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Antimicrobial peptide CAP18 and its effect on *Yersinia ruckeri* infections in rainbow trout *Oncorhynchus mykiss* (Walbaum): comparing administration by injection and oral routes

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Abstract

The antimicrobial peptide CAP18 has been demonstrated to have a strong *in vitro* bactericidal effect on *Yersinia ruckeri*, but its activity *in vivo* has not been described. In this work, we investigated whether CAP18 protects rainbow trout *Oncorhynchus mykiss* (Walbaum) against enteric red mouth disease caused by this pathogen either following i.p. injection or by oral administration (in feed). It was found that injection of CAP18 into juvenile rainbow trout before exposure to *Y. ruckeri* was associated with lowered mortality compared to non-medicated fish although it was less effective than the conventional antibiotic oxolinic acid. Oral administration of CAP18 to trout did not prevent infection. The proteolytic effect of secretions on the peptide CAP18 in the fish gastrointestinal tract is suggested to account for the inferior effect of oral administration.

Keywords: antimicrobial peptide, CAP18, rainbow trout, *Yersinia ruckeri*.

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Introduction

Antimicrobial peptides (AMPs) are small proteins (<40 amino acids) with a potential to eliminate a wide range of pathogenic microorganisms. They are encoded in the genomes of both vertebrates and invertebrates (Zaslhoff 2002; Noga *et al.* 2011) and act as important components of the innate immune system primarily by killing pathogens through disruption of the bacterial membrane although they also may display immunomodulatory activities (Ganz 2003; Hancock & Sahl 2006). A large number of these peptides have been identified in several fish species (Uzzell *et al.* 2003; Chang *et al.* 2005; Chang *et al.* 2006; Maier *et al.* 2008; Smith & Fernandes 2009; Rajanbabu & Chen 2011; Masso-Silva & Diamond 2014) suggesting an evolutionary old role in immunity. Following extensive usage of antibiotics in human and veterinary medicine for decades emergence of antibiotic resistance in a series of bacterial pathogens, some which may infect fish, has been recorded (Cabello 2006). This has raised growing concern for future control of fish diseases and new and alternative antimicrobial peptides with a therapeutic potential have therefore been in focus (Jia *et al.* 2000; Douglas 2011). It is noteworthy that development of bacterial resistance to these



peptides is less readily developed compared to conventional antibiotics (Steinberg *et al.* 1997; Cabello 2006; Noga *et al.* 2011). A range of synthetic antimicrobial peptides have been developed and clinically tested but only a limited number of them have confirmed the expected efficacy (Hancock & Sahl 2006; Zhang & Falla 2006). In addition, a limitation associated with the use of these peptides as antibiotics is the potential cytotoxicity associated with systemic application. Finally some of the peptides are not resistant to proteases potentially losing their function following oral administration (Travis *et al.* 2000; Hancock & Sahl 2006). AMP application for control and treatment of yersiniosis in rainbow trout *Oncorhynchus mykiss* (Walbaum) has not been investigated previously. Therefore, the present study was undertaken to elucidate if a synthetic AMP, belonging to the cathelicidin family, has an antibiotic effect against *Yersinia ruckeri* infection in rainbow trout. We investigated three synthetic broad-spectrum antimicrobial peptides (CAP11, CAP11-1-18 m2 and CAP18) which recently have shown *in vitro* effect against *Y. ruckeri* (Ebbensgaard *et al.* 2015). Prior to the *in vivo* experiment, the cytotoxicity of these peptides for fish cells (rainbow trout red blood cells) was assessed in a haemolytic assay and the non-toxic AMP (CAP18) was selected for further *in vivo* work comparing administration methods (intraperitoneal AMP injection and oral AMP administration in feed). Different groups of rainbow trout receiving CAP18 were then exposed to *Y. ruckeri* infection (1-h bath challenge) and the disease progression was subsequently recorded and compared to non-medicated control fish and fish receiving a conventional antibiotic (oxolinic acid).

Materials and methods

Cathelicidin peptides CAP11 (94.7% purity), CAP11-1-18 m2 (87.9% purity) and CAP18 (89.5% purity), were designed and synthesized by GenScript (USA). These AMPs showed generally high *in vitro* antimicrobial activities, that is minimum inhibitory concentration (MIC) value of 2–64 mg L⁻¹, against Gram-negative bacteria including *Y. ruckeri* (Ebbensgaard *et al.* 2015).

