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The combination effect of *N*-acetyl-D-glucosamine on oral oxytetracycline treatment against *Nocardia seriolae* infection in the yellowtail *Seriola quinqueradiata*

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ABSTRACT

Nocardia seriolae infection is one of the most serious bacterial diseases in *Seriola* species in Japanese aquaculture. In this study, the combination effect of *N*-acetyl-D-glucosamine (NAG) with oral oxytetracycline (OTC) treatment was evaluated with the experimental infection of artificially bred juvenile yellowtail *Seriola quinqueradiata*. Prior to the evaluation, the efficacy of OTC treatment against nocardiosis was assessed. The oral administration of OTC at a dose of 50 mg/kg body weight (BW) for 4 consecutive days did not show sufficient efficacy compared with that of the untreated control group, while the intraperitoneal administration of OTC, which caused no absorption loss in the gastrointestinal tract at a single dose of 50 mg/kg BW successfully suppressed mortality ($P < 0.05$). When OTC was orally administered with the same amount of NAG at a single dose of 50 mg/kg BW, the serum OTC concentrations significantly increased at 1 h and 6 h post-administration ($P < 0.05$); peak concentrations increased by 41%. In two trials evaluating the treatment of nocardiosis, the addition of NAG improved the efficacy of 5-consecutive-day oral OTC treatment by significantly reducing mortality compared with that of the untreated control group ($P < 0.05$). These results suggest that NAG can enhance the absorption of oral OTC and therefore improve the efficacy of nocardiosis treatment.

1. Introduction

The carangids yellowtail *Seriola quinqueradiata* and amberjack *S. dumerili* are the major commercially cultured fish in Japan. The annual production reached approximately 101,700 and 33,800 metric tons for *S. quinqueradiata* and *S. dumerili*, respectively, in 2015 (data from The Ministry of Agriculture, Forestry and Fisheries of Japan: http://www.e-stat.go.jp). *S. quinqueradiata* is an especially important cultured fish in Japan. However, fish nocardiosis is one of the most serious bacterial diseases in cultured *Seriola* species (Kariya et al., 1968; Kusuda and Nakagawa, 1978; Hatai and Yasumoto, 1983). The causative agent of this disease is *Nocardia seriolae*, a Gram-positive intracellular actinomycete (Kudo et al., 1988). The major clinical signs of this disease include the formation of abscesses in the epidermis and tubercles in the gills, kidney and spleen (Kariya et al., 1968; Kusuda and Nakagawa, 1978). Because no commercial vaccines for *N. seriolae* are currently available (Kato et al., 2014), sulfa drugs have been orally administered to treat nocardiosis in Japan. However, the repeated use of a single drug can generate the emergence of sulfa drug-resistant strains and the identification of other effective drugs for nocardiosis is therefore needed.

N. seriolae strains can be divided into α-glucosidase (α-glu)-positive and -negative phenotypes (Shimahara et al., 2009), and each phenotype shows different drug susceptibility patterns (Ismail et al., 2011b). All of the α-glu-positive strains are erythromycin (EM) sensitive (Ismail et al., 2011b). Most strains have been isolated from *S. dumerili*, and oral EM treatment is effective against nocardiosis in experimentally infected *S. quinqueradiata* (Hatai et al., 1984). Conversely, all of the α-glu-negative strains are oxytetracycline (OTC) sensitive (Ismail et al., 2011b), and most of the strains isolated from farmed *S. quinqueradiata* are α-glu-negative, i.e., OTC sensitive (Ismail et al., 2011a; Ismail et al., 2011b). However, Hatai et al. (1984) investigated the therapeutic effect of oral OTC treatment in experimentally infected *S. quinqueradiata* and reported that OTC was not effective against nocardiosis. This finding may have been due to the low bioavailability of orally administered OTC in addition to the intracellular bacterial characteristics of *N. seriolae*, which proliferates in host cells where sufficient drug concentrations are lacking. The bioavailability of orally administered OTC in fish has been reported to be low; for example, only 2% of OTC was found to be absorbed in the Atlantic salmon *Salmo salar* L. (Elema et al., 1996).

