Abstract of Dissertation

(論文の内容の要旨)

Title of Dissertation: Application of PCR-based techniques for evaluating the effect of chlorine, ozone and ultraviolet light on viral nucleic acid and capsid integrity

(論文題目　塩素、オゾンおよび紫外線がウイルス核酸およびカプシドに及ぼす影響の PCR に基づく手法による評価)

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Abstract（本文）

Water contaminated with pathogen has been a significant cause of human decease around the world (WHO 2002). Water treatment and disinfection has been essential and required to mitigate the occurrence of waterborne pathogen. Among the disinfection methods, chlorine, ozone, and ultraviolet (UV) light are typical disinfectants that are currently used in the wastewater and water purification plants. The disinfection can inactivate microorganisms in various ways. The disinfection mechanisms and their effects on microorganism are depended on the types of disinfectants and microorganisms. This research was carried out for evaluating the effect of chlorine, ozone, and UV light on viral nucleic acid and capsid integrity by PCR based techniques. Cell culture, ethidium monoazide treatment coupled with real-time polymerase chain reaction (EMA-PCR), and real-time polymerase chain reaction (PCR) were used to detect the infectivity, damage on viral genome, and damage on viral nucleic acid that inactivated or destroyed by disinfectants. The different kinds of microorganisms were investigated that were poliovirus,

The structure of this dissertation is: the background and literature reviews were in Chapter 1 and 2. These chapters described the research objective and strategy, the background from the previous researches, as well as the importance and requirement of our research. Chapter 3 described the analytical methods that were applied in our study. The preliminary investigations and some development of the methods, which were applied in the inactivation studies of viruses, were described in Chapter 4 and 5. The Chapter 6, 7, and 8 related to the study of disinfection mechanisms of viruses by chlorine, ozone, and UV light, respectively. Finally, Chapter 9 described the overall conclusions and recommendations from this study. The obtained results from each chapter were explained below.

Chapter 4 was separated into two main parts. The first part related to the establishment of cell culture for adenovirus that would be applied to study the inactivation adenovirus in the Chapter 7 and 8. The Hep-2 cell and A549 cell line were cultivated and used as a host cell for adenovirus. The adenovirus 2, 3, 5, 7, 40, and 41 types were tested with those two host cells. The results showed that adenovirus 5 was able to propagate in these Hep-2 and A549 cell while other types of adenoviruses were not. The plaque could not be observed when adenovirus 5 was tested with Hep-2 cell line. However, we could observe plaque clearly when A549 was used as a host cell for adenovirus 5. The plaque assay for adenovirus 5 was successfully established and would be applied in other Chapters. The second parts of this Chapter focused on the investigation of the effect of ethidium monoazide (EMA) treatment on bacteriophage Qβ, MS2, and T4, poliovirus 1 (PV1), murine norovirus (MNV), and adenovirus 5 (Ad5). This investigation was done before the EMA treatment was applied in Chapter 6, 7, and 8. The EMA could penetrate to intact bacteriophage Qβ and MS2 and inactivate these bacteriophages at the EMA concentration of 5, 10, and 50 μg/mL. So it was not applicable to apply EMA treatment with two types of bacteriophages. The EMA did not have an effect on intact poliovirus 1, murine norovirus when the EMA treatment at the concentration of 5, 10, and 50 μg/mL was applied. In the case of adenovirus 5, the EMA treatment did not showed the effect on the intact adenovirus 5 at the concentration of 5 and 10 μg/mL but it inactivated adenovirus 5 at the concentration 50 μg/mL. When the EMA-PCR and PCR were used to measure the concentration of PV1, MNV, and Ad5, the results from these two assays was quite similar. EMA treatment did not affect the PCR analysis, which consisted of RNA/DNA extraction, reverse transcription, and polymerase chain reaction. In addition, results implied that our virus stocks consisted of integrity virus and these viruses could protect viral genome from EMA treatment. The EMA treatment at the concentration of 50 μg/mL would be applied with PV1 and MNV, at the concentration of 10 μg/mL would be applied with Ad5 in the following Chapters.

In Chapter 5, the continuous quench flow system was developed for study the rapid ozone disinfection kinetics in Chapter 8. A CQF reactor was established for studying the viral inactivation. A two-channel peristaltic pump was used to drive both ozone and viral solution into reaction chamber (mixer), which was made from T-connecter (inlet diameter 1 mm; inside diameter 4 mm; outlet diameter 2 mm) with magnetic stirrer placed inside. The reacting mixture then flowed through Teflon tube for prolonging contact time before the mixture flowed to collection tubes containing quenching solution. The samples were separately collected for measuring residual ozone concentration and microbes. The contact time between two substances was controlled by adjusting the flow rate of pump and the length of the Teflon tube. The complete mixing between two solutions was ensured by observing changed color of base and acid indicators. The mixing ratio between two solutions was verified by investigating the sodium chloride concentration reducing to half of initial concentration. Before this established reactor was applied to study the viral inactivation by ozone, this CQF reactor was applied to study the viral inactivation by chlorine first. In order to verify the consistence of CQF reactor to measure the inactivation at very short contact time, the disinfection kinetics of bacteriophage Qβ after chlorination was investigated by both CQF and batch reactor. The inactivation of bacteriophage in CQF reactor was investigated from contact time varying from 0.7 second to 10.2 seconds and this inactivation was compared with inactivation results from batch reactor at longer contact time. The results showed that the inactivation rate fitted to linear regression between these two reactors was not significant difference based on the statistical analysis ($p > 0.05$). These results implied that the inactivation results from CQF reactor were

consistent with inactivation results from batch reactor. The inactivation data at short contact time is warranted.

