공학석사 학위논문

Elucidating Virus Disinfection Mechanism for Future Water Management: Comparison of Whole Viral Genome Damage with Nucleotide Reactivity for Free Chlorine and Ozone

미래 물관리를 위한 바이러스 소독 기작 규명: 염소와 오존을 이용한 바이러스 유전자 손상과 뉴클레오티드의 소독반응성 비교

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채승태의 공학석사 학위논문을 인준함

2022년 1월

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Abstract

Waterborne viruses transmitted to human and caused respiratory diseases like pneumonia. Viruses are easily spread even with the small amounts. Untreated water could be the source of viruses. Viruses in water source can be inactivated in disinfection process in water treatment. Since there are various serotypes in the virus, CT values of virus were different even when their shapes were similar. Mechanisms of virus disinfection were studied by many researchers. They elucidated that viral protein was modified by disinfection and this type of modification caused virus inactivation. However, the effect of disinfection to viral genome was not fully investigated yet.

This study focused on the reactivity of single nucleotide with free chlorine and ozone to quantify the CT values of nucleotide. Nucleotides (Adenosine 5'-monophosphate, Thymidine 5'-monophosphate, Guanosine 5'-monophosphate, Cytidine 5'-monophosphate) had different CT values with disinfectants (0.6 to 8.175 $M^{-1}s^{-1}$). Thymidine 5'-monophosphate was not reacted during free chlorine disinfection. As sequence of viral genome had different nucleotides property, viral genome was expected to have vulnerable site during disinfection. L4 gene and L5 gene in adenovirus type 2 DNA was selected to identify the sequence and base property effect.

In this study, viral genome damage during disinfection was analyzed by qPCR analysis. Disinfected viral genome was digested to single nucleosides to quantify base degradation in viral genome. Base degradation was analyzed by HPLC-UV.

Degradation of nucleotide in viral genome followed the tendency of nucleotide disinfection kinetics.

This study had environmental implications of waterborne virus treatment. Free chlorine and ozone could damage the viral genome during disinfection process. Base degradation in viral genome was occurred in free chlorine disinfection and ozone disinfection. Compared with viral genome damage analysis and viral genome digestion analysis, base degradation during disinfection process should be major source of genome damage.

Virus disinfection study helps to understand the optimization of disinfection process during water treatment. Also, elucidating genome damage in virus helps to treat emerging virus in the future. Smart city is controlled by data to make city more efficiently. In the future, water treatment in smart city will be controlled by data from water source. Viral genome data measured by molecular methods also has possibility to predict emerging virus viability or disinfection efficiency in future water management.

Keyword : Virus; Disinfection; Chlorine; Ozone; DNA; Nucleotide
Student Number : 2020-21180

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Chapter 1. Introduction

1.1 Study Background

Waterborne viruses are often transmitted to human by the untreated water. They are usually transmitted by shapes of droplets, or aerosols. In the water, viruses are transmitted by human feces. Waterborne viruses like adenovirus, norovirus, enteroviruses (poliovirus, coxackieviruses) and hepatitis A virus can be infected to human and cause diseases. For instance, adenovirus usually can cause respiratory diseases to human. Moreover, the infection of waterborne viruses makes children in low hygiene country vulnerable to suffer from diseases like diarrhea. By the reports of UNICEF, diarrhea was the leading cause of the death of 5 aged children.[16] Even small amounts of waterborne viruses could be infected to host and be replicated exponentially. Having recognized the seriousness of these problems mentioned above, U.S. EPA made Contaminant Candidate List 4 (CCL4) and which includes several waterborne viruses. U.S EPA then continuously monitored water contaminants including these waterborne viruses. Therefore, Korea government and U.S. government regulated virus degradation should be fulfilled to 4−log degradation.

Waterborne viruses were detected in surface water and wastewater. Emerging viruses need to be monitored as water parameter. Viabilities of viruses were detected by PCR measurement of extracted viral genome in recent studies. Viral genome degradation by disinfectants could be used to expect the viability of emerging viruses. In the future, water quality data will

be collected in the forms of digital to control the city more efficiently. This type of city was called 'Smart city'. Water treatment in the smart city will be controlled by data collected from water.

There were several limitations using viral genome as data in smart city. Firstly, extraction and quantification of viral genome from virus was not cost effective. Secondly, viral genome measurement needed pretreatment. Viral genome was extracted from virus and quantified by molecular methods (cPCR, RT-PCR, qPCR, etc.).[21] Thirdly, data from viral genome continuously monitored and reflected to smart water management. However, viral genome data was not driven in real time. If these limitations were solved, viral genome data in water source will be used in prediction of emerging virus viability and disinfection efficiency in smart city.

Many researchers focused on mechanisms of disinfectants to viral proteins,[1-3] since proteins of virus protected viral genome. Previous studies elucidated that when capsid proteins of viruses are damaged, viruses are inactivated and failed to infect other host.[10] Rate constants of disinfectants with capsid protein properties were faster than viral genome properties.[6-7, 18-19] Researchers also extensively studied the impact of UV to viral genome, since UV can penetrate viral protein and attack nucleic acid directly to make byproducts (e.g., Pyrimidine-pyrimidine 6-4 photoproduct, T-T cyclobutane pyrimidine dimer). For the UV affected genome in virus, serial pyrimidine could be changed to TT dimer or C-T dimer.[4] Damaged and modified gene failed to transcribe and translate properly for making essential proteins in virus.

Qiao, et al., 2016 studied about UV impact to short strand RNA. This study showed that there was difference photo reactivity in

different regions. Susceptibilities between direct photolysis (UV_{254}) and indirect photolysis ($^1O_2$) also different by regions.[17]

Recent studies showed that free chlorine and singlet oxygen also affect virus replication process.[5] In other words, free chlorine and ozone also reacted with viral genome and inactivated viruses. However, mechanisms of viral genome damaging during disinfection were not fully elucidated yet. Nucleic acids were consisted of nucleotides which have four different types of bases: guanine, cytosine, thymine, adenine.[6,7] Each bases have different reactivity to disinfectants. As reported second order rate constants order of nucleotide, guanine was the most reactive base to disinfectants in the case of chlorine and ozone. Adenine was the least reactive to both chlorine and ozone. Because single nucleotides had different kinetics with disinfectants, viral genome damage could be different by sequence and position of genes in nucleic acid.

To elucidate viral genome reactivity to disinfectants, we choose specific viral genes (e.g., hexon, fiber) for disinfection. These genes were transcribed and translated to essential proteins in virus. Hexon protein was the major capsid protein of virus. Hexon protein encapsulated its nucleic acids. This protein was made from L4 gene that encoded of hexon properties. Fiber protein was used to binding to the receptor of host cell. If there were no fiber proteins, viruses couldn't bind the receptor. These proteins were essential for virus activities like binding and injection.

The purpose of this study was to elucidate mechanisms of viral genome damage. Understanding of mechanisms of disinfectants to viral genome may help to predict the emerging virus inactivation. Free chlorine and ozone could break viral proteins and they also affected viral genome. If there were specific regions in gene or

constituents of nucleic acids to react these disinfectants (e.g., free chlorine, ozone), it could be applied to genome in emerging viruses. To elucidate these mechanisms, specific gene sites (e.g., hexon, fiber) were disinfected by free chlorine and ozone to see the genome damage by qPCR quantitative methods. In addition, nucleotides in the viral genome also were quantified by enzyme digestion methods to identify damaged sites of disinfected viral genome.

1.2. Purpose of Research

The ultimate purpose of this research attempted to to manifest that overall DNA damage could be directly correlated with reactivity of nucleotides and their composition in DNA. To fulfill this research purpose, this research was set up to achieve several specific research objectives.

(1) To quantify the degradation of individual nucleotide with disinfectants (free chlorine, ozone) to identify the reactivity of nucleotide.

(2) To identify the whole viral DNA damage during disinfection using qPCR analysis and their nucleotides degradation using digestion analysis.

(3) To elucidate the relationship between genome damage and nucleotide degradation.

