±926.1)[b]
AL-02-36	II	1.5×10^6	92.3 (±7.7)[a]	324.5 (±85.2)[a]
ARS-1	I	7.0×10^5	46.2 (±23.0)[b]	347.5 (±243.0)[a]
HS	I	4.2×10^5	3.9 (±3.9)[c]	26.2 (±20.5)[a]
MS-02-463	I	4.3×10^5	0.0 (±0.0)[c]	12.7 (±4.6)[a]
Negative Control	None	None	0.0 (±0.0)[c]	0.0 (±0.0)[a]

* Different superscript letters indicated significant difference at $P < 0.001$.
** Different superscript letters indicate a significant difference at $P < 0.01$.

using Duncan's multiple range test (SAS, 2000) to determine significant differences ($P < 0.05$) between the means. Pearson product moment correlation analysis was based on adhesion data generated from a total of seven *F. columnare* isolates (three genomovar I and four genomovar II isolates). One-way ANOVA was also used to analyze mortality data in the fingerling experiments with differences being determined at $P < 0.05$.

ISR-SSCP profiles were analyzed using the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Following conversion, normalization, and background subtraction with mathematical algorithms, levels of similarity between fingerprints were calculated with the Dice coefficient.

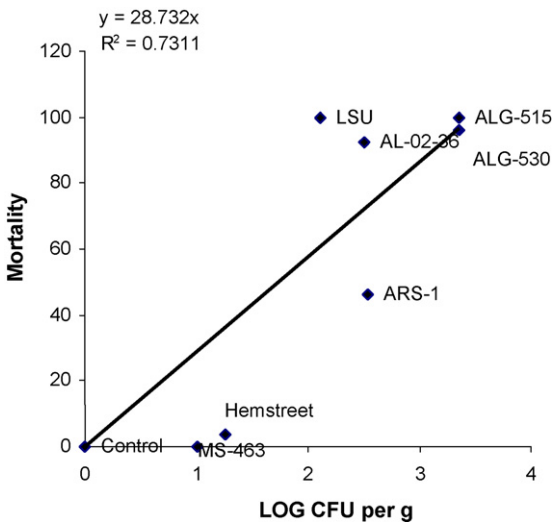

Fig. 1. Correlation of mortality versus adherence expressed as colony forming units (CFU)/g tissue for different *Flavobacterium columnare* isolates used to infect channel catfish fry.

3. Results

3.1. Experiment 1-channel catfish fry and adherence

Significantly greater mortality ($P < 0.001$) was observed in the channel catfish fry challenged with genomovar II isolates than in fry challenged by genomovar I isolates or fry that were not challenged (Table 2). Mortality ranged from 92 to 100% in fry challenged with genomovar II isolates and from 0 to 46% in fry challenged with *F. columnare* genomovar I. No mortality was observed in the non-challenged controls. *Flavobacterium columnare* cells were observed in wet mounts prepared from the dead catfish fry, and freshly dead fry were culture positive for *F. columnare* by standard microbiological methods.

Colony forming units per gram of tissue were significantly higher ($P < 0.01$) for two of the genomovar II isolates (ALG-00-515 and ALG-00-530) than for the other isolates used in the study (both genomovar I and II and the non-challenged treatment; Table 2). Mortality induced by the genomovar I and II isolates positively correlated ($r^2 = 0.73$) with the number of colony forming units of *F. columnare* recovered per gram of tissue (Fig. 1). The correlation was based on the adherence properties of seven *F. columnare* isolates (four genomovar II and three genomovar I).

3.2. Experiment 2-channel catfish fingerlings and ISR-SSCP

Mortality of fingerling channel catfish was influenced by isolate used for the challenge (Table 3).

