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Short communication

Enhanced susceptibility of channel catfish to the bacterium *Edwardsiella ictaluri* after parasitism by *Ichthyophthirius multifiliis*

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ABSTRACT

Bacterium *Edwardsiella ictaluri* and parasite *Ichthyophthirius multifiliis* (Ich) are two common pathogens of cultured fish. The objective of this study was to evaluate the susceptibility of channel catfish *Ictalurus punctatus* to *E. ictaluri* and determine bacterial loads in different fish organs after parasitism by Ich. Fish received the following treatments: (1) infected by *I. multifiliis* at 5000 theronts/fish and exposed to *E. ictaluri*; (2) infected by *I. multifiliis* alone; (3) exposed to *E. ictaluri* alone; and (4) non-infected control. *E. ictaluri* in fish organs were quantified by quantitative real-time polymerase chain reaction and reported as genome equivalents per mg of tissue (GEs/mg). The results demonstrated that the Ich-parasitized catfish showed significantly ($P < 0.05$) higher mortality (91.7%) when exposed to *E. ictaluri* than non-parasitized fish (10%). The bacterial loads in fish infected by 5000 theronts/fish ranged from 6497 to 163,898 GEs/mg which was between 40 and 2000 fold higher than non-parasitized fish (49–141 GEs/mg). Ich infection enhanced the susceptibility of channel catfish to bacterial invasion and increased fish mortality.

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1. Introduction

Host–pathogen interactions are rarely one-on-one in aquaculture systems where fish may be concurrently infected by two or more pathogens (Xu et al., 2007). There is increasing evidence that co-infections contribute to the severity of some infectious diseases, especially bacterial diseases (Busch et al., 2003). The study of parasite–bacteria interaction is gaining attention in fish health since the studies contribute to understanding epidemiology and the effect of multi-pathogens on control and prevention of disease.

Enteric septicemia of catfish (ESC), caused by the bacterium *Edwardsiella ictaluri* is responsible for approximately 50% of economic losses to catfish farmers in the United States (Shoemaker et al., 2009). Although channel catfish (*Ictalurus punctatus*), a dominant aquaculture species in the USA, is the most susceptible to infection by *E. ictaluri*, other fish species, such as white catfish *Ictalurus catus*, walking catfish *Clarius batrachus* and freshwater catfish *Pangassius hypophthalmus*, are also susceptible (Crumlish et al., 2002). Outbreaks of ESC are seasonal, occurring mainly in spring and fall with a temperature range of 22–28 °C (Tucker and Robinson, 1990).

Ichthyophthirius multifiliis Fouquet, referred to as "Ich", is a devastating fish protozoan parasite that infects most freshwater fish. The parasite damages fish gills and skin that can result in high fish mortality and heavy economic

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losses for aquaculture (Matthews, 2005). The life stages of the parasite include an infective theront, a parasitic trophont and a reproductive tomont (Hines and Spira, 1974).

There is no information available on whether Ich infection will affect the susceptibility of channel catfish to *E. ictaluri*. Therefore, the objective of this study was to evaluate the susceptibility of channel catfish to *E. ictaluri* and to determine bacterial loads in different fish organs after parasitism by Ich.

2. Materials and methods

2.1. Fish, parasite and bacterial isolation

Channel catfish (Industry pool strain) were obtained from disease-free stock from the USDA-ARS Catfish Genetic Research Unit, Stoneville, MS and reared to experimental size in indoor tanks at the USDA-ARS Aquatic Animal Health Research Unit, Auburn, AL (USDA/AAHRU). *I. multifiliis* (ARS 10-1 strain) was originally isolated from an infected pet fish and maintained by serial transmission on channel catfish held in 50 l glass aquaria as previously described (Xu et al., 2000). Trophonts were collected and theronts were cultured as described previously (Xu et al., 2000). Theronts for infection trials were enumerated with a Sedgewick-Rafter cell.

An isolate of *E. ictaluri* (AL-93-58) was originally obtained from diseased channel catfish, identified and maintained at the USDA/AAHRU (Panangala et al., 2005). The concentration (colony forming units per milliliter CFU/ml) of *E. ictaluri* was determined through serial 1:10 dilutions using standard plate-counts. Dead or moribund fish were removed twice a day during the infection trial to confirm the identity of *E. ictaluri* from the dead fish as described previously (Panangala et al., 2005).