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Guanine (99 %), cytosine (99+ %), thymine (99 %), Adenine (99 %), were purchased from Acros organics. Thymidine (99 %), Adenosine (99 %), Uracil (99+ %), Uridine (99 %) were purchased from Alfa Aesar. Guanosine (> 98%), cytidine (99 %), 2' -deoxyadenosine-5' -monophosphate (98-100 %), 2' -deoxycytidine (> 99 %), uridine 5' -monophosphate disodium salt (> 99 %), 2' -deoxycytidine 5' -monophosphate (> 95 %), Adenosine 5' -monophosphate sodium salt (> 99 %), Cytidine 5' -monophosphate (> 99 %), 2' -deoxyguanosine 5' -monophoshpate disodium salt (> 98 %), 2' -deoxyguanosine monohydrate (98-100 %) were purchased from Sigma Aldrich. All chemicals were stored at 4 ℃ and -20℃.

Sodium hypochlorite solution (5.65 %-6 %) was purchased from Alfa Aesar. It was wrapped aluminum foil to block the light. Concentration of free chlorine was measured by spectrophotometric method. Molar absorption coefficient of hypochlorite (OCl^-) at 292nm was 350 $M^{-1}cm^{-1}$ (ε_{292} = 350 $M^{-1}cm^{-1}$).[8] *N*,*N*-Diethyl-*p*-phenylenediamine sulfate(97 %) was purchased from Alfa Aesar.

Ozone contained gas (O_2+O_3) was made by ozone generator (LABS2, Ozonia, England). Pure oxygen gas (99.999 %) was injected to the ozone generator. Ozone contained gas was generated in ozone generator by corona discharge method. This ozone contained gas was dissolved in 1 L glass bottle and chilled by water circulator at 4℃. Ozone concentration in water was measured by

spectrophotometric method. Molar absorption coefficient of O_3 at 260 nm was 3,000 $M^{-1}cm^{-1}$(ε_{260} = 3,000 $M^{-1}cm^{-1}$).[9]

Adenovirus type 2 (AdV2) DNA was purchased from KBPV (Korea Bank for Pathogenic Viruses, Korea). AdV2 genome (35,937 bases; NCBI accession number J01917) was stored in lab freezer at −80 ℃ before use. Viral genome stock concentration was measured triplicate by Nanodrop One (Thermo Fisher Scientific).

2.1.2. PCR and qPCR Assay

Viral genome was amplified by PCR to make enough gene stock for experiments. Primers were designed by Primer 3 software. Designed primers were purchased from Bioneer, Korea. Primers were used to DNA amplification. PCR amplicons were covered viral genes(hexon gene, fiber gene). Thermal cycles of PCR was preceded in pre−denaturation at 95 ℃ for 3 min, denaturation for 20 sec and annealing at 95℃ for 30 sec and extension at 72 ℃ for 1 min. Cycles had been repeated 40 times. Viral gene stock concentration was measured by UV−VIS spectrometer at 260 nm.

qPCR (QIAGEN, U.S) was used for quantifying disinfected viral genome. qPCR was proceeded by 2 step procedures. Thermal cycling conditions were 95 ℃ for 3min, 72 ℃ for 40 sec and 60 ℃ for 20 sec. Cycles had been repeated 40 times. After thermal cycling, melt analysis was processed to identify amplification of target double stranded DNA.

2.1.3. Restriction Enzyme for Digestion Assay

Enzymatic digestion method was used for detecting base modification by disinfectants (FC, O_3). Nucleic acids were digested by nucleoside digestion mix (NEB #0649S, England). Digestion mix was used as following protocols of manufacturer. Viral gene was digested to single nucleoside by enzyme. 47 μL of DNA sample, 1 μL of digestion enzyme and 2 μL of digestion buffer were mixed. Viral gene contents including enzyme and digestion buffer were incubated for overnight at 37 ℃. Digested genes were stored at 4 ℃ before analyzed by HPLC-UV.

2.2. Methods

2.2.1. Kinetics of Nucleotides During Free Chlorine Disinfection

Nucleobases, nucleosides, nucleotides were eluted 50 μM in 100 mL of 10 mM phosphate buffer (pH 7.4). Dosage of free chlorine was 100 μM, 250 μM, 500 μM to each sample. Free chlorine was measured by UV-VIS spectrophotometry. Molar absorption coefficient of free chlorine was 350 $M^{-1}cm^{-1}$.[20] Experiments was proceeded in dark condition by wrapped by foil. Reactions were terminated by sodium thiosulfate as quenching agent. Its concentration was 2 times over free chlorine. Residual chlorine concentration was measured with *N,N*-diethyl-*p*-phenylenediamine (DPD) colorimetric method. DPD method in this study was slightly changed from method of Standard Methods for Examination of Water and Wastewater. DPD indicator was made by dissolving 1.1 g DPD sulfate and 8 mL of 25 % H_2SO_4 and 200 mg of disodium ethylene diamine tetraacetate dihydrate (EDTA). These reagents were made up to 1 L and stored in the amber bottle in the dark. Phosphate buffer was used for DPD method. 24 g of Na_2HPO_4 and 46 g of KH_2PO_4 was dissolved in 1L with distilled water and 800 mg of EDTA was dissolved in the bottle. DPD samples were analyzed by UV-VIS spectrometry with residual chlorine. Absorbance of samples was detected at 515 nm.[8] Residual chlorine concentration was measured using Beer-Lambert law (equation 1). Absorbance (A) was expressed by serial multiplicate molar absorption coefficient (ε), optical path length (b) and molar concentration of target (M).

$$A = \varepsilon bc \quad (1)$$

A: Absorbance

ε : Molar absorption coefficient ($M^{-1}cm^{-1}$)

b: Optical path length (cm)

c: Molar concentration (M)

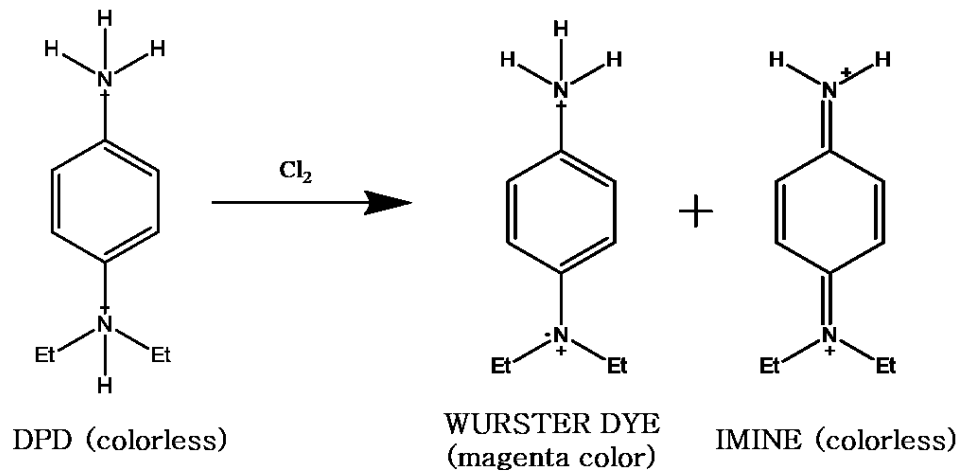

Figure 1. Principles of DPD colorimetric method

2.2.2. Nucleotide Degradation During Ozone Disinfection

Nucleotides were eluted to 50 μM in 2 mL of 10 mM phosphate buffer (pH 7.4). Ozone stock solution was made at 1 L of chilled water jacket bottle. Concentration of ozone stock solution was almost 0.5 mM analyzed by UV-VIS at 260 nm. Stock solution was diluted to 50 μM, 100 μM, 200 μM in 2 mL of reaction volume. Reactions were terminated by 250 μM of sodium thiosulfate.