Table 3
Mean cumulative percent mortality (mean ± S.E.) of channel catfish fingerlings challenged with different *Flavobacterium columnare*[*]

Isolate	Genomovar	Cumulative percent mortality ± S.E.[**]
ALM-05-182[***]	II	88.0 ± 6.1^{a}
ALG-00-530[***]	II	$76.0 \pm 8.3^{a,b}$
BGFS-27[****]	II	$60.0 \pm 10.6^{b,c}$
ALM-05-53[****]	I	46.7 ± 3.5^{c}
ALM-05-140[***]	I	44.0 ± 2.3^{c}
ATCC-23463[*****]	I	0.0 ± 0.0^{d}

[*] Tripicate tanks of 25 channel catfish were challenged with each isolate following adjustment to an OD of 0.7 at 540 nm (plate counts yielded 5×10^5 to 1×10^6 CFU/mL) for 15 min immersion exposure.
[**] Different superscript letters indicated significant difference at $P < 0.001$ for the isolate.
[***] Isolate obtained from channel catfish with lesions typical of columnaris disease.
[****] Isolate obtained from apparently healthy channel catfish (i.e., non-lesioned fish).
[*****] American type culture collection, type strain.

Significantly higher ($P > 0.001$) mortality resulted in fingerling catfish challenged with the genomovar II isolates ALM-05-182 and ALG-00-530 as compared to all genomovar I isolates. Mortality was not statistically different for genomovar II isolate BGFS-27 with one genomovar II isolate (ALG-00-530) and two genomovar I isolates (ALM-05-53 and 140). The genomovar isolate ATCC 23463 did not induce mortality in the juvenile channel catfish.

Isolate BGFS-27 was recovered after infection but could not be sub-cultured for subsequent DNA extraction. Type strain ATCC 23463 did not cause mortality in fingerling catfish and was not recovered after infection. Therefore, only four isolates were genetically compared prior to and post-challenge. Isolates ALM-05-53 and ALM-05-140 presented the typical genomovar I ISR-SSCP profile and were genetically indistinguishable (Fig. 2). Genomovar II isolates (ALM-05-183 and ALG-00-530) presented the same banding pattern with some quantitative differences that allowed us to differentiate these two strains. In all cases, recovered isolates presented the same genetic profiles prior to and post challenge thus confirming the original nature of the infectious agent (Fig. 2).

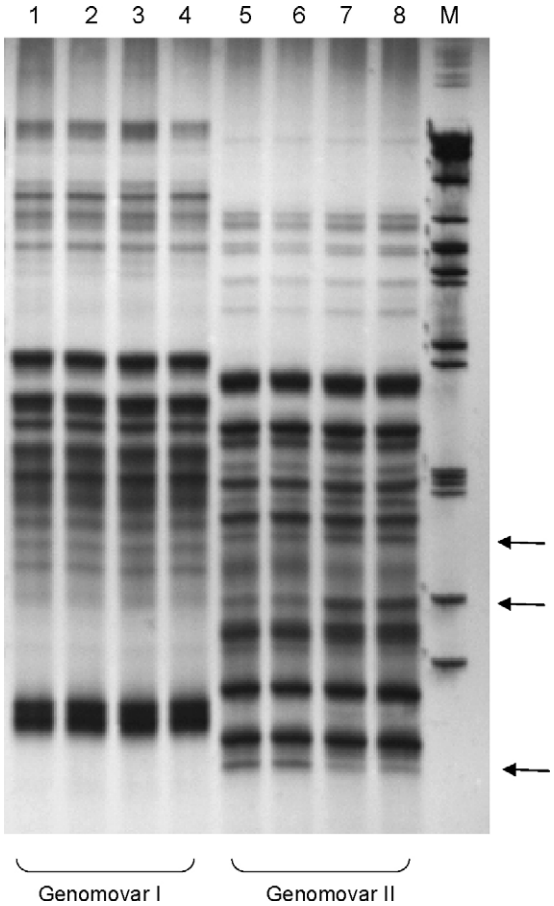

Fig. 2. ISR-SSCP profiles of *F. columnare* genomovar I and genomovar II isolates. Lane 1, ALM-05-53 post-challenge; lane 2, ALM-05-53 pre-challenge; lane 3, ALM-05-140 post-challenge, lane 4, ALM-05-140 pre-challenge; lane 5, ALM-05-182 post-challenge; lane 6, ALM-05-182 pre-challenge; lane 7, ALG-00-530 post-challenge; lane 8, ALG-00-530 pre-challenge; lane M, molecular marker.

