Expression profiles of seven channel catfish antimicrobial peptides in response to *Edwardsiella ictaluri* infection

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**Abstract**

Using quantitative polymerase chain reaction (QPCR), the relative transcriptional levels of seven channel catfish antimicrobial peptide (AMP) genes (NK-lysin type 1, NK-lysin type 2, NK-lysin type 3, bactericidal permeability-increasing protein, cathepsin D, hepcidin and liver-expressed AMP 2) in response to *Edwardsiella ictaluri* infection were determined. None of the AMP genes tested was significantly upregulated at 2 h post-infection. Hepcidin was the only one that was significantly \((P < 0.05)\) upregulated at 4, 6 and 12 h post-infection. At 24 and 48 h post-infection, four AMPs (hepcidin, NK-lysin type 1, NK-lysin type 3 and cathepsin D) were significantly \((P < 0.05)\) upregulated. Among all the AMPs that were significantly upregulated at different time points, hepcidin at 4, 6 and 12 h post-infection was upregulated the most. When catfish were injected with different doses of *E. ictaluri*, all lethal doses were able to induce significant \((P < 0.05)\) upregulation of hepcidin in the posterior kidney, whereas sub-lethal doses failed to induce any significant upregulation of hepcidin. In *in vitro* growth studies revealed that the presence of synthetic hepcidin peptide at a concentration of 16 \(\mu\)M or higher significantly inhibited the cell proliferation of *E. ictaluri*. Taken together, our results suggest that hepcidin might play an important role in the channel catfish defense against *E. ictaluri* infection.

**Keywords:** antimicrobial peptide, *Edwardsiella ictaluri*, *Ictalurus punctatus*, transcription.

**Introduction**

Enteric septicemia of catfish (ESC), the most prevalent disease affecting farm-raised channel catfish, *Ictalurus punctatus* (Rafinesque), is caused by *Edwardsiella ictaluri*, a facultative intracellular Gram-negative flagellated bacterium akin to phylogenetically related *Salmonella* (Thune, Collins & Pena 1997; Wagner, Wise, Khoo & Terhune 2002; Zhang & Arias 2007). ESC is generally an acute septicaemia that develops very quickly, especially in the temperature range of 22–28°C. Signs of the disease have been observed within 2 days after immersion challenge, and heavy mortalities have been reported as early as 4 days after infection (Newton, Wolfe, Grizzle & Plumb 1989; Wolters & Johnson 1994; Thune et al. 1997).

To control bacterial disease outbreaks such as ESC, feeding infected fish with antibiotic-medicated food is a general practice (DePaola, Peeler & Rodrick 1995). However, this practice is expensive and usually ineffective as sick fish tend to remain off feed. Furthermore, currently in the USA, there are only three FDA-approved antibiotics for use in aquaculture: oxytetracycline (Terramycin), sulphadimethoxine (Romet-30) and Aquaflor. The widespread use of the limited number of antibiotics has led to the development of antibiotic resistance in many fish pathogens worldwide (Nikaido 2009). As resistance to antibiotics by microorganisms continues to increase, the need to identify and develop new antibiotics is urgent.

In plant and animal kingdoms, antimicrobial peptides (AMPs) are evolutionarily ancient defensive weapons against bacteria, fungi and viruses (Zasloff 2002). AMPs are usually cationic, therefore binding to negatively charged phospholipids of bacterial outer membranes. In contrast, the outer
membranes of plants and animals are composed principally of lipids with no net charge because the negatively charged phospholipids are segregated into the inner membrane facing the cytoplasm (Zasloff 2002). The unique modes of action by AMPs enable them to combat resistance by microorganisms (Zasloff 2002; Boman 2003). Therefore, AMPs have been considered as templates for the development of new antibiotics.