2.2.3. Adenovirus Type 2 DNA Disinfection Measured by qPCR

Adenovirus type 2 DNA was diluted in phosphate buffer (pH 7.4, 10 mM). Initial concentration of DNA was 1.924~ 2.148 x 10^{-2} μg / mL. Free chlorine and ozone aqueous solution reacted with viral DNA in 1.5 mL of microcentrifuge tube for 2 hrs. Reaction was terminated by quenching agent ($Na_2S_2O_3$). Reacted viral DNA samples were analyzed by qPCR. Viral DNA was amplified during 40 cycles. SYBR green fluorescence was attached in double strand of DNA. Amplified DNA was proportional to degree of fluorescence. Viral DNA was quantified using $\Delta\Delta$CT method. This method used number of cycles until threshold (C_t: Cycle of threshold). Variation of sample DNA C_t and housekeeping gene C_t could be express ΔCt as equation (2). Variation between ΔCt of disinfected sample and ΔCt of control sample could be express $\Delta\Delta$Ct as equation (3).

$$\Delta Ct = Ct \text{ (gene of interest)} - Ct \text{ (housekeeping gene)} \quad (2)$$

$$\Delta\Delta Ct = \Delta Ct \text{ (disinfected sample)} - \Delta Ct \text{ (control sample)} \quad (3)$$

Theoretically during PCR cycle, template DNA was amplified twice than before cycle. Relative quantification of amplified DNA was $\log_{10}(2^{-\Delta\Delta Ct})$ in log scale plot.

2.2.4. Disinfected Viral Gene Digestion Analysis

Hexon gene segments (18,838 bp – 21,744 bp) and fiber gene segments (31,030 bp – 32,778 bp) in adenovirus type 2 DNA were amplified by PCR. Each samples were diluted by DNase free water to 10 ng / μL. Viral gene segments were reacted with free chlorine (10 ppm – 400 ppm) and ozone aqueous solution (0.5 ppm – 10 ppm) for 2 hrs. Reaction volume was 50 μL. Gene segments were digested to single nucleosides by using restriction enzyme. 1 μL of restriction enzyme and 2 μL of digestion buffer was spiked in samples. Enzyme was activated in 37 ℃ during overnight incubation. Samples were analyzed by HPLC-UV in 260 nm of wavelength. Column was Xselect HSS T3 columns from Waters. Mobile phase was 10 mM ammonium acetate and methanol.

Chapter 3. Results and Discussion

3.1. Kinetics of Nucleotide during Free Chlorine Disinfection

As mentioned in section 2.2.1. Nucleobases, nucleosides, and nucleotides reacted with free chlorine in 100 mL volume of pH 7.4 phosphate buffer. Residual chlorine was analyzed by UV-VIS spectrophotometer to calculate CT values of free chlorine disinfection. Molar ratio of nucleobase to free chlorine was 5 and molar ratio of nucleoside to free chlorine also 5. Molar ratio of nucleotide to free chlorine was 10. ([Free chlorine] / [Nucleobase] = 5, [Free chlorine] / [Nucleoside] = 5, [Free chlorine] / [Nucleotide] = 10). CT values of target materials (nucleobase, nucleoside, nucleotide) with free chlorine were fitted in second order rate constant. As a result of the experiment, rate constants that reagents containing guanine moiety were the most reactive than other base moieties. In contrast, thymine was degraded by free chlorine rapidly, but thymidine and thymidine 5' -monophosphate showed low reactivity.

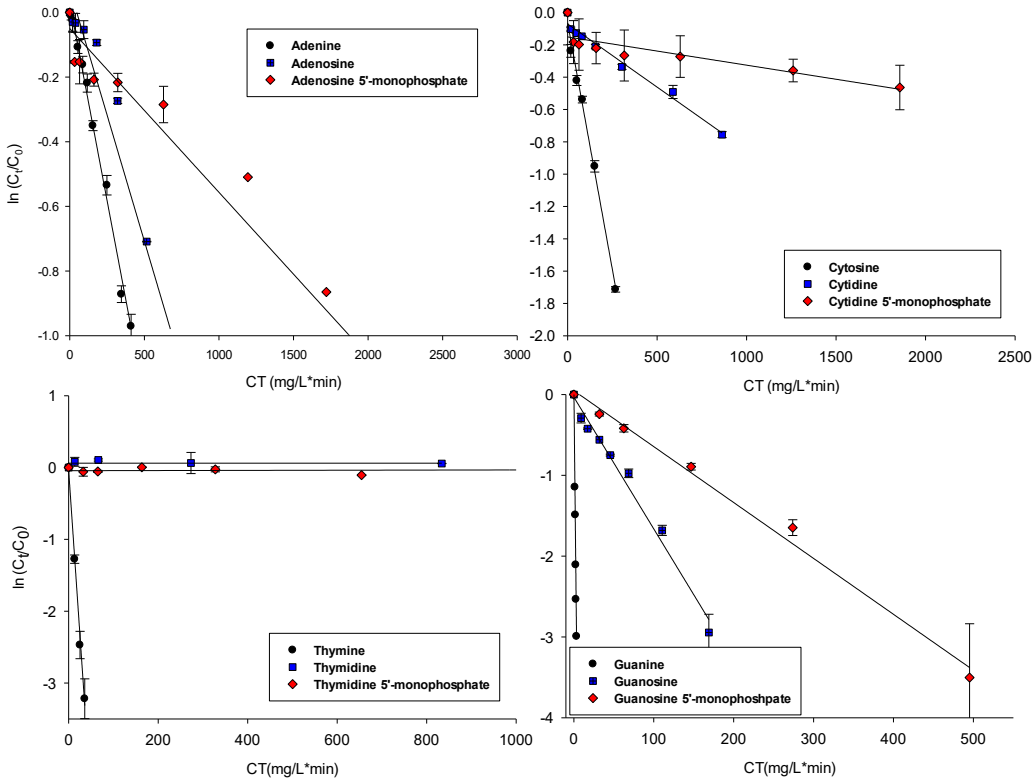

Figure 2. CT values of nucleobase, nucleoside, nucleotide: adenine, cytosine, thymine, guanine residual target materials were measured by LC-MS and residual chlorine was measured by UV-VIS photometry.

Table 1. Rate constant of nucleobase, nucleoside, nucleotide in free chlorine experiments

Rate constants ($M^{-1}s^{-1}$)					
Base	Adenine	Thymine	Guanine	Cytosine	Uracil
Nucleobase	2.89 (±0.09)	1.08 (±0.035) × 10^2	1.102 × 10^3	7.22 (± 0.09)	45.89(± 23.29)
Nucleoside	1.84 (± 0.048)	N/A	19.21 (± 1.58)	0.92 (± 0.017)	N/A
Nucleotide	0.6 (±0.042)	N/A	8.175 (± 1.48)	0.206 (± 0.011)	N/A

Adenine is purine derivatives which has 2 nitrogenous aromatic rings. Molar ratio range of target materials and free chlorine was 2

to 10. As dose of free chlorine increased, degradation of target also increased. 50 μM concentration of adenine and adenosine in pH 7.4, 10 mM phosphate buffer reacted with 100, 250 μM free chlorine. After 60 min reaction with free chlorine, 31.6 % of target was degraded with 100 μM free chlorine reaction and 60.9 % of target degraded with 250 μM free chlorine reaction. Adenosine had more resistance to free chlorine than adenine in the first 10 min. But after 60 min reaction, 50 μM adenosine lost 28 %, 66.6 % in each 100, 250 μM free chlorine reaction. Second rate constant of adenine was 2.89 (± 0.09) and rate constant of adenosine was 1.84 (± 0.048) and rate constant of adenosine 5’ −monophosphate was 0.6 (± 0.042). In the case of adenine containing materials, as molecular weight increased second order rate of target with free chlorine decreased.