Haemolysis assay

The cytotoxicity of these AMPs was determined by measuring the released haemoglobin from

rainbow trout red blood cells. Blood samples were collected by caudal vein puncture from rainbow trout juveniles (10–12 g) using heparinized syringes and transferred to Eppendorf tubes each containing 100 µL heparin. The blood sample was diluted with PBS and centrifuged at 4696 g for 5 min. The supernatant was discarded and RBC resuspended in PBS containing 100 µL heparin whereupon the cell number was adjusted to 10⁶ cells mL⁻¹. Dilution series (1:10–1:1000) of antimicrobial peptides CAP11, CAP11-1-18m2 and CAP18 (all in basic concentrations of 10 mg mL⁻¹) were prepared in a 96-well plate, and 100 µL of the RBC suspension was added to each well, which was then incubated at room temperature (18 °C) in an orbital shaker (50 rpm) for 1 h. The plate was subsequently centrifuged at 4696 g for 5 min, and the supernatant (100 µL) was transferred to another plate and read at 540 nm in ELISA plate reader, Epoch Microplate Spectrophotometer (Holm & Halby), to measure released haemoglobin. Triton X-100 (0.2%) was used as positive control, as it caused 100% haemolysis, and PBS was applied as negative control. CAP18 and CAP11-1-18m2 were dissolved in water, while dimethyl sulphoxide (DMSO) was used for dissolving CAP11. Therefore, not only PBS but also DMSO was tested alone to examine the effect of this solvent on fish RBCs.

Infection experiments

Based on the results of the haemolytic assay, CAP18 was selected because it showed the lowest haemolytic effect on fish RBCs making it the best candidate for further *in vivo* experiments. Two experiments were performed to determine the functional effective dose of CAP18. This was followed by two main experiments where the selected dose of CAP18 was used on a larger number of fish. For these trials, administration by intraperitoneal (i.p.) injection and the oral route (in feed) were tested.

Experimental fish

For all experiments, we used rainbow trout (Fousing strain) fry (2–5 g), hatched and reared in a pathogen-free facility at the Salmon Hatchery, Bornholm, Denmark (Xueqin, Kania & Buchmann 2012). Fish were transported to the university fish-keeping facility, Copenhagen, Denmark,

and acclimatized for 1–2 weeks before experimentation. Upon arrival at the university fish-keeping unit, the pathogen-free status of fish was confirmed by examining 2–3 fish for bacterial (head kidney swabs) and parasitic infection (Buchmann 2007). During the acclimatization period, water temperature was slowly increased until the desired temperature of 15–16 °C and a light–dark scheme (12/12-h light/dark) was attained. Fish were fed (1.5% of biomass per day) with commercial standard pelleted feed (Inicio 917; BioMar A/S) until challenge.

Dose adjustment experiments for i.p. injection of CAP18. In the first experiment, rainbow trout fry were anaesthetized in MS222 (50 mg L⁻¹) (Sigma–Aldrich) and intraperitoneally injected with 0.1 mL of an aqueous solution containing different amounts of the antimicrobial peptide CAP18, that is 50 µg, 100 µg, 200 µg and 400 µg per fish. Control fish were only injected with sterile distilled water. Fish from all the groups were stocked together in the same aquaria, and tagging of different groups of fish was performed by partial fin clipping (upper caudal fin, lower caudal fin or adipose fin). One hour post-injection with CAP18, fish were challenged by bath exposure to *Y. ruckeri* O1 biotype 2 (5.6 × 10⁸ CFU mL⁻¹) for 1 h. Subsequently, fish were transferred to clean water and maintained until 15 days post-infection (dpi). The fish were examined every second hour, and moribund fish were removed and killed in a lethal overdose of MS222 (300 mg L⁻¹) (according to the animal welfare guidelines of the University of Copenhagen, Denmark).