At least 800 AMPs have been reported in plants and animals (Toke 2005). In channel catfish, seven AMPs [NK-lysin type 1, NK-lysin type 2, NK-lysin type 3, bactericidal permeability-increasing protein (BPI), cathespin D, hepcidin and liver-expressed AMP 2 (LEAP2)] have been reported. The expression profiles of the three NK-lysin AMP genes and LEAP2 in various tissues of normal channel catfish have been demonstrated to be differentially expressed (Bao, Peatman, Xu, Li, Zeng, He & Liu 2006; Wang, Wang, Xu & Liu 2006). The expression profiles of hepcidin, cathespin D and BPI in response to pathogen infections at 24 h or longer post-infection have also been reported (Cho, Park, Kim, Lee, Kim & Kim 2002; Bao, Peatman, Li, He & Liu 2005; Xu, Bao, He, Peatman, He & Liu 2005). However, many other genes involved in the catfish innate immune responses are upregulated much earlier than 24 h post-challenge, including chemokines, Toll-like receptors and cytokines (Bao, Peatman, Chen, He, Kucuktas, Li, Wang, Somridhivej, Dunham & Liu 2004; Peatman, Bao, Baoprasertkul & Liu 2005; Bao et al. 2006; Peatman, Bao, Peng, Baoprasertkul, Brady & Liu 2006; Wang, Bao, Peatman, Bao & Bao 2006; Pridgeon, Russo, Shoemaker & Klesius 2010). How the seven channel catfish AMPs are regulated in response to acute bacterial infection at early infection stages (within 24 h post-infection) is currently unknown.

Using red fluorescence as a marker, in vivo localization studies have revealed that E. ictaluri is present in almost all organs of channel catfish after injection, with the greatest amount of the red fluorescence in the anterior kidney, posterior kidney and spleen (Russo, Shoemaker, Panangala & Klesius 2009). The expression of hepcidin and TLRs has been reported to be upregulated in the anterior kidney of channel catfish after E. ictaluri infection (Bao et al. 2005; Pridgeon et al. 2010), suggesting that the anterior kidney is an important organ involved in innate immunity against E. ictaluri infection. However, it is currently unknown whether AMPs are differentially regulated in the posterior kidney in response to E. ictaluri infection. Therefore, the objectives of this study were to (i) determine the expression profiles of the seven AMPs in the posterior kidney of channel catfish after E. ictaluri infection at different time points: 0-, 2, 4, 6, 12, 24 and 48 h post-infection; (ii) identify the AMP gene or genes that are upregulated the earliest and the most (in both fold and time span); (iii) determine whether there is a correlation between the upregulation of the AMP genes identified in objective (ii) and the amount of E. ictaluri in the posterior kidney; and (iv) determine whether synthetic peptide of the AMP identified in objective (ii) has any effect on cell proliferation of E. ictaluri in vitro.

**Materials and methods**

**Experimental fish and sample collection**

Fingerling channel catfish (mean weight 32.5 g) were selected from stocks maintained at the USDA-ARS-Aquatic Animal Health Research Unit at Auburn, AL. All fish were acclimatized for 7 days prior to challenge. Acclimatized fish were maintained in 185-L glass aquaria with flow-through dechlorinated tap water and constant aeration with water temperature at 28 °C. For time course studies, five fish were sampled for E. ictaluri before challenge (non-exposed control). Thirty fish were injected with 100 µL of tryptic soy broth (TSB). Thirty fish were injected with 100 µL of 10⁸ CFU mL⁻¹ of virulent E. ictaluri (AL-93-58 strain). The dose of 100 µL of 10⁷ CFU mL⁻¹ (10⁶ CFU per fish) was chosen because that was the dose that caused about 50% mortality in infected fish in 14 days. At the dose of 10⁶ CFU per fish, no fish died within 48 h during our experiment. Five fish per time point per treatment (TSB or E. ictaluri injection) were sampled at 2, 4, 6, 12, 24 and 48 h post-injection (hpi). Fish were anaesthetized in a 300 mg L⁻¹ solution of MS-222 before collection of posterior kidney. For correlation studies, six doses (0, 10⁴, 10⁵, 10⁶, 10⁷ and 10⁸ CFU per fish) of E. ictaluri were used. Twenty-five fish per dose group were injected, and posterior kidney samples from five fish were collected from the control (TSB) fish group or infected fish group at 6 h post-injection. The rest of the twenty fish were used to determine the 14-day cumulative mortality caused by the six doses used. At all doses, no fish died within 6 h during our experiment. All samples were...
flash frozen in liquid nitrogen and then stored at −80 °C until DNA or RNA extraction.