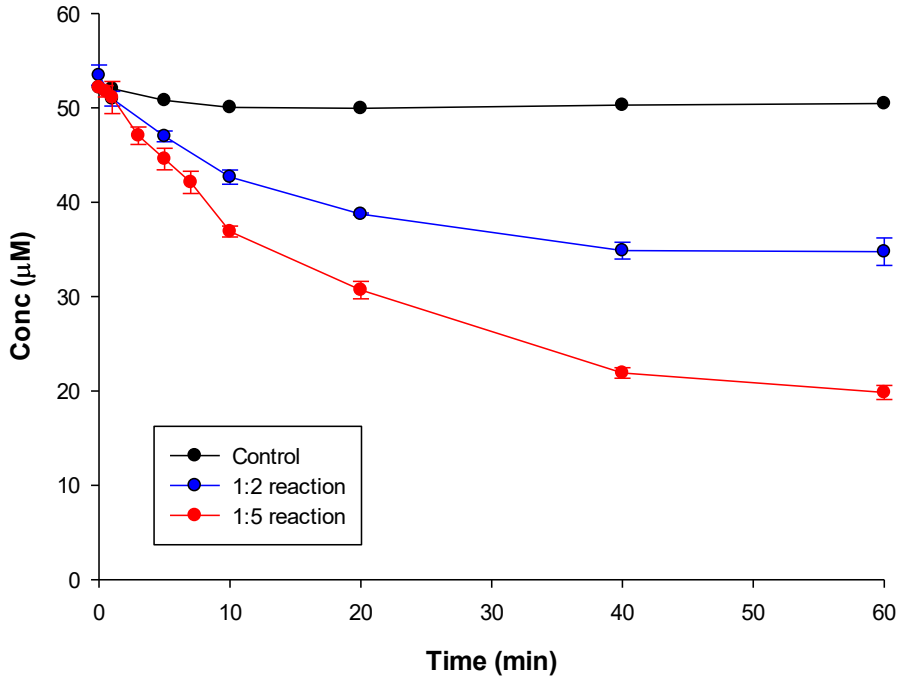

Figure 3. Adenine degradation with free chlorine. $[Adenine]_0 = 50\ \mu M$ in phosphate buffer; pH 7.4

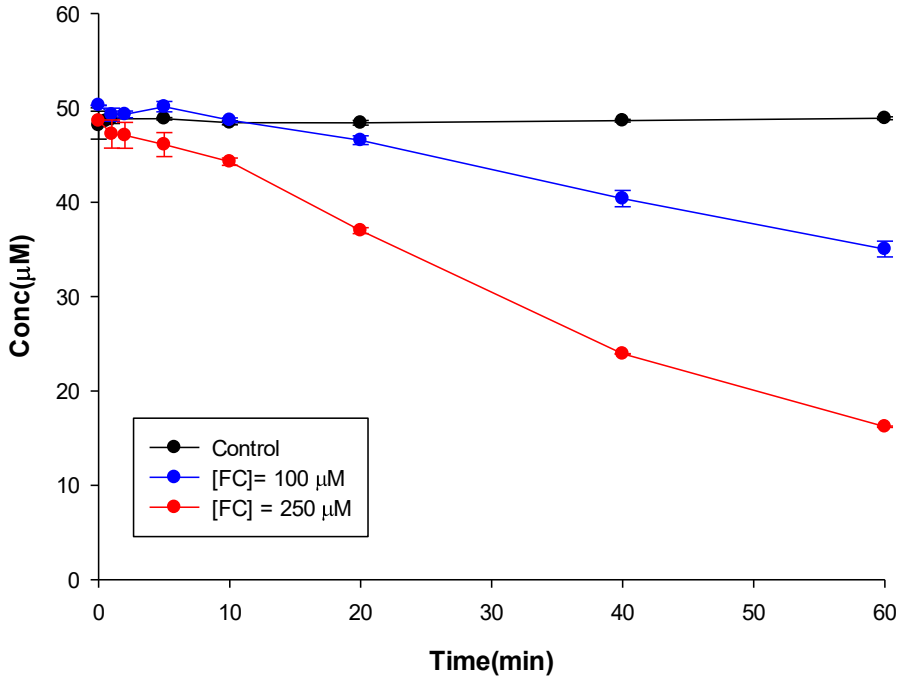

Figure 4. Adenosine degradation with free chlorine. $[Adenosine]_0$ = 50 μM in phosphate buffer; pH 7.4

Cytosine is pyrimidine derivatives containing nitrogenous aromatic ring. It has amine functional group as substituents. Target materials containing cytosine base (cytosine, cytidine, cytidine 5'-monophosphate) reacted to free chlorine under pH 7.4, 10 mM phosphate buffer condition. Molar ratio of target and free chlorine was 5 to 10. Cytosine reacted with 250 mM free chlorine and its reaction was terminated before 40 min.

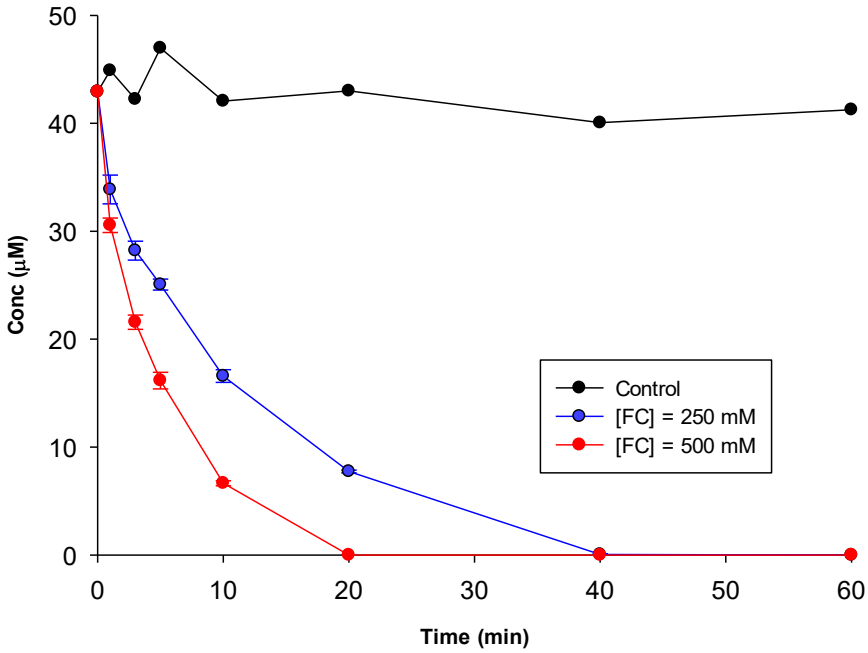

Figure 5. Cytosine degradation with free chlorine. $[Cytosine]_0 = 50\ \mu M$ in phosphate buffer; pH 7.4

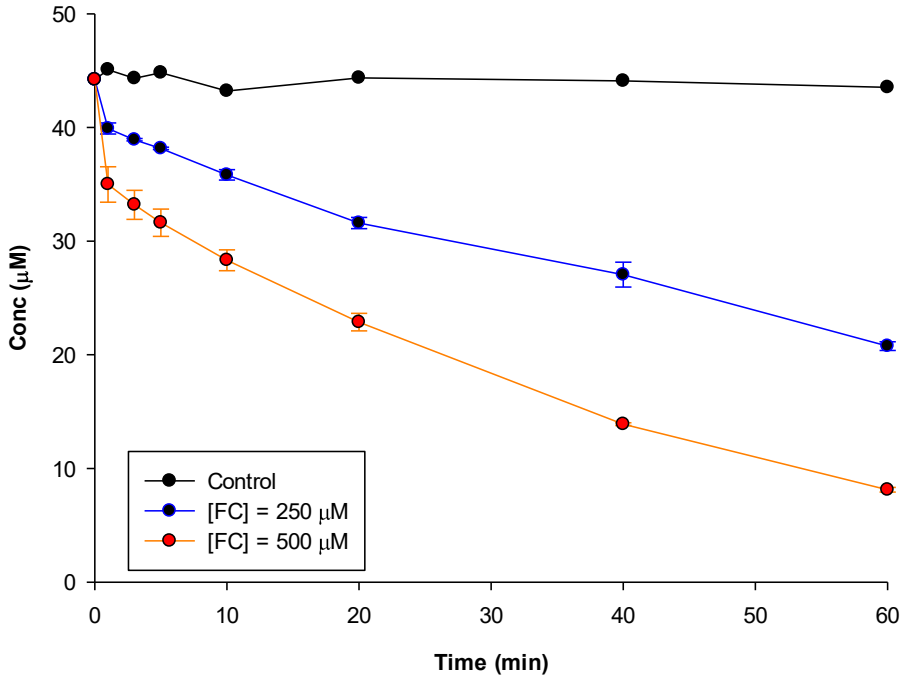

Figure 6. Cytidine degradation with free chlorine. $[Cytidine]_0 = 50\ \mu M$ in phosphate buffer; pH 7.4

Thymine degraded faster than adenine or cytosine, as kinetics of thymine was 1.08 (± 0.035) × 10^2 $M^{-1}s^{-1}$. Thymine included methyl group and amine group where free chlorine could be reacted. Because of these chemical structure characteristics of thymine, free chlorine easily reacted with thymine. Thymine was chlorinated during chlorine disinfection.[6] In case of thymidine and thymidine 5' −monophoshphate, free chlorine rarely reacted with target materials. During free chlorine reaction for 2 hours, thymidine and thymidine 5' −monophosphate were not degraded by free chlorine. thymine base was connected to ribose or deoxyribose by N−glycosidic bond. This bond resisted free chlorine reaction with amine group in thymine base.