Chapter 6 related the usefulness of EMA-PCR and PCR for the evaluation of damage on viral genome and capsid by chlorine. The viral infectivity, damage on viral genome, and damage on viral nucleic acid were evaluated by Cell culture, ethidium monoazide treatment coupled with real-time polymerase chain reaction (EMA-PCR), and real-time polymerase chain reaction (PCR). The chlorine disinfection at different concentration of 0.1, 0.25, 0.5, and 1 mg/L was applied with PV1 and at concentration of 0.25, 0.5 and 1 mg/L was applied with MNV. After PV1 and MNV were inactivated by chlorine, the inactivation rate results obtained from EMA-PCR was higher than to the results obtained by PCR at the initial chlorine concentration of 0.25, 0.5, and 1 mg/L for PV1 and 0.5 and 1 mg/L for MNV, respectively. At this rate, the disinfection mechanism of chlorine was mainly on the damage of viral capsid. The viral inactivation rate detected by plaque assay was lower than the reduction detected by EMA-PCR. Chlorine might react with viral particle and lead to the loss of viral infectivity but it did not cause enough damage on viral capsid, which let EMA penetrate to viral capsid. For instance, the damage might cause at the viral attachment site. At the clorine concentration 0.1 mg/L for PV1 and 0.25 for MNV, the reduction of viruses was not observed by EMA-PCR and PCR at short contact time. Chlorine might not cause severe damage on viral capsid. The capsid still protected the viral genome from EMA. However, the loss of viral infectivity might come from the destruction of antigenicity of virusese and viruses unable to attach to the host cell.

The EMA-PCR and PCR were used for evaluating the effect of ozone on viral nucleic acid and capsid integrity in Chapter 7. In order to investigate the rapd inactivation by ozone, a continuous quench-flow (CQF) reactor, which was established in Chapter 5, were applied to study the disinfection kinetic of microbes by ozone at very short contact time e.g. 0.7 second in this chapter. By applying CQF reactor, the positive results of remaining virus after ozonation could be obtained. The CQF reactor was appropriate for studying the fast inactivation rate of microbes by both chlorine and ozone at the contact time as short as 0.70 second. By using the CQF reactor to study the inactivation of viruses at short contact time, we could elucidate the inactivation of viruses clearer than what we learned from batch reactor. Ozone had an ability to cause damage to viral capsid and genome of PV1. The destruction by ozone seemed to primary on viral capsid as we could observe from the higher reduction rate of viruses from EMA-PCR than PCR.

Chapter 8 described the investigation of damage on viral capsid by low-pressure UV lamp (LP UV lamp) and medium-pressure UV lamp (MP UV lamp). When polioviruses and adenoviruses were inactivated by LP UV lamp, the inactivation results detected by PCR and EMA-PCR showed no difference even at high UV fluence of 1000 mJ/cm2. However, the results of EMA-PCR showed higher inactivation rate than the results of qPCR when the polioviruses and adenoviruses were inactivated by MP UV, just at UV fluence above 300 mJ/cm2. Additionally, the difference became larger as the UV fluence was increased. At the UV fluence of 500 mJ/cm2, the difference was more than 0.4-log and 1-log for the poliovirus and adenovirus, respectively. The difference of results between PCR and EMA-PCR implied that EMA could penetrate to inactivated damaged viral capsid, which had complete viral nucleotide, and bind to viral DNA/RNA. Therefore, the difference between inactivation rate of viruses after MP UV lamp observed by EMA-PCR and PCR meant that MP UV

lamp has a potential to cause the damage on viral capsid. The MP UV lamp emitted broad wavelength spectrum from 200 to 600 nm while LP UV lamp mainly emitted UV light at a wavelength of 254 nm. Therefore, the effectiveness of UV wavelength on viral capsid was investigated. The difference between PCR and EMA-PCR at the UV wavelength around 230 to 240 nm is larger than others. The reasons might be that the composite of viral capsid is protein. This data agreed with the UV absorbance profile of proteins and amino acids, which showed high absorbance at low UV wavelength. The previous study found that MP UV lamp enhanced the inactivation of adenovirus over LP UV lamp. Based on our study, MP UV possibly caused damage on viral capsid, which may enhance the inactivation of adenovirus. This finding contributes to better understanding in UV disinfection mechanism and leads to the improvement of UV disinfection in the future.

In this study, the EMA-PCR and PCR had a merit to investigate the damage on viral genome and capsid of viruses. The understanding of disinfection mechanisms from this study has a benefit to provide an effective disinfection method to control viruses. In addition, it could use as an information for develop PCR-based method to selectively detect infectious viruses in the future.