論文の内容の要旨

Cryptosporidium and Virus Concentration Method with Hydroxylapatite and Ethidium Monoazid-PCR for Selective Detection of Intact Virus

(ハイドロキシアパタイトを用いたクリプトスポリジウムとウイルスの濃縮およびエチヂウムモノアザイド PCR を用いたウイルスの選択的検出)

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According to the epidemiological studies, consumption of contaminated water or food has been associated with waterborne disease which is caused by the pathogens including a wide variety of viruses, bacteria and protozoa. Especially, the *Cryptosporidium* species and the enteric viruses have been recognized as major causes of waterborne outbreaks. Their incidence and behavior in water environments differ due to the differences in size, structure, composition and excretion by humans or animals. Further complications make it harder to detect for the waterborne pathogens. Therefore the specific detection methods are required for detecting those pathogenic microorganisms. The detection methods for pathogenic microorganisms are basically composed of three steps; concentration, purification and quantification. The filtration is one of the concentration methods used most widely. However, filtration methods have limitations such as low recovery yields and low sampling amount. Recently, a new filtration method using hydroxylapatite (HAP) as a filter media was published. This method has advantages in recovering the concentrated *Cryptosporidium* oocysts as well as volume of water sample. As a next step, a concentration method for diverse microorganisms is required in the field of water quality monitoring. PCR detection methods have been used to quantify the pathogenic microorganisms because of its sensitivity and quick output, it is often unable to discriminate between intact and damaged virus. The objective of this study was to develop a concentration method by using HAP filtration for *Cryptosporidium* oocysts (oocysts) and enteric viruses from water samples and to develop a discrimination method between intact and damaged viruses by using ethidium monoazide (EMA)-PCR.

In Chapter 4, the development of HAP filtration was described. This study began with HAP layer filtration formed on a nylon net filter with a pore size of 11 um and applied for concentrating viruses and oocysts, simultaneously. To evaluate the HAP layer, the magnetic beads with size-distribution of 4±1.4 μm was used with respect to the mean size of oocysts. The obtained recovery ratios of magnetic beads and oocysts were 98.8% and 72%, respectively,where no oocysts or magnetic beads were detected in the filtrate. From the results, the HAP layer was considered to be a good filter media for concentrating oocysts. To understand the interaction between virus and HAP layer, the samples of viruses (bacteriophage Qβ (Qβ), poliovirus type I (PV1) and murine norovirus (MNV)) were filtered with various conditions. The processes of HAP layer filtration with viruses consisted with three steps; sample filtration, acid rinse and alkali elution. Comparing to the filtrate and the acid rinsed sample, the elution of viruses was observed in the alkali elution. The HAP filtration system was designed as combination of HAP layer and negatively charged membrane with a pore size of 0.45 um. Although the use of HAP layer for concentrating and recovering viruses was not offered good recovery yield, the HAP filtration system showed improved recovery yields in the alkali elution. Finally, the simultaneous concentration was carried out with oocyt and PV1. The mixed sample of oocysts and PV1 was tested for HAP filtration system. As a result, oocysts and PV1 were recovered with 76% and more than 100% of recovery yield from the HAP layer and from the eluant of alkali elution, respectively. The HAP filtration system can be applicable to concentrate, simultaneously, pathogenic protozoa and viruses.

In Chapter 5, the EMA-PCR method was developed to discriminate intact viruses from damaged viruses. The important factors studied in the EMA treatment for viral single-stranded (ss) RNA were the conditions of light irradiation and the EMA dose. The remaining concentration of viral ss RNA was not decreased by only light irradiation dose of the 650 W halogen lamp for less than 5 min exposure. The EMA dose alone did not affect to viral ss RNA in various intensity of EMA dose (0.001 - 10 μg/mL), as well. light exposures (30 - 300 sec) and EMA dose (0.001 - 10 μg/mL) were optimized for the EMA-PCR. Approximately 10^6 PDU/mL of extracted viral ss RNA could be diminished by 10 μg/mL of EMA dose with 300 sec of light irradiation. Here the conditions of the light irradiation for 3 min and 10 μg/mL of EMA dose were selected. The inhibition effect for RT-PCR by residual EMA was removed by using spin-column gel chromatography with Sephacril S-300. Use of QIAamp® Viral RNA mini kit for extracting the viral RNA was also effective showing no remarkable differences between

with and without additional purification. The processes of EMA-PCR (light exposure, EMA dose and light exposure + EMA dose) were applied to Qβ, PV1 and norovirus.. From only the EMA dose, no remarkable degradation was observed in plaque assay result. From this result, intact viruses in the sample were stable during the EMA treatment. In the RT-PCR assay results, the decreases in the remaining ratios were observed, which was thought to be caused by the presence of non intact virions in the laboratory stoch solutions. From these results, the EMA treatment with the process of the light exposure and the EMA dose was considered to be able to detect selectively intact viruses when applied to ss RNA virus samples.

EMA-PCR was applied to the thermal treated viruses and compared with conventional RT-PCR. The EMA treatment could reduce the RNA of thermal damaged PV1. The remaining ratios by EMA-PCR, PCR and plaque assay were compared. The observed slope was 0.91 (r^2=0.99) by the logarithm of the remaining ratios of the EMA-PCR and the plaque assay, showing a good correlation. On the other hand, low relationship was observed between the remaining ratios of the EMA-PCR and the PCR assay with 0.01 of the slope. The EMA treatment reduced the false positive background of PCR assay such as naked RNA. The thermal treatment was also applied to NoV. The observed remaining ratio was 0.99, 0.86, 0.74, 2.48×10^{-3} and 0.88 by PCR assay at 45℃, 55℃, 65℃, 75℃ and 95℃ for 10 min, respectively. In the EMA-PCR, 0.97, 1.17, 0.15, 1.33×10^{-5} (detection limit) and 7.42×10^{-3} of remaining ratio was observed at 45, 55, 65, 75, and 95℃, respectively. Reduction of NoV RNA may be due to denature of the free RNA of dead viruses by RNase like inhibitor because NoV stock solution was purified from human fecal sample. In conclusion, the EMA-PCR could be used as a tool for quantifying the thermal treated viruses instead of plaque assay.

In this study, HAP filtration system for concentrating pathogenic microorganisms and EMA-PCR for selective detection of intact virus were developed and applied to laboratory scale. Obtained results from those detection methods is required for using as a factor or coefficient for risk assessment.