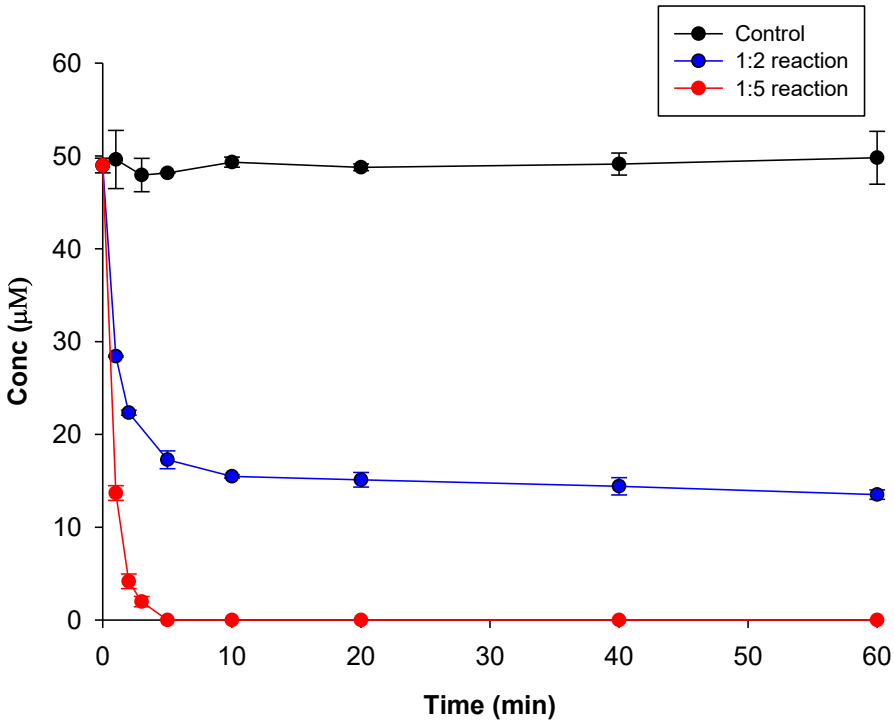

Figure 7. Thymine degradation with free chlorine. $[Thymine]_0 = 50\ \mu M$ in phosphate buffer; pH 7.4

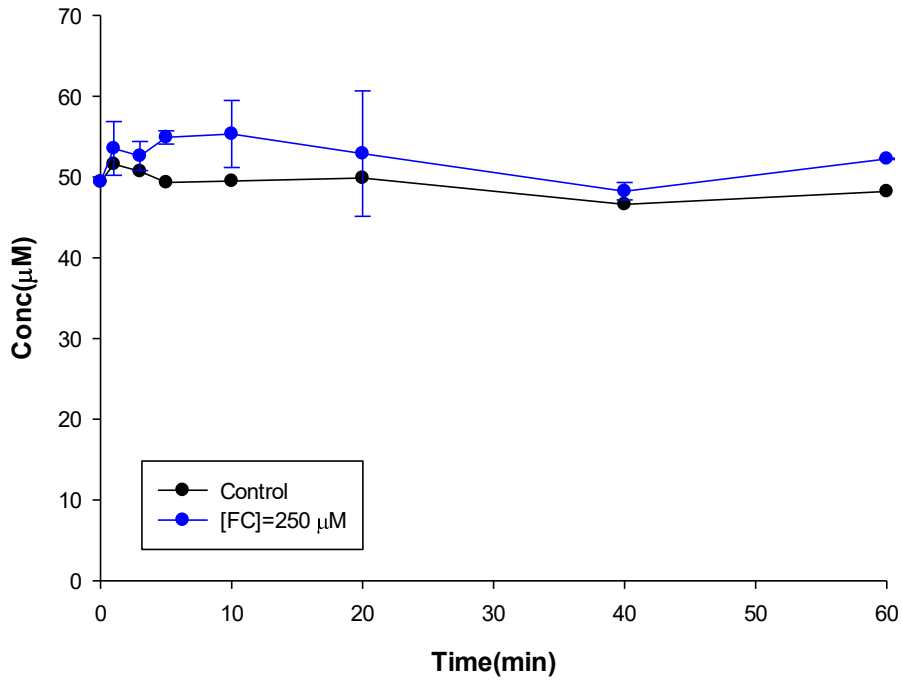

Figure 8. Thymidine degradation with free chlorine. $[Thymidine]_0 = 50$ μM in phosphate buffer; pH 7.4

Guanine moieties were most reactive with free chlorine. Kinetics of guanine with free chlorine was 1.102×10^3 $M^{-1}s^{-1}$. Guanine is purine derivatives. Chlorination site of guanine was carbon in heterocyclic compound. Guanosine and guanosine 5 ' – monophosphate were also reacted easily with free chlorine. Because of high reactivity of guanine with free chlorine, initial concentration of guanine was 5 μM and concentration of free chlorine was 5 μM to 10 μM. As target molecular weight increased (nucleobase to nucleoside, nucleoside to nucleotide), kinetics was decreased.

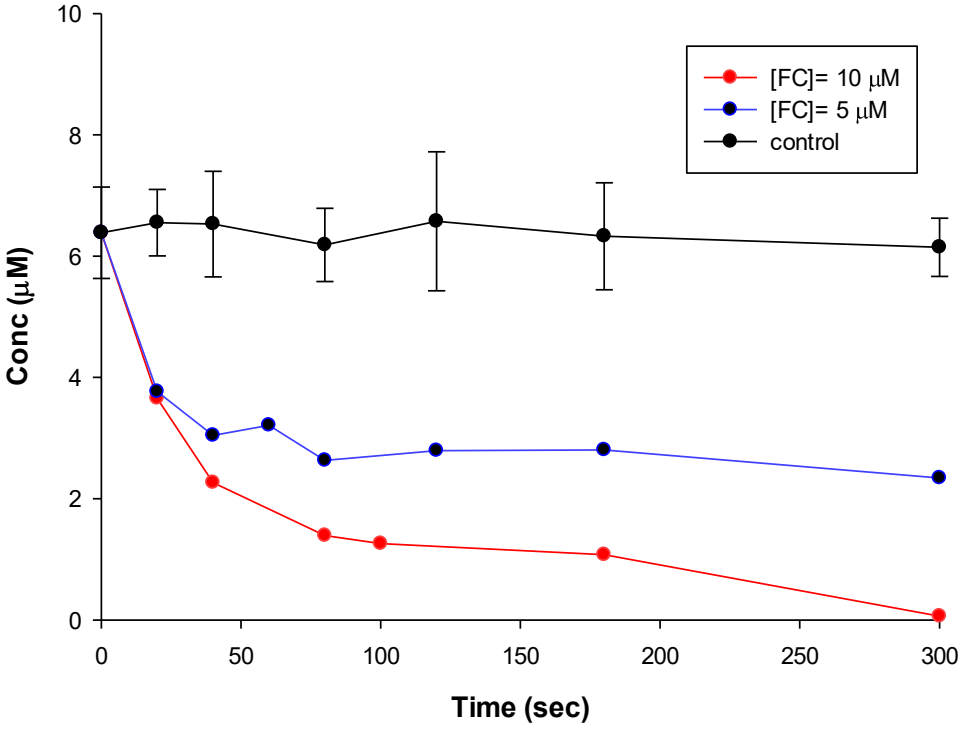

Figure 9. Guanine degradation with free chlorine. $[Guanine]_0 = 50\ \mu M$ in phosphate buffer; pH 7.4