Efficacy testing experiment. According to the results from the preliminary experiments, the effective dose of 400 µg CAP18 per fish was selected for the main experiment. Groups of fish receiving the antibiotic oxolinic acid (at 200 mg kg⁻¹ body weight) were included as positive controls. A total of six treatment groups with replicate tanks for each group were set up. Rainbow trout fry were anaesthetized in MS222 and intraperitoneally injected (0.1 mL) with the antimicrobial peptide CAP18 (400 µg per fish) or the antibiotic oxolinic acid (200 mg kg⁻¹ body weight). One hour post-injection with AMP or oxolinic acid, fish were challenged by bath exposure to *Y. ruckeri* O1 biotype 2 (4 × 10⁶ CFU

mL⁻¹) in 5 L water volume for 6 h, and then, the fish tank water level was raised up to 20 L diluting the bacterial concentration to 1 × 10⁶ CFU mL⁻¹. Fish were allowed to swim in this bacterial solution for 18 h after which water was totally replaced with fresh tap water and fish were maintained until 14 dpi. The fish were examined every second hour, and moribund fish were killed and recorded. Three control groups comprising unmedicated fish, CAP18-injected fish and oxolinic acid-injected fish were kept uninfected.

Oral administration (CAP18 in feed)

Based on the results of the i.p. injection experiment, the dose of 400 µg CAP18 per fish was chosen for the in-feed-based delivery of AMP. Due to the risk of proteolytic degradation of AMPs in the fish digestive tract and risk of CAP18 leaching into the water, we also tested a higher dose, 800 µg CAP18 per fish. Eight experimental groups with replicate tanks were used. Fish were fed (1.5% of biomass) with different diets during the experiment. The uninfected and infected control fish were fed commercial standard non-medicated feed (BioMar A/S) during the entire experiment.

The feed composition for the different treatment groups is shown in Table 1. The positive control, receiving conventional antibiotic, was fed with commercial standard feed until challenge but received oxolinic acid containing feed from the day of challenge until 14 dpi. Four groups were fed with CAP18-coated feed, and they all received this diet from a week prior to challenge. One group was fed with a dose of 400 µg g⁻¹ feed for only 1 week until challenge, after which the fish were offered non-medicated feed. A second group

Table 1 Composition of feed in the different treatment groups and resulting mortality following *Yersinia ruckeri* challenge (bath exposure)

Groups	Survival (%)
Infected unmedicated control	50
CAP18-coated feed (400 µg g ⁻¹) [for 1 week]	54
CAP18-coated feed (400 µg g ⁻¹) [for 3 weeks]	48
CAP18-coated feed (800 µg g ⁻¹) [for 1 week]	38
CAP18-coated feed (800 µg g ⁻¹) [for 3 weeks]	58
Oxolinic acid-coated feed (1.25 g kg ⁻¹ feed)	98
CAP18 injection (400 µg fish ⁻¹)	58
Uninfected control	100

was fed with the same dose for 3 weeks; that is, feeding was continued after challenge until the end of the experiment. The remaining two groups were fed with the high dose, $800 \mu\text{g g}^{-1}$ feed, correspondingly.

Preparation of medicated feed

To avoid leaching of CAP18 from the pellets into the tank water during feeding, the feed was top-coated with CAP18 and fish oil under vacuum allowing the AMP/oil to penetrate pores in the feed pellet. Dry powder of oxolinic acid (1.25 g kg^{-1} feed) was mixed with fish oil (at room temperature), and feed was top-coated by spraying the mixture on 1.5-mm pelleted feed (BioMar A/S) while stirring. The medicated feed was stored at 4°C .

Challenge of fish receiving CAP18 in feed

After 7 days of feeding with CAP18-coated feed, fish were challenged. Fish from all groups (except uninfected control fish) were challenged by bath exposure to *Y. ruckeri* O1 biotype 2 ($1 \times 10^6 \text{ CFU mL}^{-1}$) by the procedure described above for efficacy testing. The fish were examined every second hour, and moribund fish were removed, killed and recorded. Water temperature was measured on a daily basis in the course of experiment with a mean of 15°C . In all infection experiments, bacteria were re-isolated on blood agar (head kidney swabs) from moribund fish (2 fish per tank) to confirm that the bacterium causing disease was identical to the challenge strain.