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Welch et al. (1958) reported that serum OTC/tetracycline (TC) level enhancement was observed in humans when OTC/TC was orally administered with the same amount of D-glucosamine hydrochloride. Weber and Ridgway (1967) developed a method to mark the skeletal tissues of Pacific salmon administered oral TCs, and reported the effectiveness of D-glucosamine addition to enhance the detectability of such marks. Thus, absorption-enhancing agents such as D-glucosamine are anticipated to improve the efficacy of oral OTC treatment against nocardiosis.

In the present study, the efficacy of intraperitoneal injection vs. oral administration of OTC against nocardiosis in *S. quinqueradiata* was first assessed with a challenge trial. Then, the combination effect of *N*-acetyl-D-glucosamine hydrochloride (NAG, a derivative of D-glucosamine hydrochloride) with OTC was investigated by analyzing serum OTC levels, and oral treatment against nocardiosis in *S. quinqueradiata* was evaluated in challenge trials.

2. Materials and methods

2.1. Fish

One-year-old, artificially bred juvenile *S. quinqueradiata* (500 individuals) weighing 5 g each were obtained from a local juvenile producer in Kagoshima Prefecture. The fish were maintained in a 1000-l tank with sand-filtered and UV-irradiated running seawater. The water temperatures were between 15 and 25 °C. The fish were fed a commercial expanded 1- to 3-mm pellet diet (Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan) each day depending on fish size. The daily feeding rate was 3% of the body weight (BW). Parasites, bacteria and viruses were checked according to a guide to diseases of *Seriola* species (Sheppard, 2004), and pathogens and symptoms of diseases were not recognized until the experiments began. Prior to each experiment, the fish were acclimated at 25 °C for 2 weeks and were starved for 2 days before the administration of the drugs.

2.2. Bacteria

The α-glu-negative and OTC-sensitive *N. seriolae* strain 07O003 was used for the experimental infection. This strain was isolated from the spleen of farmed *S. quinqueradiata* with clinical nocardiosis signs in Miyazaki Prefecture in 2008 and was identified as *N. seriolae* by PCR (Miyoshi and Suzuki, 2003). The bacteria were stored in cryotubes with 10% skim milk (Becton, Dickinson and Company, Franklin Lakes, USA) at − 80 °C until use. The α-glu activity of this strain was checked with ChromID MRSA (bioMérieux, Marcy l'Etoile, France) as previously described (Ismail et al., 2011a). The minimum inhibitory concentration (MIC) for OTC of this strain was determined by the broth microdilution method (JSAA, 2003), and the strain was classified as OTC sensitive (MIC; 2 μg/ml). Bacteria were grown in BHI broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and incubated at 25 °C for 6 days on a horizontal shaker at 200 rpm. The number of the bacteria used for the exposure was quantified by 10-fold serial dilutions of the inoculum on Middle brook 7H11 agar supplemented with OADC enrichment (Becton, Dickinson and Company, Franklin Lakes, USA).

2.3. Assessment of the effect of intraperitoneal injection and oral administration of OTC on N. seriolae infection

Forty-five fish weighing 91.4 ± 4.3 g were randomly divided into 3 groups and were placed in 200-l polycarbonate tanks (15 fish each). The water temperature was 24.0–24.6 °C, and the water renewal rates were maintained at 83.3%/h throughout the experiment. Each group of fish was exposed to *N. seriolae* by adding a bacterial suspension to the seawater at a final concentration of 1.1×10^4 CFU/ml, and the seawater supply to each tank was stopped for 1 h. At 24 h post-exposure, 2 groups were treated with oxytetracycline hydrochloride (20% OTC formulation, Bio Science Co., Ltd., Aich, Japan) as follows: (1) intraperitoneal injection at a dose of 50 mg OTC/kg BW on day 1; (2) oral administration at a dose of 50 mg OTC/kg BW/day, with 3% BW of the 3-mm pellet diet, for 4 consecutive days. The injection solution was made by dissolving OTC with sterile 0.9% sodium chloride solution at a concentration of 25 mg OTC/ml. For the oral OTC administration, OTC was dissolved in 35% reduced starch hydrolysate liquid (SE-30, B Food Science Co., Ltd., Aich, Japan), in a volume that was 5% of the diet weight, and then the solution was mixed with the pellet diet. The remaining group served as the untreated control group, and the fish were fed the same diet mixed with the same quantity of 35% SE-30 without OTC. Each group of fish was fed daily, and the daily feeding rate was 3.5% of the BW, except during the treatment period (3% of the BW).