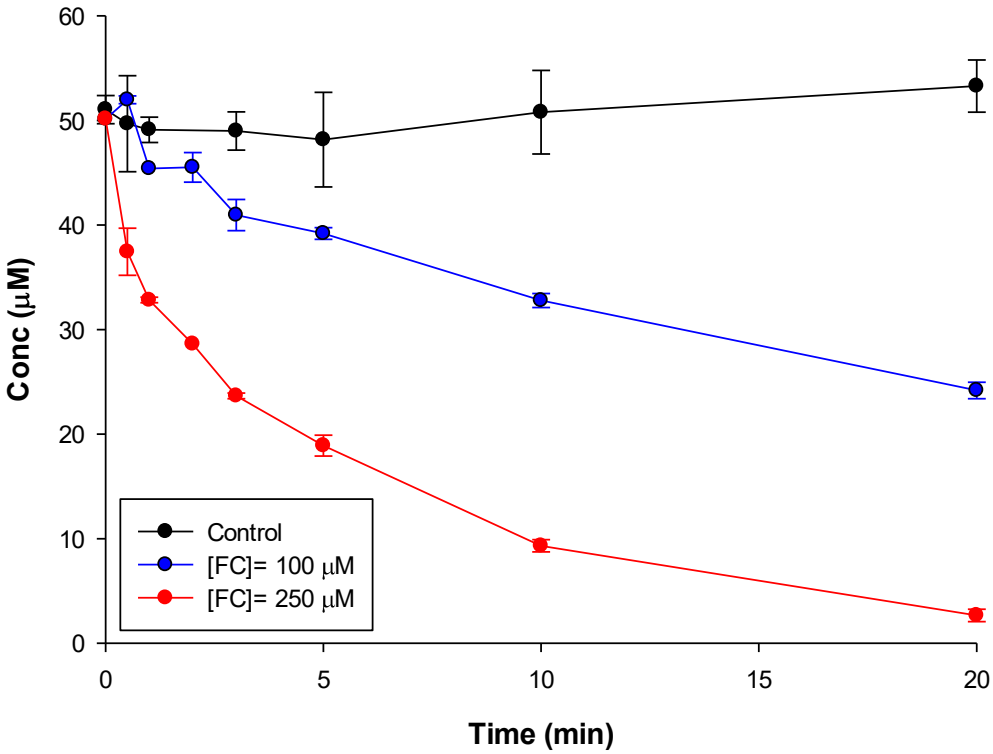

Figure 10. Guanosine degradation with free chlorine. $[Guanosine]_0$ = 50 μM in phosphate buffer; pH 7.4

To sum up the results of free chlorine disinfection experiment, each nucleotide was degraded by free chlorine except for thymidine and thymidine 5' -monophosphate. There were differences in kinetics between nucleotides. Guanine had 2-3 orders fast kinetics than adenine and cytosine. Another point to note was that all nucleotides were reacted with 500 μM free chlorine except thymidine 5'-monophosphate. Rate constant of guanosine 5' -monophosphate (8.175 (± 1.48)), adenosine 5' -monophosphate (0.6 (± 0.042)), cytidine 5' -monophosphate (0.206 (± 0.011)). Thymidine 5' -monophosphate was not degraded by free chlorine. Lastly, nucleotides containing guanine moiety had more reactivity

than other nucleotides (Adenosine 5' -monophosphate, Thymidine 5' -monophosphate, Cytidine 5' -monophosphate). Target materials containing thymine moiety except thymine had low reactivity with free chlorine. Prütz (1996) showed different result with this study that rate constant of thymidine 5' -monophosphate (k: 4.3×10^3)[6].

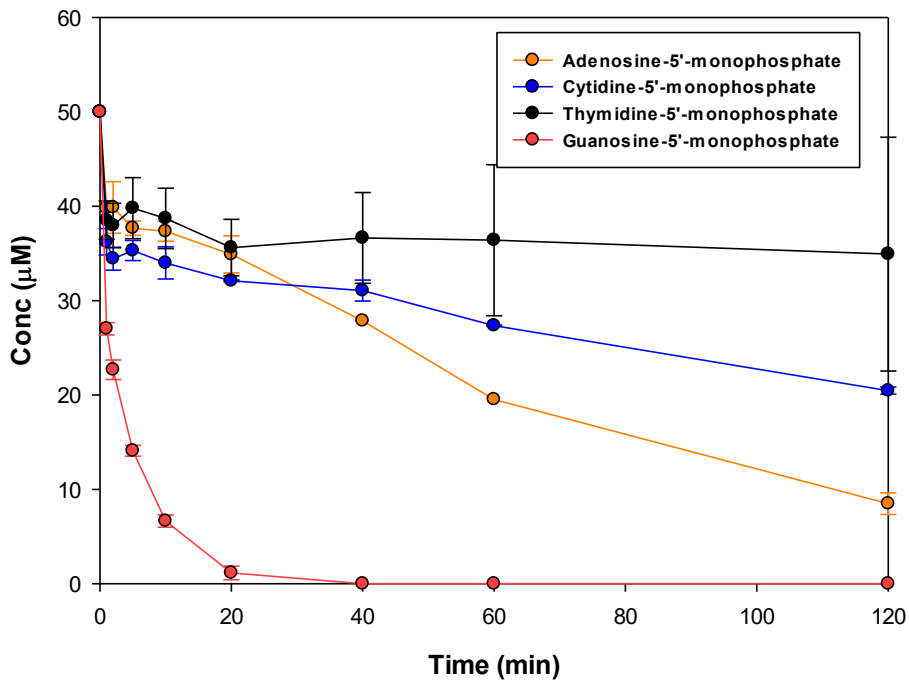

Figure 11. Nucleotide degradation with free chlorine. $[Nucleotide]_0$ = 50 μM. Molar ratio of nucleotide with free chlorine was 10 ([Free chlorine] / [Nucleotide] = 10).

3.2. Nucleotide and Deoxyribonucleotide degradation during Ozone Disinfection

The first finding of ozone disinfection experiment was that types of ribose did not affect the degradation of nucleotides. Former researchers like Ishizaki, et al. 1984, Matsui et al., 1991, and Theruvathu, et al., 2002 studied that ozone was more reacted with base moieties than ribose moieties. Target materials containing pyrimidine base (Cytidine 5'-monophosphate, deoxycytidine 5'-monophosphate, Thymidine 5'-monophosphate and Uridine 5'-monophosphate) reacted with ozone were degraded more than materials containing purine base (adenosine 5'-monophosphate, guanosine 5'-monophosphate, deoxyadenosine 5'-monophosphate, deoxyguanosine 5'-monophosphate). Thymidine 5'-monophosphate and Uridine 5'-monophosphate were the most degraded materials in 50 μM of ozone reaction.