### RNA extraction, cDNA synthesis, primer design and QPCR

Total RNA was isolated from the posterior kidney tissues using Trizol Reagent (Invitrogen). All RNA was treated with DNA-free (Ambion) and quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). A total of 2 μg of total RNA was used for cDNA synthesis. The first strand cDNA used for QPCR was synthesized using AMV reverse transcriptase (Invitrogen). Briefly, a 2-μg aliquot of total RNA was reverse transcribed in a 20-μL reaction volume using a Clone AMV First Strand cDNA Synthesis Kit (Invitrogen). The reaction was terminated by heat inactivation at 95 °C for 5 min. The cDNA was then diluted with 80 μL water, and 1 μL of diluted cDNA was used in QPCR. For each cDNA sample, channel catfish 18S ribosomal RNA primers were included as an internal control to normalize the variation of cDNA amount (Pridgeon et al. 2010). Sequences of AMP genes deposited at GenBank were used to design gene-specific primers by using Primer3 program (http://frodo.wi.mit.edu/primer3). Primers used for amplification of the 18S ribosomal RNA gene and seven AMPS are listed in Table 1. To determine the specificities of the primers, PCR was performed using uninfected channel catfish posterior kidney cDNA as template, and PCR products were subjected to sequencing at USDA-ARS Mid South Genomic Laboratory with an ABI 3730 Genetic Analyzer (Applied Biosystems). Sequences were analysed using the National Center for Biotechnology Information (NCBI) BLAST program to search for sequence homologies. All primers were confirmed to be specific through sequence analysis. All QPCRs were performed on an Applied Biosystems 7300 Real-Time PCR System (ABI) using Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen) in a total volume of 12.5 μL. The QPCR mixture consisted of 1 μL of cDNA, 0.5 μL of 5 μM gene-specific forward primer, 0.5 μL of 5 μM gene-specific reverse primer and 10.5 μL of 1× SYBR Green SuperMix. The QPCR thermal cycling parameters were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All QPCR was run in duplicate for each cDNA sample, and three samples at each time point were used for QPCR. The relative transcriptional level of AMP was determined by subtracting the cycle threshold (Ct) of the sample by that of the 18S rRNA, the calibrator or internal control, as per the formula: 

$$
\Delta C_t = C_t (\text{AMP}) - C_t (18S \text{ rRNA})
$$

Relative expression level of specific AMP at certain time points post-injection of TSB or *E. ictaluri* was then calculated by the formula of 2^ΔΔCt where 

$$
2^{\Delta \Delta C_t} = 2^{(\Delta C_t \text{ (time point 0)} - \Delta C_t \text{ (time point x)})}
$$

as described previously (Pridgeon et al. 2010). Relative expression level of specific AMPs in *E. ictaluri*-infected fish compared to that in TSB-treated fish was then calculated by the formula 2^{ΔΔCt} where

<table>
<thead>
<tr>
<th>Gene name (accession no.)</th>
<th>Primer name</th>
<th>Primer sequence (5'–3')</th>
<th>Annealing temperature (°C)</th>
<th>Product size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s rRNA (BE469353)</td>
<td>18S-F</td>
<td>ATGGCCGTTCTTAGTTGGTG</td>
<td>59.99</td>
<td>220</td>
</tr>
<tr>
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<td>18S-R</td>
<td>TAGTGAACCACACGCTGATCG</td>
<td>60.03</td>
<td>235</td>
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<tr>
<td>NK-lysin type 1 (AY934592)</td>
<td>NKL1-F</td>
<td>GGGCCATGAAGAAAGTGAAGA</td>
<td>60.05</td>
<td>158</td>
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<tr>
<td></td>
<td>NKL1-R</td>
<td>GCTTGGAAACATCCAGCAT</td>
<td>60.08</td>
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<tr>
<td>NK-lysin type 2 (DQ153186)</td>
<td>NKL2-F</td>
<td>TGTAAGTGGGGCGATGAACAA</td>
<td>59.96</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td>NKL2-R</td>
<td>TCCTCGAACAAGGTATCCCA</td>
<td>60.31</td>
<td></td>
</tr>
<tr>
<td>NK-lysin type 3 (DQ153187)</td>
<td>NKL3-F</td>
<td>GGGCTGACAAACTCCACAGT</td>
<td>60.16</td>
<td>157</td>
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<tr>
<td></td>
<td>NKL3-R</td>
<td>GGATCAACTCCACAGTGTC</td>
<td>59.99</td>
<td>183</td>
</tr>
<tr>
<td>BPI (AY816351)</td>
<td>BPI-F</td>
<td>TGTTGGCCCTTGGCTCCTTT</td>
<td>59.99</td>
<td>183</td>
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<tr>
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<td>BPI-R</td>
<td>TGGCTATGGGGGAGAGCCTTC</td>
<td>60.07</td>
<td>162</td>
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<tr>
<td>Cathepsin D (GUS88646)</td>
<td>CathD-F</td>
<td>CTGGAGAGAAATGTCGCA</td>
<td>60.75</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>CathD-R</td>
<td>GGTGAGAAACCAGGCCCTA</td>
<td>59.82</td>
<td></td>
</tr>
<tr>
<td>Hepcidin (AY834209)</td>
<td>Hepc-F</td>
<td>TGCAACTTTACACTTGAGG</td>
<td>59.02</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>Hepc-R</td>
<td>AGGTGACTCTTGACGCTCTCG</td>
<td>60.06</td>
<td></td>
</tr>
<tr>
<td>LEAP2 (AY845141)</td>
<td>TLR5-F</td>
<td>TTGGAAGGCCCTCAAATCTC</td>
<td>59.85</td>
<td>212</td>
</tr>
<tr>
<td></td>
<td>TLR5-R</td>
<td>ACCGGAGGTGAATAATC</td>
<td>60.02</td>
<td></td>
</tr>
</tbody>
</table>

