Novel treatment methods of columnaris disease

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Abstract

Columnaris disease, caused by *Flavobacterium columnare*, is an urgent problem in Finnish fish farming. The occurrence of disease outbreaks has increased significantly at fish farms producing salmonid fingerlings in the inland freshwater area. The reasons for this are unclear, but we have previously suggested that the ecological and epidemiological factors of intensive farming, together with climate warming may have selected for higher virulence in *F. columnare* [1]. The suggestion was supported recently, when we isolated several *F. columnare* strains directly from a river upstream of a fish farm, from free water, biofilm and dead wild fish. The bacteria isolated from environment were less virulent than those isolated from a disease outbreak [2]. Therefore, the source of columnaris disease is most likely in the income water of farms. Furthermore, we have found that *F. columnare* can survive in the water outside the fish host for several months, and use dead fish material as a nutrient resource [3]. These factors make the prevention and treatment of columnaris disease challenging in the farming environment.

Currently, fish infected with columnaris disease are treated with antibiotics. The effectiveness of antibiotic treatment has declined during the last years although resistant strains have not been isolated. When minimum inhibitory concentration tests (MIC) in isolates from upstream, within and downstream of a fish farm were performed, the tolerance against tetracyclin was found to be significantly higher in downstream isolates. The threat of development of antibiotic resistant strains has led to a need for new methods to prevent and treat columnaris disease.

Phages (bacteriophages) are bacterial viruses that infect only bacteria, not eukaryotes. They are the most numerous biological entities on our planet, and theoretically destroy half of the earths bacterial population every second day [4]. In the life cycle of lytic phages, the virus binds to the phage receptor on the bacterial surface and invades the cell. The virus is replicated inside the bacterial cell and when the viral progeny is released from the cell the host cell erupts and dies. The therapeutic use of phages against bacterial diseases, i.e. phage therapy, was invented by Felix d´Herelle in 1919. However, during the Second World War antibiotics were taken into use and phage therapy was forgotten. Since then the western medicine has relied on antibiotics, but Eastern Europe (Poland and Russia) continued to develop phage therapies. Nowadays, when antibiotic resistant bacterial strains are becoming more common, phage therapy is in it´s new rise (see reference 5 for an overview). Phage therapy is used in experimental treatment of human infections (clinical trials with *Pseudomonas aeruginosa*, *Escherichia coli*, MRSA, reviewed in reference 6), and it has been shown that phages could specifically be used to kill antibiotic

resistant bacteria [7]. Phages are generally considered safe, because they are only infecting bacteria and do not replicate in eukaryotes.

A number of successful reports on phage therapy against fish diseases have already been published [8,9], including phages against *Flavobacterium psychrophilum* [10,11]. In these studies, promising results have been obtained when the phages are applied in fish feed or injected into fish. We have previously isolated phages that infect and lyse flavobacteria [12]. The phages were isolated from environmental waters and from fish farming. Phages infecting *F. columnare* were isolated only from fish farming environment, and they were found to be strictly host-specific, whereas the host range of phages infecting other *Flavobacterium* species was broad. Some of these broad host range phages also prevented the growth of *F. columnare* although they did not replicate [12]. Because the symptoms of columnaris disease are external and the bacteria transmit through water flow, phages could be used as biological tools to kill the bacteria without causing harm to the fish or the environment.

In this study, we explored the phage-host interaction in *F. columnare* with emphasis on virulence management in the outside-fish environment. We exposed four *F. columnare* isolates to three lytic phages on an agar plate assay. When exposed to their phages, *F. columnare* bacteria developed phage resistance, which led to a dramatical change in colony morphology of the bacteria from original rhizoid (Rz) to rough (R). Because our previous studies have indicated that colony morphology is an important indicator of virulence in *F. columnare*, we tested the virulence of the original and phage-resistant morphotype variant by exposing zebrafish (*Danio rerio*) to the bacteria. The ancestral Rz morphotype caused 25-100 % mortality in the zebrafish infection model whereas the R type, parallel to gaining phage resistance, had lost their virulence. In addition for losing their virulence, the R morphotype were deficient in gliding motility and organized biofilm formation compared to the ancestral Rz type [13].

In another experiment, we exposed both zebrafish and rainbow trout (*Oncorhynchus mykiss*) to *F. columnare* by bathing. After challenge phages were added in the aquaria in different concentrations (10^6 – 10^8 colony forming units per ml), and fish mortality and the amount of bacterial cells and phages remaining in the aquarium water were monitored (for rainbow trout). In both fish species the addition of phages prevented the development of columnaris disease and fish mortality, whereas the group without phages suffered from 100% (zebrafish) and 40% (rainbow trout) mortality. *F. columnare* was isolated from water 4h after addition of phages and not later (vs. 3 days in the control group), but the majority of the bacteria were of the non-virulent R morphotype. Phages remained in the system for 2-3 days in the magnitude of 10^3 plaque forming units per ml.

Our results indicate that phages can cause phenotypic changes in *F. columnare* that have correlated effects on its virulence in the outside-host environment. Together with our previous studies [14,15] our results suggest that virulence of *F. columnare* is linked with gliding motility. It is also possible that phages also hamper the biofilm formation of *F. columnare* which makes the colonization of the fish host difficult.
Indeed, the addition of phages into the aquaria holding experimentally infected fish prevented the development of columnaris disease in two fish species. Phages are able to efficiently lyse the bacteria, and the majority of the bacteria remaining in the aquaria were of the non-virulent R

morphotype. As biological entities, the phages degraded 2-3 days after they were added in the aquaria because no bacterial hosts were available for reproduction.

Although the fish experiments were a pilot study, the results are encouraging. We suggest that enrichment of phages in rearing units could be selectively used to eradicate *F. columnare* from the system. This approach could benefit the fish farming industry by killing or altering the virulence of *F. columnare* bacteria present in the water bodies of fish tanks and thus reduce the risk of infections. However, the phage-bacterium relationship still needs to be characterized further in order to find the optimal conditions for phage therapy. Furthermore, the challenges of the narrow host range of the *F. columnare* phages and how to sustain them in flow-trough water at farms need to be tackled. We aim to characterize the phage-bacterium relationship in detail to find tools to biologically treat columnaris infections at fish farms.

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