2.2. Water quality

During trials, dissolved oxygen (DO) and temperature in tanks were measured using an YSI 85 oxygen meter (Yellow Spring Instrument, Yellow Springs, OH). The pH, hardness, ammonia and nitrite were determined using Hach CEL/890 Advanced Portable Laboratory (Loveland, Colorado). The mean ± SEM of dissolved oxygen was 6.1 ± 0.2 mg/l, temperature was 24.6 ± 0.3 °C, pH was 7.0 ± 0.1, ammonia was 0.39 ± 0.1 mg/l, hardness was 80.2 ± 4.8 mg/l, and nitrite concentration was 0.01 ± 0 mg/l.

2.3. Experimental design

A total of 274 channel catfish with an average total length of 11.4 ± 0.3 cm (mean ± SEM) and average weight of 12.5 ± 0.5 g were used. All fish treatment protocols were approved by Institutional Animal Care and Use Committee at the Aquatic Animal Health Research Unit. Ten catfish were examined and cultured to verify pathogen free status of parasites and bacteria prior to the trial. All fish were negative for Ich and *E. ictaluri*. Fish were distributed into 11 tanks with 24 fish/tank and received the following treatments: (1) infected by *I. multifiliis* at 5000 theronts/fish and exposed to *E. ictaluri*; (2) infected by *I. multifiliis* at 5000 theronts/fish alone; (3) infected by *E. ictaluri* alone; and (4) not infected by *I. multifiliis* and *E. ictaluri*. There were 3 tanks per treatment in groups 1–3 and two tanks in group 4. Water was lowered to 10 l in each tank prior to Ich exposure. For fish infected with Ich, Ich theronts were added to each tank at 5000 theronts per fish for 1 h with aeration. The fish in the remaining tanks were not exposed to Ich theronts but kept in 10 l water for 1 h. When fish showed visible "white spots" (a gross clinical sign characteristic of Ich infection) 5-day post theront exposure, each tank of fish were moved to a bucket with 5 l water with aeration for *E. ictaluri* challenge. Briefly, fish were immersed in water with *E. ictaluri* at a concentration of 3.64×10^6 CFU/ml for 30 min. Fish not exposed to the bacterium were kept in water with BHI broth for the same time. After challenge, fish were returned to the tanks and flowing water resumed at 0.5 l/min. Fish mortality was monitored daily after *E. ictaluri* challenge. Two fish were sampled from each tank to quantify *E. ictaluri* in fish organs 3 and 6 days post *E. ictaluri* challenge. After fish were anesthetized with 300 mg/l tricaine methanesulfonate (MS-222), the brain, gill, liver, and kidney were collected from fish using aseptic technique.

2.4. Genomic DNA isolation from bacteria and fish organs

The genomic DNA (gDNA) of *E. ictaluri* (AL-93-58) was extracted and purified using DNeasy tissue kit following the manufacturer's instructions (Qiagen, Valencia, CA, USA). The DNA from the bacteria used in the specificity test was isolated using the same protocol as for *E. ictaluri*. For standards, 10-fold serial dilutions from 5 ng/μl to 5 fg/μl of the gDNA of *E. ictaluri* were made in sterile water or tissue extracts (brain, gills, kidney, and liver). The fish organs used to quantify *E. ictaluri* with qPCR were weighed (approximately 20 mg) and macerated with sterilized Kontes disposable pestles in a microcentrifuge tube. Total gDNA of *E. ictaluri* in fish organs was extracted by the DNeasy Tissue kit and eluted with a volume of water equal to 10 μl water per mg tissue.

2.5. Quantitative real-time PCR

One-step qPCR was performed as described by Bilodeau et al. (2003) on an Applied Biosystems 7500 Real-Time PCR machine (ABI, Foster City, CA) using Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA). Two *E. ictaluri*-specific primers (forward 5′-ACTTATCGCCCTCG-CAACTC-3′ and reverse 5′-CCTCTGATAAGTGGTTCTCG-3′) and a dual-labeled probe (5′-CCTCACATATTGCTTCA-GCGTCGAC-3′) described by Bilodeau et al. (2003) were used for specific detection of *E. ictaluri*. Extracted DNA from fish organs (1 μl) was used as template in qPCR and the DNA concentration in fish organs was determined via the standard curve (threshold cycle (Ct) values vs. DNA concentration of *E. ictaluri*). Since 1-μl of eluted sample was run in qPCR, the amount of bacterial DNA in each mg of tissue was equal to bacterial DNA concentration (pg/μl) × eluted volume/tissue weight (mg). Bacterial DNA in each mg of tissue was calculated as genome equivalents

Table 1
Cumulative mortality of channel catfish infected by parasite *Ichthyophthirius multifiliis* (Ich) and bacterium *E. ictaluri*. Fish mortality (±SEM) was the mean of 40–60 fish from 2 to 3 tanks and observed for 17-day post challenge with *E. ictaluri* by immersion for 30 min. Within a given column, means followed by different superscript letters are statistically different ($P < 0.05$).