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Table 1 Primers used in quantitative polymerase chain reaction analysis of antimicrobial peptide genes in channel catfish

BPI, bactericidal permeability-increasing protein.
\[ \Delta C_t = \Delta C_t (E. \text{ ictaluri}) - \Delta C_t (\text{TSB}) \] as described previously (Pridgeon et al. 2010).

**Amount of E. ictaluri in the posterior kidney at 6 h post-injection**

To determine whether there is a correlation between gene upregulation and bacterial amount in the posterior kidney samples, three catfish fingerlings were exposed to *E. ictaluri* by intraperitoneal injection at six doses (0, 10⁴, 10⁵, 10⁶, 10⁷ and 10⁸ CFU per fish). At 6 h post-injection, posterior kidney samples were collected and weighed. Posterior kidney samples were then ground in TSB containing 0.1% Triton X-100 and serially diluted in TSB. One hundred microlitres of serially diluted samples was then plated onto TSA plates. After 24-h incubation at 28 °C, the number of CFU was counted and the average concentration in the posterior kidney (CFU mg⁻¹) was calculated for each dose injected.

**Synthesis of mature hepcidin peptide and in vitro effect on cell proliferation of E. ictaluri**

The protein sequence of hepcidin deposited at GenBank (accession no: AAX39713) was subjected to sequence analysis, and the signal peptide sequence (first 70 amino acids) was predicted by SignalP at http://www.cbs.dtu.dk/services/SignalP (Nielsen, Engelbrecht, Brunak & von Heijne 1997; Wang, Cai, Cai, Qu, Yang & Zhang 2009). The predicted mature peptide (RQSHLSLCRYCCN CCKNKGGFCRDF) was then synthesized by Biosynthesis. MALDI-TOF mass spectrometry and HPLC analyses were performed to identify the synthetic peptides. The effect of the synthetic hepcidin peptide on the proliferation of *E. ictaluri* was performed according to published procedures (Wang et al. 2009) with slight modifications. Briefly, the synthesized hepcidin peptide was dissolved in Milli-Q water to make a stock solution of 10.24 mg mL⁻¹. A twofold serial dilution of the hepcidin peptide was performed in a 96-well plate to make a series of concentrations (0.5–512 μM final concentration in a total volume of 100 μL). Logarithmic-phase *E. ictaluri* bacterial cultures were diluted in TSB to make a final concentration of 1.0 × 10⁶ CFU mL⁻¹ based on published procedures (Wang et al. 2009). The assay mixture contained 50 μL diluted hepcidin and 50 μL diluted bacterial culture in TSB. Plates were incubated at 28 °C. The number of viable cells in each well was determined by CellTiter 96® AQeuous Non-Radioactive Cell Proliferation Assay (MTS) (Promega). Briefly, 20 μL of solution containing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt] (MTS) and an electron coupling reagent phenazine methosulphate was added to each well. The MTS was then bioreduced by viable cells into a formazan product for 15 min. The absorbance of the formazan product at 15 min post-MTS addition was then measured at 490 nm. The optical density of the 96-well plate before and after incubation at different time points was measured at 490 nm using a BioRad 680 microplate reader (Bio-Rad). Relative increased optical density (OD) value was calculated using the following formula: Increased OD value = OD value after incubation – OD value before incubation. Mean increased OD data were then determined by analysis of variance (ANOVA) using SigmaStat statistical analysis software (Systat Software). Minimum inhibitory concentration (MIC) was calculated as the lowest hepcidin concentration that significantly (P < 0.05) decreased the ΔOD compared to that of TSB negative control (no hepcidin present).

