Electronic supplementary material

Minor environmental concentrations of antibiotics can modify bacterial virulence in co-infection with a non-targeted parasite

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Bacterial strains and culture conditions

F. columnare strains B375, B374 and B351 used in this study were originally isolated from fish farms, and their genetic characteristics and virulence have been characterized in a previous study [1]. The bacteria were stored in -80 °C with 10% glycerol and 10% fetal calf serum. For the growth and virulence assays, the bacteria were revived from the stock by culturing overnight in Shieh medium [2] and enriched for 21 h (25 °C, 110 rpm).

Resistance of the strains to tetracycline (defined as minor inhibitory concentration, MIC) was tested on agar plates using the Liofilchem MIC test strips on Shieh agar. Bacterial MIC values for tetracycline (using the strip test) were 1.5 μg for B375, 0.38 μg for B374 and 0.19 μg for B351. Based on the MIC, the strains were designated as not resistant, intermediate or resistant to 1 μg ml^{-1} antibiotic concentration (respectively) used in the experiment (see below). However, because it is possible that the tetracycline resistance on plate culture does not directly correlate with oxytetracycline (OTC) resistance in liquid, we also tested bacterial resistance to 1 μg ml^{-1} OTC in liquid culture (Supplementary Figure 1). Growth of the no resistance, intermediate and resistant strain in presence of the antibiotic was significantly different ($F = 845.7$, $df = 2$, $p < 0.001$). Pairwise LSD post hoc tests indicated that all strains differed from each other ($p < 0.001$ for all).

Flukes

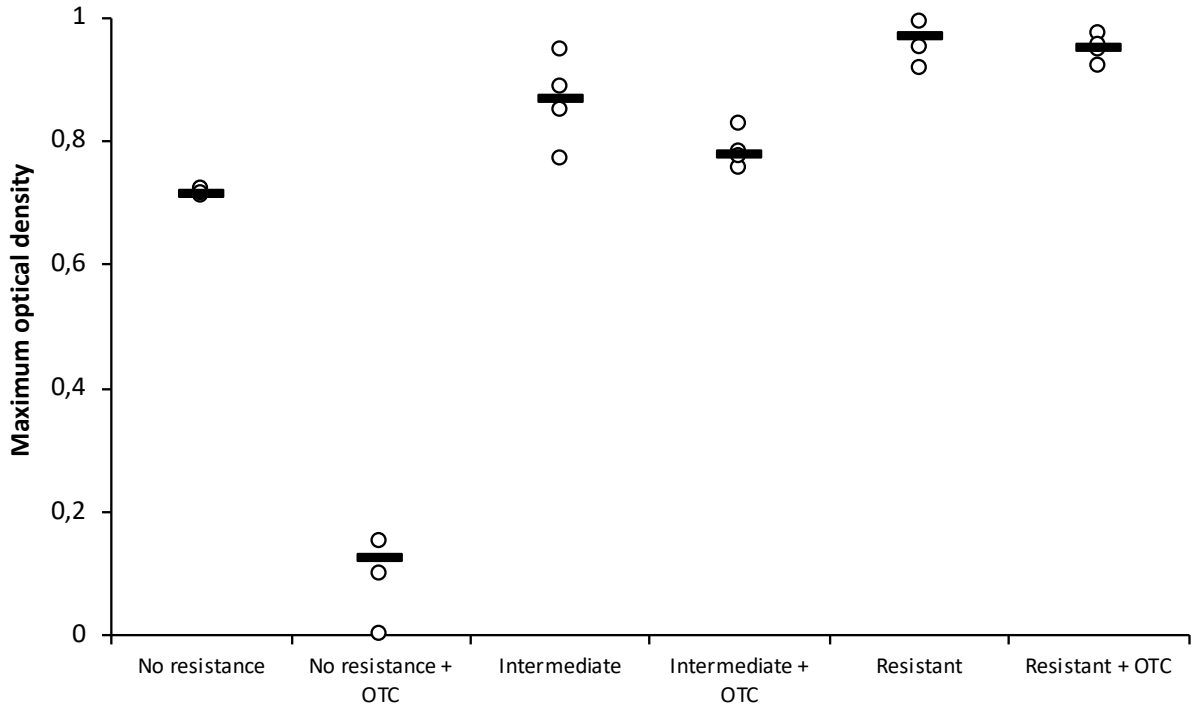
Infected *Lymnaea stagnalis* snails ($n = 42$), intermediate hosts for *D. pseudospathaceum*, shedding clonal fluke larvae were collected from Lake Vuojärvi (62° 24' 54" N, 25° 56' 14" E), Finland in June 2014. Snails were stored individually in 1 l of water at 6 °C and fed *ad libitum* with lettuce until the beginning of the experiment. Snail were allowed to produce cercariae for the infection trials for 3 h before the experiment. Cercarial suspension was then combined from 10 snails and cercarial density was determined from ten 1 ml samples.