Theront concentration	*E. ictaluri* (CFU/ml)	Tank number	Fish number	Dead number	Mortality (%)
5000/fish	3.64×10^6	3	60	55	91.7 ± 1.6^a
5000/fish	BHI broth	3	60	47	78.3 ± 6.7^b
0/fish	3.64×10^6	3	60	6	10.0 ± 5.0^c
0/fish	BHI broth	2	40	2	5.0 ± 5.0^c

per mg of tissue (GEs/mg) based on the genome size of *E. ictaluri* = 3.8 fg/cell (Bilodeau et al., 2003).

2.6. Sensitivity and specificity of the qPCR assay

The detection limit was evaluated from 5 ng/μl to 5 fg/μl of the gDNA of *E. ictaluri*. Specificity of the qPCR was determined by performing the assay on DNA extracted from following 17 cultures of bacteria: *E. ictaluri* (Eic2; ALG-03-58; AL93-58; S94-1051 & S99-1908), *Aeromonas caviae* (ATCC 15468), *Aeromonas hydrophila* (AL09-71 & AL98-C1B), *Aeromonas sobria* (ATCC 43979), *Edwardsiella tarda* (Eta8 & AL-98-87), *Flavobacterium columnare* (AL0236 & AL6-00530), *Pseudomonas aeruginosa* (ATCC-27853), *Streptococcus agalactiae* (Sg10) and *Streptococcus iniae* (Sin17 & Uruguay1). Most bacteria in the list were originally isolated from diseased fish, identified to species using standard methods and maintained at the USDA/AAHRU (Panangala et al., 2005).

2.7. Statistical analysis

All data were analyzed with SAS software (SAS Institute, 1989). The levels of *E. ictaluri* DNA in organs (GEs/mg) from different treatment groups were compared with Duncan multiple range tests. The fish mortality was transferred to the natural logarithm and analyzed with Poisson regression. *P*-values of 0.05 or less were considered statistically significant.

3. Results

3.1. Bacterial isolation, Ich infection and fish mortality after exposure to E. ictaluri

No *E. ictaluri* was isolated from dead fish not exposed to *E. ictaluri*. All dead fish from the *E. ictaluri* exposed treatments were culture positive from both the liver and the kidney. Parasites were not observed on fish that were not exposed to Ich theronts. Fish exposed to 5000 theronts per fish showed medium infection (50–100 trophonts/fish) 5 days post Ich infection (DPI) and heavy infection (>100 trophonts/fish) 11 DPI. At the end of the experiment (22 DPI) no parasites were seen on surviving fish. Ich-parasitized fish showed significantly ($P < 0.05$) higher mortality (91.7%) when exposed to *E. ictaluri* than non-parasitized fish (10%, Table 1). Fish infected by Ich alone at 5000 theronts per fish showed 78.3% mortality (Table 1). Two fish in one of the control tanks died 8 and 10 days, respectively after start of the trail. No parasite was observed under microscope and no bacteria were isolated from the liver and kidney of the dead fish. The death of 2 fish in the control tank was not caused by the parasite or bacterial infections.

3.2. Sensitivity and specificity of the qPCR

The qPCR detected 10-fold serially diluted samples containing 5 ng to 50 fg of *E. ictaluri* DNA. Six DNA dilutions equivalent to 1.32×10^1–1.32×10^6 genome equivalents of *E. ictaluri* were used to generate a standard curve. The standard curve revealed a linear correlation between Ct values (Y) and log amount (X) of nucleic acid ($Y = -4.1367X + 20.414$, $R^2 = 0.99$). Using specific qPCR primers of *E. ictaluri* (Bilodeau et al., 2003), a 178 bp amplified product was observed in all 5 isolates of *E. ictaluri* by gel electrophoresis confirming specificity (data not shown). The 178 bp product was not observed in isolates of *A. caviae*, *A. hydrophila*, *A. sobria*, *E. tarda*, *F. columnare*, *P. aeruginosa*, *S. agalactiae* and *S. iniae* (data not shown).