**Data analysis**

All data used in statistical comparison were tested for normality and equal variance using SigmaStat statistical analysis software (Systat Software). After normality and equal variance tests were passed, data were then determined by analysis of variance (ANOVA). P-values of 0.05 or less were considered statistically significant. Pearson’s correlation analysis between the amount of *E. ictaluri* in the posterior kidney at 6 h post-injection and the amount of *E. ictaluri* injected into fish or the transcriptional level of hepcidin in infected fish was calculated using the SigmaStat statistical analysis software.

**Results**

**Expression profiles of the seven AMP genes in uninfected channel catfish**

In uninfected channel catfish at all time points, the cycle threshold values for all seven AMPs studied were higher than that of 18S rRNA, indicating that all seven AMPs were less abundant than 18S rRNA. In uninfected fish, relative cycle threshold values for
any given AMP at all seven time points (0, 2, 4, 6, 12, 24 and 48 h post-TSB injection) were not significantly different. The relative cycle threshold of the seven AMPs compared with 18S rRNA at 0 h is shown in Fig. 1. Of the seven AMPs studied, two (cathepsin D and hepcidin) were significantly $(P < 0.001)$ less abundant than the other five (NK-lysin type 1/NK L-1, NK-lysin type 2/NK L2, NK-lysin type 3/NK L-3, BPI and LEAP2) (Fig. 1).

Expression profiles of the seven AMP genes in response to infection at different time points

The expression profile of the seven AMP genes in response to infection is summarized in Fig. 2. At 48 h post-injection, the transcriptional level of NK-lysin type 1 in *E. ictaluri*-infected fish was significantly $(P < 0.05)$ higher than that in TSB-injected control fish (Fig. 2a). However, the transcriptional level of NK-lysin type 2 in infected fish at all time points was not significantly different from that in TSB-injected control fish (Fig. 2b). At 24 and 48 h post-injection, the transcriptional level of NK-lysin type 3 in infected fish was significantly $(P < 0.05)$ upregulated (Fig. 2c). At 24 h post-injection, the transcriptional level of BPI in infected fish was significantly $(P < 0.05)$ upregulated (Fig. 2d). At 24 h post-injection, the transcriptional level of cathepsin D in infected fish was significantly $(P < 0.05)$ upregulated (Fig. 2e). At 4, 6, 12 and 24 h post-injection, the transcriptional level of hepcidin in infected fish was significantly $(P < 0.05)$ upregulated (Fig. 2f). However, the transcriptional level of LEAP2 at all time points post-infection was not significantly different from that of control fish (Fig. 2g). When the transcriptional levels of significantly induced AMP identified were compared with each other, the transcriptional level of hepcidin at 6 h post-*E. ictaluri* infection was upregulated the highest (Fig. 2h).

Expression profiles of hepcidin in response to *E. ictaluri* infection at different doses

When six doses (0, 10⁴, 10⁵, 10⁶, 10⁷ and 10⁸ CFU per fish) of *E. ictaluri* were injected to channel catfish, the 14-day cumulative mortalities were 0%, 0%, 25%, 75%, 100% and 100%, respectively. At a sublethal dose of 10⁴ CFU per fish, the transcriptional level of hepcidin in infected fish was not significantly different from that in uninfected fish (Fig. 3a,b). At a low lethal dose (25% mortality) of 10⁵ CFU per fish, the average transcriptional level of hepcidin in infected fish was higher than that in uninfected fish (Fig. 3). However, the difference was not statistically significant $(P = 0.054)$. At a lethal dose of 10⁶ CFU per fish (50% mortality), the transcriptional level of hepcidin in infected fish was significantly $(P < 0.001)$ higher than that in uninfected fish (Fig. 3). At a lethal dose of 10⁷ CFU per fish (100% mortality), the transcriptional level of hepcidin in infected fish was also significantly $(P < 0.001)$ higher than that in uninfected fish (Fig. 3). At a lethal dose of 10⁸ CFU per fish (100% mortality), the average transcriptional level of hepcidin in infected fish was higher than that in infected fish at doses of 10⁶ and 10⁷ CFU per fish, but that increase was not statistically significant $(P > 0.05)$ (Fig. 3). At all doses, the remaining six AMPs were not significantly upregulated in the posterior kidney of infected fish (data not shown).

