DOCTOR DISSERTATION

Establishment of a new approach for determining hygiene standard values for fecal pollution based on the acceptable risk of pathogen infection

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Conventional fecal pollution indicators, including coliforms, fecal coliforms, *Escherichia coli*, and *Enterococcus* spp. have enormously contributed to the control and maintenance of public health. However, fundamental shortcomings of these conventional indicators are emphasized. Firstly, conventional indicators have an inability to identify sources of fecal contamination in water, because they are present in animal feces and soil. To identify sources of fecal contamination in water, diverse alternative indicators of fecal contamination have been developed. Especially, genetic markers of human enteric bacteria are being used as alternative indicators for identifying fecal pollution sources with a library- and culture-independent way. The quantitative PCR (qPCR) assay for 16S rRNA of *Bacteroides-Prevotella* has been constructed for that purpose and most widely used. For application of *Bacteroides-Prevotella* 16S rRNA genetic marker as a fecal pollution indicator, the developments of specific detection method of each contamination sources such as human, cow, pig and bird feces are needed. Quantification method of these genetic markers should be standardized for practical use; those enable us to check whether the sample processing performed normally and to compare results obtained from different laboratories.

The second fundamental shortcoming of conventional indicators is that the current water hygiene standards using conventional indicators were not determined based on the possible adverse effects on human health. In the other words, it is not clear that the density of indicator microorganisms below the current value of water hygiene standards is meaning the low risk of infection by pathogenic microorganisms. Therefore, it is very critical to establish evidence-based water hygiene standards based on the acceptable risk of infection. In addition to it, very poor correlations have been found between the amount of bacterial indicator microorganisms and that of pathogenic microorganisms. Concentrations of bacterial indicators and pathogens are independently fluctuated, owing to the difference in biological and physicochemical properties of these microbiological entities. This makes it difficult occasionally to predict the presence of pathogens in water by detecting conventional indicators. For microbiologically safe water management, it is critical to understand the occurrence characteristics of pathogens in water and to establish the water hygiene standards in consideration of concentration fluctuation.

Based on these background, the final goal of this study is to construct the framework to determine the hygiene standard values based on the acceptable risk of pathogen infection. To achieve this final goal, some small goals were set. Firstly, *Bacteroides-Prevotella* 16S rRNA genetic markers were developed for each contamination source. Secondly, to use these genetic markers for the detection and the monitoring of fecal contamination in environmental water, quantification method of them was improved. Thirdly, the acceptable risks of pathogen infection were calculated by the point estimation of quantitative microbial risk analysis. Fourthly, the quantitative relationships of indicators and pathogens were calculated by using surveillance data of pathogen and indicator concentrations in water. Finally, candidates of hygiene standard values were determined based on the acceptable risk of pathogen infection.

This thesis is composed of the following chapters:

Chapter 2 (Literature review) summarized literature findings regarding the fecal indicators, the standardization and normalization of quantification process, and the hygiene standard values.

Chapter 3 (Development of the Chicken- and duck-associated *Bacteroides–Prevotella* genetic markers for detecting fecal contamination in environmental water) described the development of real-time PCR assays for chicken- and duck-associated *Bacteroides-Prevotella* 16S rRNA genetic markers in order to quantitatively evaluate avian fecal contamination in water.

Chapter 4 (Improvement of the quantification method of alternative indicator for fecal contamination "Genetic marker") showed the development of genetically-engineered strain of *E. coli* (designated as strain MG1655 Δlac::kan) as a sample process control (SPC) for qPCR assays using propidium monoazide treatment to accurately quantify *Bacteroides-Prevotella* gene markers in environmental water samples. In addition, an internal amplification control (IAC) was also constructed and applied to further improve the measurement accuracy.

Chapter 5 (Effects of temperature and predator on the persistence of host-specific *Bacteroides-Prevotella* genetic markers in water) showed the fate of some human-specific genetic markers in river water was compared with that of conventional indicator microorganisms at various water temperature conditions. In addition to that, the possible effect of predators on the persistency of bacterial genetic markers in natural water environment was also investigated.

Chapter 6 (Acceptable concentration of pathogens in environmental water derived by Quantitative Microbial Risk Analysis (QMRA)) showed the calculation of the acceptable concentration of pathogens for determination of the hygiene standard values based on the acceptable risk of pathogen infection.

Chapter 7 (Development of the hygiene standard value of conventional and alternative indicators based on the acceptable risk of pathogen infection) described the framework to determine the hygiene standard values based on the acceptable risk of pathogen infection. Firstly, concentrations of pathogens and indicators in environmental water are quantified in multiple sites for a period of time. Secondly, Bayesian model is used to estimate the concentration distribution of pathogens, indicators and the distribution of the ratio of a pathogen to an indicator. Thirdly, the quantitative relationship between indicator and pathogen is calculated by using the distribution of the ratio of a pathogen to an indicator. Finally, the quantity of indicator in water ensuring that the pathogen concentration is significantly below the acceptable concentration of pathogen is determined as the water quality standard value by using the quantitative relationship between indicator and pathogen and the acceptable concentration of pathogens in river water (Chapter 6).

Chapter 8 (Conclusions) summarized the findings of this study and provided future perspectives.