Data analysis

Mortality in the experimental groups is presented as cumulative mortality. Differences among mortality of different groups were tested using Kaplan–Meier plots and one-way ANOVA with Tukey's post hoc test after combining data from the replicates.

Results

Haemolysis assay

The antimicrobial peptide CAP18 showed a minimal haemolytic activity (15.4%) at 10-fold dilution, reducing to zero at further dilutions tested in this study. The other antimicrobial peptides, CAP11 and CAP11-1-18m2, showed a marked haemolytic activity at 10-fold dilution (96.3% and 71.6%, respectively) although the haemolytic activity of these antimicrobial peptides decreased considerably at subsequent dilutions. The solvent DMSO had an adverse effect on blood cells as it elicited a haemolysis of 30% (Fig. 1). This compound was only used as solvent for CAP11, whereas other AMPs were dissolved in water. Due to the low CAP18 toxicity, this peptide was selected for further analysis.

In vivo experiments

Intraperitoneal injection. Preliminary experiments. In the first challenge experiment with *Y. ruckeri*, the highest survival (70%) was recorded

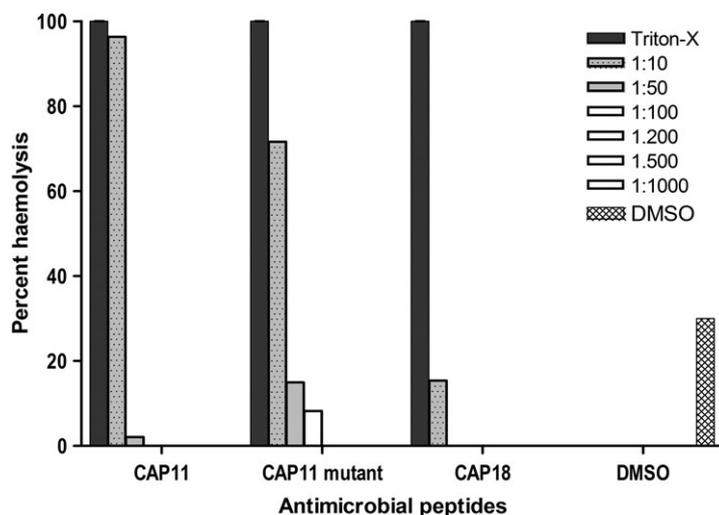


Figure 1 Haemolytic activity of different antimicrobial peptides (CAP11, CAP11 mutant and CAP18) on rainbow trout red blood cells (RBCs).

in fish injected with 400 μg of CAP18 followed by fish injected with 200 and 100 μg CAP18 (30% survival). The mortality in the 200- μg CAP18 group did not stabilize during the experimental period and must be regarded as a minimum mortality. The exposed but unmedicated control group showed a survival of 20%, and all fish died in the 50- μg CAP18-injected group. The onset of mortality was delayed 1 day in the CAP18-injected group compared to the control group (Fig. 2). *Y. ruckeri* was isolated from all killed fish (removed due to disease progression) following challenge.

Main experiment. In the *Y. ruckeri* infection, the highest survival was recorded in the oxolinic acid-injected group (80%) followed by the CAP18-injected (37%) and the control group (13%).

Survival rates of fish groups receiving CAP18 and oxolinic acid were significantly higher ($P < 0.05$) than the control group (Fig. 3). No mortalities were observed in naïve uninfected control group during the course of the experiment (Fig. 3). *Y. ruckeri* was isolated from all killed fish following challenge.

Oral administration of CAP18

In the feeding experiment, the highest mortalities were recorded in the group fed with CAP18 800 $\mu\text{g g}^{-1}$ for 1 week (62%), the group receiving CAP18 400 $\mu\text{g g}^{-1}$ for 3 weeks (52%) and CAP18 400 $\mu\text{g g}^{-1}$ for 1 week and the infected control (50%). The group fed with CAP18 800 $\mu\text{g g}^{-1}$ for 3 weeks had lower mortality rates

Figure 2 Cumulative mortality (%) of rainbow trout fry intraperitoneally injected with antimicrobial peptide CAP18 or antibiotic oxolinic acid following exposure to *Y. ruckeri*.