Bacterial concentrations in the posterior kidney at 6 h post-injection of *E. ictaluri* at different injection doses

As hepcidin was upregulated the highest at 6 h post-injection of *E. ictaluri* at a dose of 10⁶ CFU per fish, this time point was chosen to study the expression of hepcidin in response to *E. ictaluri* infection at different doses. Posterior kidney sam-

![Figure 1](https://via.placeholder.com/150)

**Figure 1** Relative cycle threshold of antimicrobial peptide (AMP) gene compared with 18S rRNA in control fish. The relative cycle threshold was calculated using the formula $\Delta C = C_\text{AMP} - C_{18S\ rRNA}$. Data are presented as mean ± standard deviation (SD) from three replicates.

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Transcription of AMPs in response to E. ictaluri infection

(a) Tryptic soy broth
(b) E. ictaluri

(c) Tryptic soy broth
(d) E. ictaluri

(e) Tryptic soy broth
(f) E. ictaluri

(g) Tryptic soy broth
(h) E. ictaluri

* P < 0.05
* P = 0.003
* P < 0.05
* P < 0.05
samples were used to estimate the concentrations of *E. ictaluri* at 6 h post-injection. Plate count results revealed that all posterior kidney samples of the fish injected with *E. ictaluri* were positive for the bacterium, whereas the control TSB-injected fish were negative for *E. ictaluri*. The amount of *E. ictaluri* in the posterior kidney increased when injection doses were increased (Table 2). Correlation analysis revealed that the amount of bacteria in the posterior kidney significantly (\( P = 0.0418 \)) correlated with the amount of bacteria injected into the fish (\( R^2 = 0.892 \)). However, there was no significant correlation between expression levels of hepcidin and concentrations of *E. ictaluri* in the posterior kidney (\( P > 0.05 \)).

**Effect of synthetic hepcidin peptide on the in vitro cell proliferation of *E. ictaluri***

The effect of synthetic hepcidin peptide at different concentrations on in vitro cell proliferation of *E. ictaluri* is summarized in Figs 4 (low doses) and 5 (high doses). At a concentration of 8 \( \mu \)M or lower, the synthetic hepcidin failed to significantly decrease the in vitro cell proliferation of *E. ictaluri* at all time points (Fig. 4). On the other hand, lower concentrations of hepcidin significantly (\( P < 0.05 \)) increased the cell proliferation of *E. ictaluri* at 60 min post-incubation and afterwards (Fig. 4). The MIC of hepcidin against *E. ictaluri* cell proliferation was 16 \( \mu \)M, at which concentration the synthetic hepcidin significantly (\( P < 0.05 \)) decreased the in vitro cell proliferation of *E. ictaluri* at 30 min post-incubation (Fig. 5a). However, from 45 to 90 min post-incubation, there was no significant difference in cell proliferation of *E. ictaluri* in the presence or absence of 16 \( \mu \)M hepcidin.

**Figure 2** Relative transcriptional level of antimicrobial peptide (AMP) in the posterior kidney of *Edwardsiella ictaluri*-infected fish compared to that in control fish at different time points. The induced transcriptional level is calculated using the formula of \( 2^{\Delta C_t} \), where \( \Delta C_t = C_t \) (infected or control) - \( C_t \) (average control) and \( \Delta C_t = C_t \) (AMP) - \( C_t \) (18S rRNA). Data are presented as mean ± standard deviation (SD) from five replicates. (a) NK-lysin type 1; (b) NK-lysin type 2; (c) NK-lysin type 3; (d) BPI; (e) cathepsin D; (f) hepcidin; (g) LEAP2; (h) comparison. BPI, bactericidal permeability-increasing protein; LEAP2, liver-expressed AMP 2.