4. Discussion

Multiple studies have examined genetic diversity (Triyanto and Wakabayashi, 1999; Michel et al., 2002; Arias et al., 2004; Thomas-Jinu and Goodwin, 2004; Schneck and Caslake, 2006; Suomalainen et al., 2006b; Zhang et al., 2006; Olivares-Fuster et al., 2007a), virulence properties (Altinok and Grizzle, 2001; Stringer-Roth et al., 2002; Suomalainen et al., 2006c) and adherence (Decostere et al., 1999a,b,c) of *F. columnare* isolates to various fish species. However, no studies have attempted to correlate mortality with

adherence (*in vivo*) to channel catfish fry based on genetically characterized *F. columnare*. The results of the present study demonstrated that adherence of *F. columnare* isolates to young channel catfish fry correlated with mortality (i.e., the more adherent the more virulent). Decostere et al. (1999a,b,c) clearly demonstrated the association of virulence with adherence to fish and that differences in virulence of *F. columnare* in black mollies (*Poecilia sphenops*) was related to adherence (Decostere et al., 1998). Previous studies have suggested differences in virulence and adhesion of *F. columnare* based on as few as two isolates (Suomalainen et al., 2006c) that were not genetically characterized.

Thomas-Jinu and Goodwin (2004) suggested that no clear association was made to virulence and genetic group based on RAPD analysis of 17 *F. columnare* (6, 8 and 3 strains based on RAPD grouping). However, interpretation of their results is difficult because the number of bacteria used for the challenge experiments was not quantified and fish were subjected to temperature stress. More recently, Schneck and Caslake (2006), Suomalainen et al. (2006b), Olivares-Fuster et al. (2007b) suggested that there are genetic differences among *F. columnare* isolated from diverse fish species. Suomalainen et al. (2006c) demonstrated by automated ribosomal intergenic spacer analysis (ARISA) in eight defined genomovar I isolates that differences in mortality for rainbow trout, *Oncorhynchus mykiss* (Walbaum) existed. The authors suggested that increased chondroitin AC lyase activity of the four virulent isolates was related to the increased mortality of the *F. columnare* studied. While we did not analyze AC lyase activity in the present study, this may have resulted in the increased mortality noted for the genomovar II isolates that caused mortality but were not as adherent to fry. Alternatively, there may be a critical threshold of adherent bacteria (i.e., greater than 100 CFU's) needed to induce mortality.

Suomalainen et al. (2006c) point out the difficulties in establishing columnaris disease by immersion challenge infection in rainbow trout. The same difficulty in establishing columnaris disease in channel catfish has been documented. Previous studies have used abrasion (Bader et al., 2006), temperature stress (Thomas-Jinu and Goodwin, 2004) or feed deprivation (Shoemaker et al., 2003) to induce mortality. Our results are the first to demonstrate that genomovar significantly influences mortality of channel catfish in immersion challenge studies. A molecular typing method (ISR-SCCP) demonstrated that the isolates used to infect the channel catfish were the same genotype as the isolates recovered from the moribund fish, at least with respect to the isolates that caused mortality and were re-isolated in pure culture (Fig. 2). This is particularly important when challenging fish with *F. columnare* since channel catfish can be asymptomatic carriers for *F. columnare* and therefore might harbor additional strains of the pathogen. Interestingly, isolates obtained from apparently healthy (i.e., no clinical signs of disease) wild channel catfish (i.e., Mobile River basin with a water temperature ranging from 22 to 26 °C; Table 1) were virulent in laboratory studies. Suomalainen et al. (2006c) suggest that *F. columnare* is part of the normal aquatic bacterial community (Rickard et al., 2003; Arias et al., 2006) and probably the source for pathogenic *F. columnare*. Our results suggest that although both genomovars are present in the aquatic environment and routinely isolated from diseased channel catfish, genomovar II appears to be more pathogenic for channel catfish in immersion challenge studies.

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