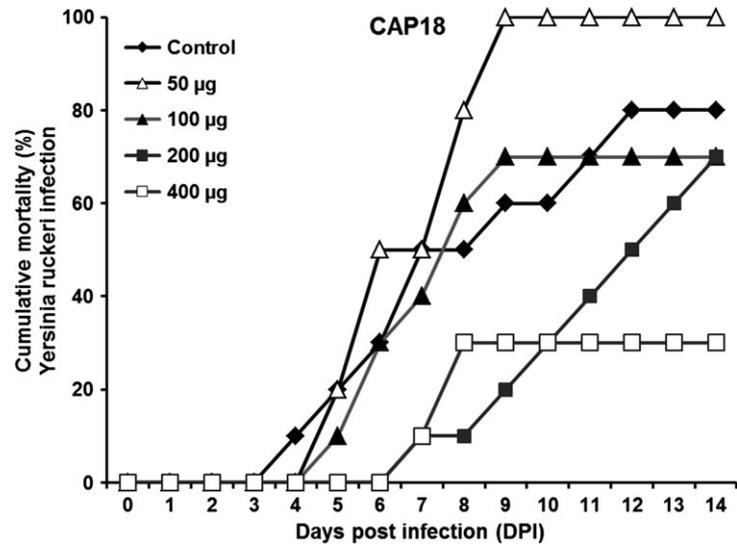
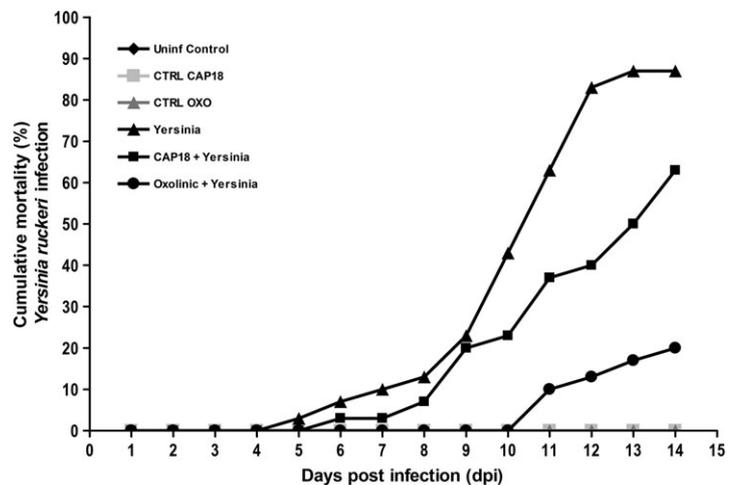


Figure 3 Cumulative mortality (%) of rainbow trout fry intraperitoneally injected with antimicrobial peptide CAP18 or antibiotic oxolinic acid and bath-exposed to *Y. ruckeri*.



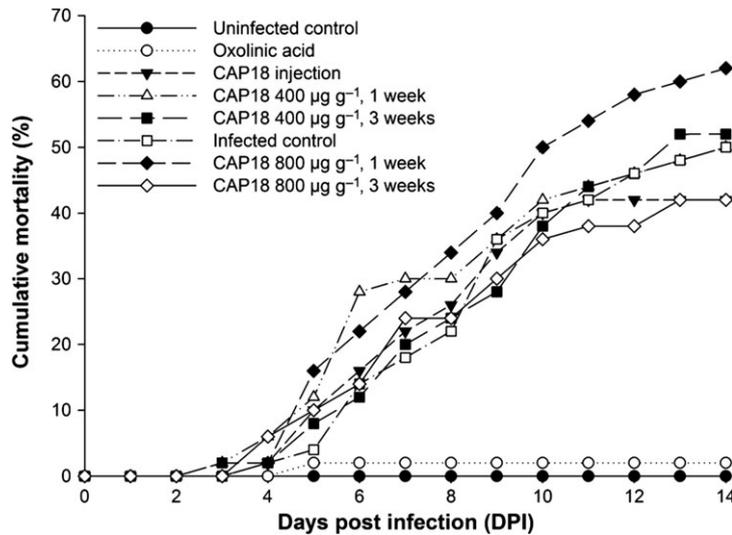


Figure 4 Cumulative mortality (%) of rainbow trout fingerlings fed with antimicrobial peptide CAP18 ($400 \mu\text{g g}^{-1}$ and $800 \mu\text{g g}^{-1}$ feed) or antibiotic oxolinic acid or injected with CAP18 ($400 \mu\text{g fish}^{-1}$) following exposure to *Y. ruckeri* O1 biotype 2 by bath exposure for 6 h.