**Figure 3** Relative transcriptional level of hepcidin in the posterior kidney of channel catfish at 6 h post-injection of *Edwardsiella ictaluri* at different injection doses. The induced transcriptional level was calculated using the formula 2\( ^{\Delta C_t} \) where \( \Delta C_t = C_t \) (infected) - \( C_t \) (control) and \( \Delta C_t = C_t \) (hepcidin) - \( C_t \) (18S rRNA). Data are presented as mean ± standard deviation (SD) from five replicates.

**Table 2** Amount of *Edwardsiella ictaluri* in the posterior kidney of channel catfish at 6 h post-injection of *E. ictaluri* at different doses

<table>
<thead>
<tr>
<th>Dose injected (CFU per fish)</th>
<th>Amount of <em>E. ictaluri</em> in the posterior kidney (CFU mg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 \times 10^4</td>
<td>Fish 1 19 Fish 2 14 Fish 3 23 Average (SD) 19 ± 5</td>
</tr>
<tr>
<td>1 \times 10^5</td>
<td>163 67 137 182 ± 126</td>
</tr>
<tr>
<td>1 \times 10^6</td>
<td>561 855 1403 940 427</td>
</tr>
<tr>
<td>1 \times 10^7</td>
<td>94 737 94 444 54 546 81 242 23 120</td>
</tr>
<tr>
<td>1 \times 10^8</td>
<td>157 895 150 000 125 000 144 298 ± 7172</td>
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</table>

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**Figure 4** Effect of low concentrations of synthetic hepcidin peptide on in vitro cell proliferation of *Edwardsiella ictaluri*. Relative cell proliferation of *E. ictaluri* was indicated by relative increase of optical density values at 490 nm (OD\(_{490\text{ nm}}\)) before and after incubation in the presence of hepcidin. The relative OD\(_{490\text{ nm}}\) increase was calculated using the formula ΔOD = OD\(_{\text{after incubation}}\) - OD\(_{\text{before incubation}}\). Data are presented as mean ± standard deviation (SD) from four replicates.
At 3 and 5 h post-exposure, the synthetic hepcidin at 16 lM significantly increased the in vitro cell proliferation of *E. ictaluri* (Fig. 5a). At concentrations of 32 and 64 lM, the synthetic hepcidin significantly decreased the in vitro cell proliferation of *E. ictaluri* at 30, 45 and 60 min post-exposure (Fig. 5b,c). However, at 90 min post-exposure, there was no significant difference in cell proliferation of *E. ictaluri* in the presence or absence of hepcidin. At 5 h post-exposure, the synthetic hepcidin significantly increased the in vitro cell proliferation of *E. ictaluri* (Fig. 5b,c). At a concentration of 128 lM, the synthetic hepcidin significantly decreased the in vitro cell proliferation of

Figure 5 Effect of higher concentrations of synthetic hepcidin peptide on *in vitro* cell proliferation of *Edwardsiella ictaluri*. a: 16 lM; b: 32 lM; c: 64 lM; d: 128 lM; e: 256 lM; f: 512 lM. Relative cell proliferation of *E. ictaluri* was indicated by relative increase of optical density values at of 490 nm (OD$_{490}$ nm) before and after incubation in the presence of hepcidin. The relative OD$_{490}$ nm increase was calculated using the formula $\Delta$OD = OD$_{after \, incubation}$ - OD$_{before \, incubation}$. Data are presented as mean ± standard deviation (SD) from four replicates.
**E. ictaluri** up to 90 min post-exposure (Fig. 5d). At a concentration of 256 μM, the synthetic hepcidin significantly decreased the *in vitro* cell proliferation of *E. ictaluri* up to 180 min post-exposure (Fig. 5e). At a concentration of 512 μM, the synthetic hepcidin significantly decreased the *in vitro* cell proliferation of *E. ictaluri* up to 300 min (Fig. 5f, post-exposure).