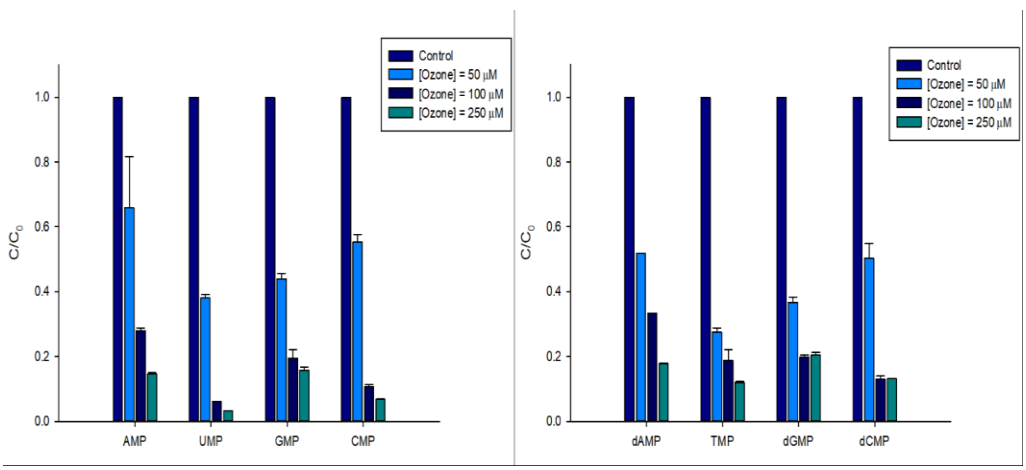

Figure 12. Ozone disinfection with nucleotide and deoxyribonucleotide. $[Nucleotide]_0$ = 50 μM. Molar ratio of nucleotide with ozone was 1, 2, 5 ([Ozone] / [Nucleotide] = 1, 2, 5)

The second finding of Ozone disinfection experiment was that

ozone was unstable in pH 7.4. Ozone aqueous solution in pH 7.4 was decomposed to hydroperoxide radical (HO_2•), superoxide radical ions (O_2•), hydroxyl radical (OH•). All nucleotides and deoxyribonucleotides were degraded with ozone aqueous solution. Because there was limitation of reaction volume, kinetics of ozone reaction with nucleotides were not described in this study. Kinetics of ozone with nucleotides were described by Ishizaki, et al., 1984.[10] Difference between ribose and deoxyribose was not shown in HPLC-UV analysis.

Table 2. Reported rate constant of ozone with nucleotide

Reported rate constants ($M^{-1}s^{-1}$) in pH 7					
Base	Adenine	Thymine	Guanine	Cytosine	Uracil
nucleotide	2.0×10^2	1.6×10^4	5.0×10^4	1.44×10^3	6.5×10^2

To sum up the result of ozone disinfection with nucleotides experiments, there were small difference in reactivity between nucleotides and deoxyribonucleotides. All types of bases were reacted with ozone aqueous solutions. Typically, nucleotides containing Guanine and thymine (uracil) more degraded than other nucleotides (adenine, cytosine) in 50 μM of ozone solution.

3.3. Adenovirus Type 2 DNA disinfection Measured by qPCR

As mentioned in introduction, viral genome reactivity with disinfectants was not yet fully elucidated. Adenovirus type 2 DNA was used to seek the viral genome damage with disinfectants (Free chlorine, Ozone).

Adenovirus type 2 DNA was diluted in phosphate buffer (10 mM, pH 7.4) to make 1.924~ 2.148 x 10^{-2} μg / mL. Hexon gene and fiber gene were amplified using RT-qPCR. Because of amplification efficiency, each gene site was segmented by 400 ~ 450 bp. Free chlorine (0.2 ~ 50 μM) and ozone (1 ~ 30 μM) were spiked in DNA and reactions were terminated by quenching agent ($Na_2S_2O_3$). Disinfected DNA samples were analyzed by qPCR. Damage of viral DNA was calculated by $\Delta\Delta$CT methods.

The first finding of viral DNA disinfection experiment was that low dose of free chlorine and ozone could not damage the viral DNA efficiently. Free chlorine ranging 0.2 μM to 2 μM damaged viral DNA under 10 % of genome. The second finding of this experiment was that high dose of free chlorine (5 μM to 50 μM) damaged viral DNA effectively. There was DNA damage gap between low dose of free chlorine and high dose of chlorine. More than 10 μM of free chlorine and ozone could damage the viral genome 1-log degradation.

Nucleotides were connected by phosphodiester bond in nucleic acid. DNA has double strand, connected by hydrogen bond between complementary nucleotides. Due to these properties, viral DNA has stability. Low dose of disinfectants unfulfilled to destroy the viral genome. Ozone disinfection couldn't damage effectively in low doses (0.2 ~ 2 μM). Ozone disinfection also needed more than 5

μM of ozone solution to damage the viral gene more than 1-log degradation. Viral gene damage was increased as ozone dose increased.

Viral genome was quantified by qPCR analysis. If there were base modifications or phosphodiester bond cleavage in viral genome, viral genome couldn't be amplified in thermal cycling process and SYBR Green fluorescence couldn't bind in double strand of amplified DNA. This study showed that free chlorine and ozone caused base modification in viral genome restricting qPCR amplification.

To sum up the results of viral genome disinfection experiments, viral genome damaged by disinfectants in low dose to high dose of disinfectants. Low dose of disinfection could damage the viral genome slightly, but it could not damage effectively. More than 1-log degradation was needed to molar ratio of viral genome to disinfectants 134,661 ([disinfectants] / [viral genome]).

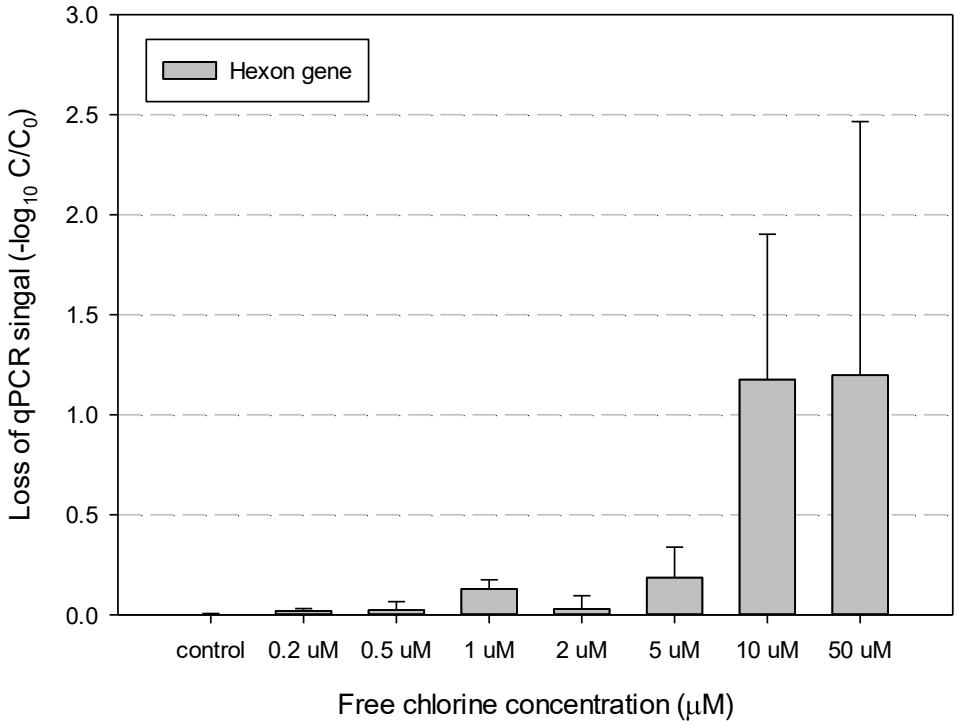

Figure 13. Hexon gene damage with free chlorine measured by qPCR. Hexon gene was segmented by 7 parts. $[\text{Viral genome}]_0 = 1.924 \sim 2.148 \times 10^{-2}$ μg / mL. Hexon gene segments were averaged.