(42%). Fish receiving oxolinic acid showed the lowest mortality (2%) (Fig. 4; Table 1). There was a delay in the onset of disease in the fish fed with oxolinic acid-coated feed (5 dpi) compared to the other groups (Fig. 4). No disease was observed in naïve uninfected control group during the course of the experiment. Mortality in the oxolinic acid group was significantly lower ($P < 0.001$) than in the other groups, while there were no significant differences ($P > 0.05$) between the mortality of the infected control and the other treated groups. The slightly lowered mortality in the CAP18-injected group (42%) compared to the infected control (50%) was not significant ($P > 0.05$) due to variation of the replicates (mortality rates of 28% and 56%).

Discussion

In the present study, the chemotherapeutic potential in rainbow trout of three cathelicidin antimicrobial peptides, CAP11, CAP11-1-18m2 and CAP18, was evaluated. It was previously shown *in vitro* that these three AMPs have a bactericidal effect (Ebbensgaard *et al.* 2015). However, cytotoxicity studies revealed that CAP11 and CAP11-1-18m2 were able to elicit lysis of rainbow trout RBCs after which these AMPs were excluded from the study. Only CAP18 (18-kDa cationic antimicrobial protein originally isolated and described from rabbit leucocytes) showed high bactericidal activity and low cytotoxicity and was therefore considered a suitable candidate for the *in vivo*

experiments. Earlier studies have also indicated CAP18 to be a promising antimicrobial compound among a range of cationic antimicrobial proteins (Mason *et al.* 1997; Travis *et al.* 2000). We tested both intraperitoneal and oral administration of this antimicrobial peptide and compared it with the action of a well-characterized antibiotic, oxolinic acid. Compared to the non-injected fish, we found that intraperitoneal injection of CAP18 was associated with a significantly lower mortality of rainbow trout exposed to *Y. ruckeri*. The antimicrobial activity was indicated and corresponded to the action of other AMPs shown to protect fish against other Gram-negative bacteria (Jia *et al.* 2000; Pan *et al.* 2007). When testing oral delivery of CAP18, a high and prolonged dose of $800 \mu\text{g g}^{-1}$ applied for 3 weeks led to a slightly higher survival than the other groups orally treated with CAP18, although it was not statistically significant ($P > 0.05$). In the feeding experiment, fish received AMPs a week prior to infection, but it was obvious that this administration method was not sufficient for prevention of mortality associated with *Y. ruckeri* exposure. An earlier study on oral administration of AMPs (epinecidin-1) starting 30 days before the experimental bacterial infection with *Vibrio vulnificus* showed a significantly higher survival in host fishes from 13 dpi (Pan *et al.* 2012). Thus, it cannot be excluded that an even longer premedication period based on the administration of CAP18 as feed supplement could lead to improved effect against *Y. ruckeri*. However, proteolytic

degradation in the fish gastrointestinal tract of the peptides in feed is likely to play a role for the limited effect observed in the present experiment. As these peptides are vulnerable to proteolytic degradation in the gut environment, it may be suggested that the orally administered CAP18 was degraded following ingestion. Therefore, for future studies it may be suggested to improve stability and protease resistance of this peptide by adding compounds such as chitosan or alginate to the feed (Hancock & Sahl 2006; Douglas 2011). In addition, although vacuum and oil coating was used for optimizing AMP adhesion to feed pellets, it cannot be excluded that part of the CAP18 leached from the feed following feeding. The suboptimal effect of CAP18 when used in feed together with the high cost of AMP compounds may at present challenge usage of oral CAP18 administration in the aquaculture industry. In conclusion, the results of this study showed that antimicrobial treatment via intraperitoneal injection of CAP18 was associated with an increased survival in rainbow trout infected with *Y. ruckeri*, whereas oral administration (in-feed administration) was unsuccessful.

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