3.3. Amount of E. ictaluri in fish organs

No *E. ictaluri* was detected in organs of parasitized or non-parasitized fish prior to exposure to *E. ictaluri*. All organs from fish exposed to the parasite only showed no *E. ictaluri* during the trial (Table 2). *E. ictaluri* was detected in brain, gill, kidney and liver of fish exposed to *E. ictaluri* only. The bacterial loads ranged from 26 to 141 GEs/mg at 3 and 6 days post exposure (dpe) to *E. ictaluri* in different organs of fish exposed to *E. ictaluri* only (Table 2). The bacterial loads increased significantly ($P < 0.05$) in the organs of parasitized fish compared to non-parasitized fish after exposure to *E. ictaluri*. All parasitized fish showed higher bacterial loads in organs at 6 dpe to *E. ictaluri* than 3 dpe to *E. ictaluri* (Table 2).

4. Discussion

Few studies have used molecular methods to quantify the amount of bacteria in fish organs following concurrent infection. This trial demonstrated that Ich parasitized fish had a higher load of *E. ictaluri* in organs than non-parasitized fish. The bacterial load in fish infected by Ich (109–347 GEs/mg) was 2–10 fold higher than non-parasitized fish (26–58 GEs/mg) 3 dpe to *E. ictaluri*. The bacterial increment in organs became more pronounced 6 dpe to *E. ictaluri* suggesting replication. The bacterial loads ranged 49–141 GEs/mg for non-parasitized fish and 6497–163,898 GEs/mg for parasitized fish. The bacterial loads in fish infected by theronts were roughly 50–2300

Table 2
The average genome equivalent of *Edwardsiella ictaluri* (±SEM) in organs of channel catfish (GE/mg) co-infected by *Ichthyophthirius multifiliis* at 5000 theronts/fish and *E. ictaluri*. Within a given sampling day, means followed by different superscript letters are statistically different ($P < 0.05$).

Organs	Theronts/fish	*E. ictaluri* (CFU/ml)	Sample number	Day 3 GE/mg	Day 6 GE/mg
Brain	5000	BHI broth	8	0 ± 0^{a}	0 ± 0^{a}
	0	3.64×10^{6}	12	26 ± 18^{a}	141 ± 47^{a}
	5000	3.64×10^{6}	12	248 ± 68^{b}	6497 ± 152^{b}
Gill	5000	BHI broth	8	0 ± 0^{a}	0 ± 0^{a}
	0	3.64×10^{6}	12	50 ± 34^{a}	58 ± 39^{a}
	5000	3.64×10^{6}	12	225 ± 85^{b}	$26{,}323 \pm 3478^{c}$
Kidney	5000	BHI broth	8	0 ± 0^{a}	0 ± 0^{a}
	0	3.64×10^{6}	12	58 ± 25^{a}	70 ± 35^{a}
	5000	3.64×10^{6}	12	347 ± 86^{c}	$163{,}898 \pm 7535^{d}$
Liver	5000	BHI broth	8	0 ± 0^{a}	0 ± 0^{a}
	0	3.64×10^{6}	12	53 ± 23^{a}	49 ± 21^{a}
	5000	3.64×10^{6}	12	109 ± 46^{d}	$78{,}692 \pm 14851^{e}$

fold higher than non-parasitized fish in the different organs. The kidneys of fish co-infected by 5000 theronts and 3.64×10^{6} *E. ictaluri* had the most *E. ictaluri* at 6 dpe *E. ictaluri* among 4 organs studied. The results in this study are in agreement with previous findings. Russo et al. (2009) used a red fluorescent protein as a marker and studied the distribution of *E. ictaluri* in fish organs. They reported the greatest amount of bacteria in kidneys when compared to gills, skin, fins, muscle, spleen, liver, stomach, intestine, heart and brain. The high amount of bacteria in the kidney may relate to the immune and blood filtering functions of the kidney (Russo et al., 2009) and also suggests bacterial replication.

The study of multi-pathogen interaction and their influence on the efficacy of disease control and pathogenesis is gaining interest in human and animal diseases (Cattadori et al., 2007). Compared to studies on multi-pathogen interaction in human and other animal infectious diseases (Cattadori et al., 2007), co-infection studies for aquatic animals are limited. This study demonstrated that parasitic infection in fish resulted in increased bacterial loads and caused high fish mortality.

5. Conclusions

In summary, channel catfish co-infected by Ich and *E. ictaluri* showed higher mortality than fish infected by *E. ictaluri* only, fish infected by parasite only, and non-infected fish. The loads of *E. ictaluri* in brain, gill, kidney, and liver of Ich-parasitized fish were significantly (50–2300 fold) higher than non-parasitized fish after exposure to *E. ictaluri*. This work suggests that prevention of parasite infection in fish will not only reduce the direct damage caused by the parasite but will also reduce fish mortality due to bacterial co-infection.

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