Experimental exposure of rainbow trout

Clinically healthy, naïve rainbow trout fry were obtained from a fish hatchery in Central Finland in early spring, and maintained in six 80 l aquaria (250 fish in each) with constant flow-through of aerated ground water (16 °C) for 20 days prior the experiments. Fish were fed daily with commercial feed (2% of body weight). Six days prior to the beginning of the experiment, feed for the fish in three of the aquaria was changed to antibiotic feed containing 30 g of Orimycin (commercial oxytetracycine used as veterinary product) per 1 kg feed. At the same time, water temperature was slowly raised to 25 °C in all aquaria. Fish were kept unfed for one day before the experimental exposures.

In the experiment, fish were individually placed is small plastic aquaria containing 500 ml of water (25 °C). All fish were haphazardly assigned to treatment groups. The experimental set-up included three groups of fish: fish without an antibiotic treatment, fish pre-treated with antibiotic in feed, and fish that received Oxytetracycline (Sigma) directly in the water at the beginning of the experimental infection with a final concentration of 1 μg OTC ml $^{-1}$. Next, fish in all three treatment groups were exposed individually to three *Flavobacterium* strains and *Diplostomum*, in single *Flavobacterium*, single *Diplostomum*, and in coinfection combinations including both *Flavobacterium* and *Diplostomum*, totaling seven different exposure types for each of the three treatment groups. Negative controls with neither bacterial nor parasite infection were also included with and without the antibiotic treatments. Bacteria and parasites were pipetted directly in the aquaria to reach infective doses of 5 x 10^3 colony forming units per ml of bacteria and 40 *Diplostomum* parasites per aquarium.

From the fish removed from the experiment, random 30% were sampled for presence of *F. columnare* on the skin and gills by culturing a sample on Shieh agar supplemented with tobramycin [3]



Supplementary Figure 1. Effect of oxytetracycline (OTC) antibiotic (0 or 1 ug ml $^{-1}$) on maximum population size (measured as maximum optical density after 24 h culture) of *F. columnare* strains designated as sensitive (no resistance), intermediate or resistant to the 1 ug ml $^{-1}$ OTC concentration. Lines indicate means of individual measurements (circles) (see [4]).

Supplementary references

1. Sundberg, L.-R., Ketola, T., Laanto, E., Kinnula, H., Bamford, J. K. H., Penttinen, R. & Mappes, J. 2016 Intensive aquaculture selects for increased virulence and interference competition in bacteria. *Proceedings of the Royal Society B: Biological Sciences* **283**, 20153069. (doi:10.1111/j.1365-2109.2010.02671.x)

2. Song, Y. L., Fryer, J. L. & Rohovec, J. S. 1988 Comparison of six media for the cultivation of *Flexibacter columnaris*. *Fish Pathology* **23**, 81–94.

3. Decostere, A., Haesebrouck, F. & Devriese, L. 1997 Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from diseased fish. *Journal of Clinical Microbiology* **35**, 322–324.

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