**Discussion**

Channel catfish, the predominant fish species cultured in the southern United States, is particularly susceptible to ESC. Under artificial challenge conditions, ESC is responsible for high mortality levels well before the adaptive immunity starts to respond (Wolters & Johnson 1994). Therefore, the innate immune response of channel catfish to ESC and other bacterial infections is critical in preventing the onset of infection. This study compared the expression profiles of seven AMPs in response to acute *E. ictaluri* infection at different time points after infection. Our results revealed that five AMPs (NK-lysin type 1, NK-lysin type 3, cathepsin D, BPI and hepcidin) were significantly upregulated within 48 h post-infection, suggesting that these AMPs play important roles in the early defence against infection. Of the five AMPs, hepcidin was upregulated the earliest (within 4 h post-infection), the most (more than 100-fold) and the longest (4, 6, 12 and 24 h post-infection). Significant upregulations of hepcidin in channel catfish at 24 h post- *E. ictaluri* infection (Bao *et al.* 2005) and in orange-spotted grouper, *Epinephelus coioides* (Hamilton), at 4, 6, 8, 12 and 24 h post-*Vibrio vulnificus* infection (Zhou, Wei, Xu, Cui, Yan, Ou-Yang, Huang, Huang & Qin 2011) have been reported previously. Taken together, our results suggest that hepcidin plays an important role in innate immunity against *E. ictaluri* at the early infection stage.

Dose-dependent immune-related gene regulation has been reported previously. For example, immune genes such as interleukin-1β, TNFα1, serum amyloid A and interferon-γ in rainbow trout, *Oncorhynchus mykiss* (Walbaum), were all significantly upregulated at 24 h post-infection by *Vesuvius ruckeri* (the causative agent of enteric red mouth disease) at lethal doses of 50 000 and 500 000 CFU per fish (Wiens & Vallejo 2010). However, at a sublethal dose of 50 CFU per fish, none of the immune-related genes was significantly upregulated (Wiens & Vallejo 2010). Our results revealed that at a sublethal dose of 10⁴ CFU per fish (no fish mortality), hepcidin was not upregulated. However, at a dose of 10⁵ CFU per fish that caused 25% mortality, the upregulation of hepcidin occurred in the majority (80%, four of five) of infected fish. At a dose of 10⁶ CFU per fish that caused 50% mortality, hepcidin was significantly upregulated in all infected fish. Taken together, our results suggest that infection dose has to reach a certain level in the host to induce the upregulation of hepcidin.

In general, the concentrations of AMPs needed to inhibit growth of microbes are at micromolar concentrations (Zasloff 2002). For example, synthetic large yellow croaker hepcidin (PC-hepc) has been reported to exhibit different activities against different bacteria, with MICs ranging from 3 to 24 μM (Wang *et al.* 2009). The MICs of synthetic bass hepcidin against different bacteria have been reported to range from 11 to 44 μM (Lauth, Babon, Stannard, Singh, Nizet, Carlberg, Ostland, Pennington, Norton & Westerman 2005). The MIC of synthetic hepcidin-1 from orange-spotted grouper against the Gram-negative bacterium *V. vulnificus* and the Gram-positive bacterium *S. aureus* is 50 μg mL⁻¹ or higher (equivalent to 20 μM) (Zhou *et al.* 2011). The MIC of hepcidin-2 from orange-spotted grouper against *V. vulnificus* is reported to be 400 μg mL⁻¹ (equivalent to 160 μM) (Zhou *et al.* 2011). Our results revealed that the MIC of the synthetic channel catfish hepcidin against *E. ictaluri* was 16 μM, suggesting that the activity of channel catfish hepcidin is similar to most fish hepcidin, but higher than that of hepcidin-2 from orange-spotted grouper. However, MIC assay incubation time and pathogen amount used in the assay will affect the MIC results. Whether the activity of hepcidin from one fish is higher than that from other fish species needs to be determined under the same assay conditions against the same pathogen.

In summary, the relative transcriptional levels of seven channel catfish AMP genes in response to acute infection of *E. ictaluri* were determined. Among the AMPs that were significantly upregulated at different time points, hepcidin at 4, 6 and 12 h post-infection was upregulated the most. When catfish were injected with different doses of *E. ictaluri*, all lethal doses were able to induce significant (*P* < 0.05) upregulation of hepcidin in the posterior kidney, whereas sublethal doses failed to induce any significant upregulation of hepcidin. *In vitro* growth studies revealed that the
presence of synthetic hepcidin peptide at concentrations of 16 μM or higher significantly inhibited the growth of *E. ictaluri*. Taken together, our results suggest that hepcidin might play an important role in channel catfish against *E. ictaluri* infection.

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**References**


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