

論文の内容の要旨

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Studies on the characterization of mesophilic *Aeromonas* species isolated from diseased fresh water aquarium fish using molecular approaches

(罹病淡水観賞魚から単離された中温性エロモナスの分子生物学的特性解析)

The growing interest in keeping aquarium/ornamental fish as a hobby has resulted in a significant rise in the number of countries culturing and exporting ornamental fish. Today, the international trade of ornamental fish has become a vast, lucrative global business with a great potential for development in most parts of the world. At present, over 1 billion ornamental fish worth of US\$200–300 million are traded globally each year involving more than 100 countries. As the industry expands, the disease incidences and associated costs have become hindrances to the profitable culture of ornamental fish. A thorough understanding of the pathogens associated with the commercial production of ornamental fish is the prerequisite to develop more effective diagnostic techniques targeted at the field level pathogen identification and to improve the disease control strategies.

Through this dissertation we investigated into the aspects of aetiology (causative agents) and the diagnosis of motile aeromonas septicaemia (MAS) in fresh water ornamental fish, a disease that received a least priority and attention despite its common occurrence and potential adverse impacts on ornamental fish industry. In an attempt to identify the diverse *Aeromonas* species that could possibly be involved in MAS in ornamental fish, we sampled tropical fresh water ornamental fish showing signs of septicaemia from aquaria in geographically diverse locations in Sri Lanka. Aseptically isolated *Aeromonas* sp. from diseased fish were then characterized using a polyphasic approach that included conventional phenotypic identification, 16SrDNA-Restriction Fragment Length Polymorphism (RFLP) analysis, molecular fingerprinting and the sequence analysis of *gyrB* and *rpoD* (two housekeeping genes that have already been used successfully as accurate, unequivocal molecular chronometers for the phylogenetic identification of the members of the genus *Aeromonas*). Through this approach we aimed to prevent the possible misidentification of aeromonads at the species level, attributed to the extremely complicated taxonomy of the genus *Aeromonas*. Our

results indicated the diverse range of aeromonads that could potentially be associated with motile aeromonad septicaemia in ornamental fish. The isolates characterized in this study (n=53) were originated from 10 different species of ornamental fish and the phylogenetic characterization showed that they belong to 6 different species of motile *Aeromonas* species adding more evidence to the wide host range and geographical distribution of this bacterium. Our findings emphasize the emerging role of *Aeromonas veronii* as an ornamental fish pathogen, in contrast to the long standing view that *A. hydrophila* is the classical pathogenic motile aeromonad in fish. We also isolated *A. dhakensis*, which the first isolation of this recently described species from a clinical case (a septicaemic ornamental fish) since its original description from the same host. Isolation of diverse species of *Aeromonas* that are genetically heterogeneous in terms of their virulence profiles (genetic determinants of virulence), with no clonal relationships with each other proves the role of mesophilic aeromonads as opportunistic pathogens in ornamental fish. This highlights the importance of fish health management in first place, since the opportunistic pathogenicity could have been the end result of the loss of fine balance between the fish, pathogen and the environment, but not a direct effect of the mere presence of virulent bacteria in the culture environment.

Our study also added evidences to the poor correlation between phenotypic and genotypic characterization of *Aeromonas* spp emphasizing the need for incorporating polyphasic molecular approaches in precise species identification since phenotype alone will often lead to misleading results as shown by our findings. While the exact taxonomic position of fish-pathogenic *Aeromonas* species might not be of interest to all fish pathologists, its use is invaluable in epidemiological studies and in recognizing new pathogenic species and subspecies. Resistance of *Aeromonas* species to commonly used antibiotics is an emerging problem in the ornamental fish industry. Antimicrobial susceptibility testing of *Aeromonas* spp. isolated in the present study provided interesting insights into the emerging nature of development of antibiotic resistant phenotypes in bacteria dwelling ornamental aquaculture settings. Presence of antibiotic resistant *Aeromonas* species harboring multiple virulence genes and transferable genetic elements such as integrons, in aquarium waters and ornamental fish raises concerns about the possible treatment failures in fish disease outbreaks and the public health threats they may pose, given the importance of aeromonads as emerging human pathogens. The relatively high prevalence of antimicrobial-multi resistance observed among our isolates (49%), could likely be a result of their use in the aquarium fish industry, however, resistance can also arise from gene mutations or by acquisition of transferable genetic elements such as integrons.

In the recent past, high throughput whole genome sequencing has emerged as a rapid means of understanding the biology, evolution, virulence and diversity of bacterial pathogens. Genome sequencing could be considered as a promising avenue for solving many mysteries surrounding the genus *Aeromonas* and its genetic heterogeneity. The first complete genome of an *Aeromonas* strain, *A. hydrophila* ATCC 7966 isolated from a tin of milk with fishy odour has been sequenced in 2006. Whole genome sequences of *Aeromonas* species published since then have been able to reveal important inferences regarding the importance of lateral gene transfer as a governing mechanism that determines the emergence of virulent strains of *Aeromonas*. Genomic differences among different *Aeromonas* strains with respect to their toxin production, biofilm formation, antibiotic resistance, and other virulence properties

are believed to be a result of bacteriophage or hypothetical genes. Despite the growing interest and economic importance of global aquarium fish trade, there is a dearth of information on the genome sequence of *A. hydrophila* isolated from tropical ornamental fish. Therefore, as a part of this dissertation, we sequenced *A. hydrophila* strain Ae34, an isolate recovered from the kidney of a septicemic koi carp (*Cyprinus carpio koi*) with the view of identifying what specific genetic features would differentiate a 'fish pathogenic strain' from their 'commensal' and 'environmental' counterparts (by comparing this genome to that of *A. hydrophila* ATCC 7966).

Making a prompt and accurate diagnosis is vital for successful treatment and long term prevention of fish diseases. As the clinical signs of MAS are rarely pathognomonic, the diagnosis is accomplished by the isolation of the pathogen from the internal organs and identification using biochemical characterization which is laborious, time consuming and needs microbiology expertise. In a typical outbreak of septicemia in fish, especially in motile *Aeromonas* septicemia, symptoms occur gradually with an increasing number of fish becoming affected. To reduce the mortalities and to prevent the spread, it is important to identify the problem as early as possible to take corrective measures (both therapeutic interventions and environmental manipulations) within the shortest possible time. Molecular methods are rewarding in this respect, but the high cost limits their practical use in routine fish disease diagnosis. Loop mediated isothermal amplification (LAMP) is an auto-cycling strand displacement DNA synthesis performed under isothermal conditions. This methodology is widely accepted as a rapid, easy to perform, economical and specific method of identifying pathogens and have been used successfully for the fish and shellfish pathogen identification at the field level. Using the draft genome sequence of *A. hydrophila* Ae34 and the other publicly available genomes of *Aeromonas*, we attempted to evaluate the specificity of GCAT (glycerophospholipid cholesterol acyl- transferase) gene which is reported to be present in >98% of aeromonads, as a target gene sequence specific for the genus *Aeromonas*. Using a combination of whole genome informed target gene sequences we aim to develop a LAMP technique to detect the presence of *Aeromonas* in the kidney tissues of infected fish and to extend the protocol to differentiate *A. hydrophila* and *A. veronii*, which are the two commonest mesophilic, motile aeromonads causing septicemia in in fresh water ornamental fish.