Mortality was recorded daily in each group, and the dead fish were dissected for confirmation of the clinical signs of nocardiosis. At 25 days post-exposure, all surviving fish were sampled under anesthesia and dissected. The gills, kidneys and spleens were examined for tubercles, and the number of tubercles located in the 1-cm-square surface of the most affected organ was counted. The clinical severity of each surviving fish was classified into 4 grades according to the following criteria: (1) none: no tubercles; (2) mild: at least 1 but < 5 tubercles; (3) moderate: 5 but no > 20 tubercles; (4) severe: > 20 tubercles.

2.4. Forced oral administration of OTC and NAG for the serum OTC level analysis

Forced oral OTC administration was conducted based on the method described by Uno et al. (1992), with modifications. Thirty-six fish weighing 216 ± 16.2 g were divided into 2 groups and placed in 200-l polycarbonate tanks (18 fish each). The water temperature was 24.3–24.9 °C. One group received 50 mg/kg BW of OTC, and the other group received 50 mg/kg BW of both OTC and NAG. These chemicals were mixed with the grinded powder of a 3-mm pellet diet suspended in a 1.75 volume (*w/v*) of distilled water, which was orally administered by a catheter to fish anesthetized with 2-phenoxyethanol (0.5 ml/l of seawater). Three fish from each group were sampled at 1, 3, 6, 12, 24 and 48 h post-administration. The blood was collected from the caudal vein and was stored at 4 °C overnight. Heparin was not used because OTC might partially counteract the anticoagulant action of heparin sodium (Hirsh et al., 1998). The serum was isolated from the blood sample by centrifugation at 1200 × *g* for 30 min and was stored at − 30 °C until analysis. Before the fish were assigned into 2 groups for administration, serum was sampled from 3 fish that were of the same origin to serve as 0 h blank samples.

2.5. Serum OTC level analysis

OTC was extracted from each serum sample by solid phase extraction using an Oasis HLB Cartridge (1 cm^3/30 mg, Waters Corp., Milford, USA). The cartridges were preconditioned with 1 ml of methanol, 1 ml of ultrapure water and 0.5 ml of saturated disodium ethylenediaminetetraacetate (Na_2EDTA). Then, 100 μl of each serum sample was added to the column, and the column was rinsed with 1 ml of ultrapure water followed by a nitrogen purge. OTC was eluted from the column with 1 ml of methanol and collected in a 5-ml cryogenic tube. The eluate was evaporated to dryness under nitrogen. The residue was dissolved in 400 μl of 5 mM oxalic acid solution. The solution was passed through a 0.45-μm filter (Millex LH; Merk KGaA, Darmstadt, Germany) and injected into the HPLC system (1200 Series; Agilent Technologies Inc., Santa Clara, USA). OTC was separated using a Discovery HS F5 HPLC column (5 cm × 2.1 cm, 3 μm; Sigma Aldrich Co., LLC., St. Louis, USA) with a Discovery HS F5 Supelguard cartridge (2 cm × 2.1 cm, 4 μm) and was detected at the wavelength of 353 nm. The column was maintained at 35 °C with a flow rate of 0.4 ml/min. The mobile phase was composed of ultrapure water (A), acetonitrile (B) and 50 mM oxalic acid (C), and the composition was altered during the analysis as