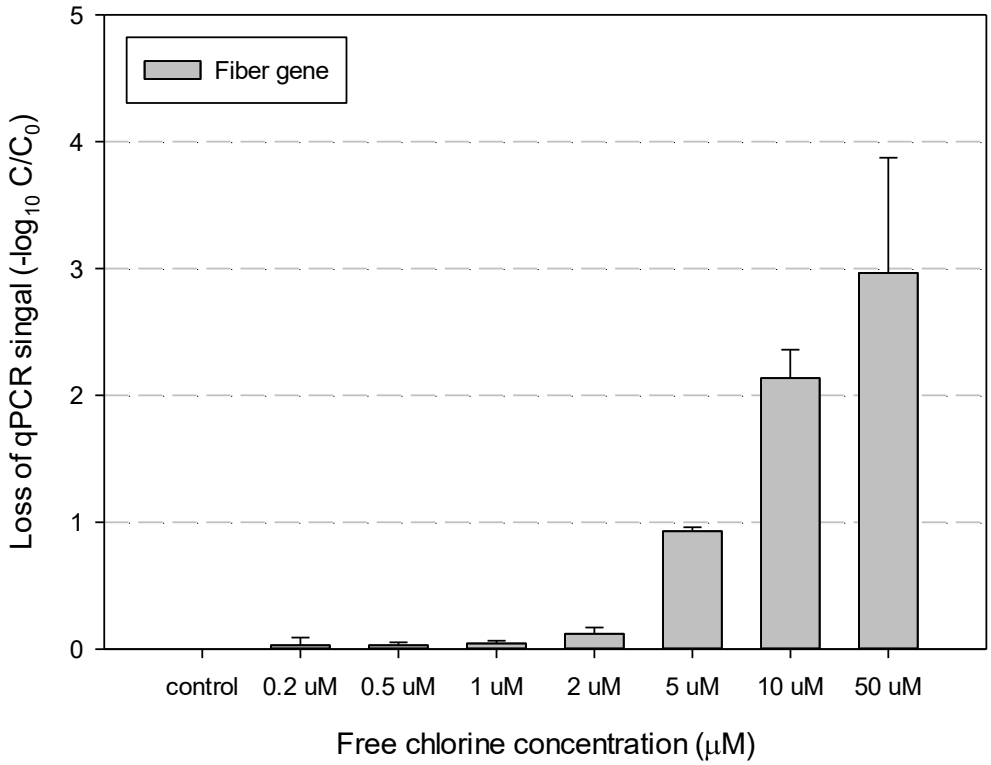

Figure 14. Fiber gene damage with free chlorine. Fiber gene was segmented by 4 parts. $[\text{Viral genome}]_0 = 1.924 \sim 2.148 \times 10^{-2}$ μg/mL. Fiber gene segments were averaged.

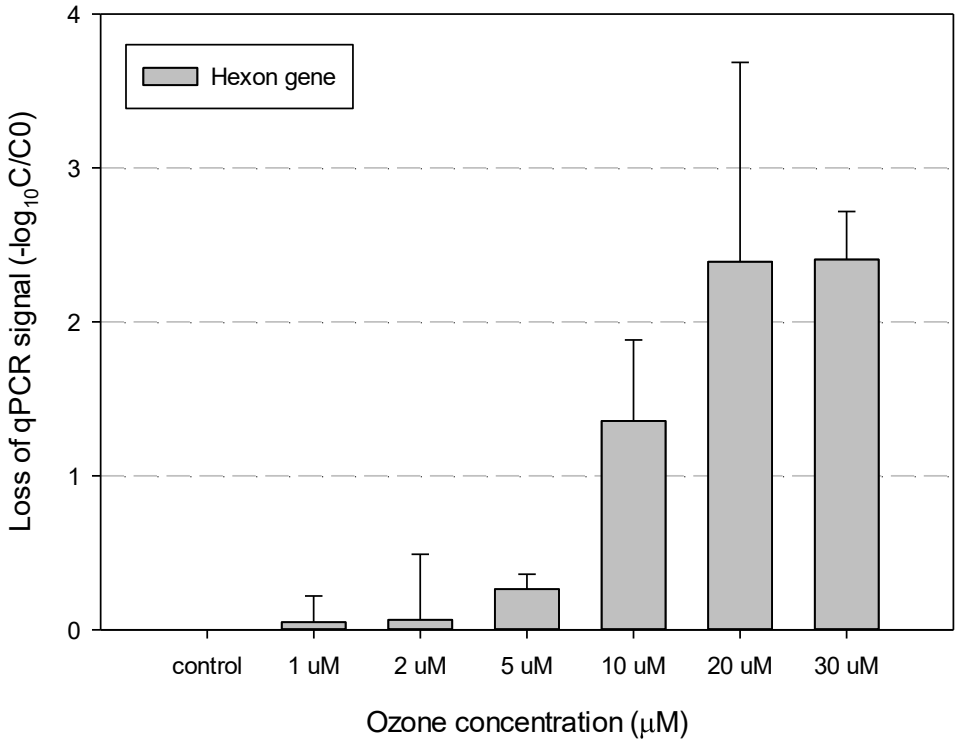

Figure 15. Hexon gene damage with ozone. Hexon gene was segmented by 7 parts. $[\text{Viral genome}]_0$ = 1.924~ 2.148 x 10^{-2} μg / mL. Hexon gene segments were averaged.

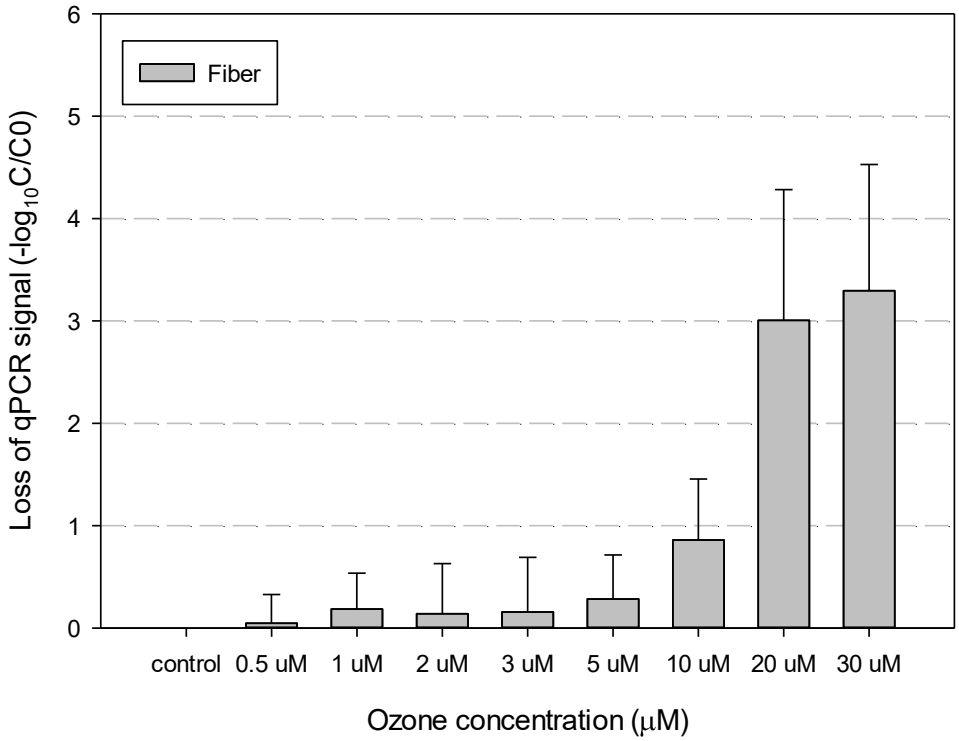

Figure 16. Fiber gene damage with ozone. Fiber gene was segmented by 4 parts. $[\text{Viral genome}]_0$ = 1.924~ 2.148 x 10^{-2} μg / mL. Fiber gene segments were averaged.

3.4. Viral Gene Disinfection and Digestion Analysis by Restriction Enzyme

To find out base modifications during disinfection, disinfected viral genome was digested by restriction enzyme. Viral gene (hexon, fiber) was disinfected by free chlorine and ozone. Hexon gene was segmented to 7 parts and fiber gene was segmented to 4 parts. Segment length was about 400 to 450 bp. Viral gene segments were amplified by PCR and its concentration was analyzed in UV-VIS spectrophotometer at 260 nm. Initial concentration of viral gene was 10 ng / μL. Gene segments were reacted with disinfectants for 2 hours. Disinfected viral genes were digested by nucleoside digestion mix to make viral gene to single nucleosides. After overnight incubation at 37 ℃, all samples were analyzed by HPLC-UV at 260 nm wavelength. Gene segment reacted with free chlorine (10, 20, 50, 100, 200, 400 ppm).

The first finding of digestion experiment was that single nucleosides remained (except segment 5) during low dose of free chlorine reactions (FC: 10 - 20 ppm). Reacted with over 50 ppm of free chlorine showed that adenosine, guanosine, cytidine degraded to LOQ.

The second finding of digestion experiment was that thymidine was still detected by HPLC over 50 ppm chlorine reaction. This result showed that thymidine was the most resistant to free chlorine disinfection. Viral gene digestion experiments also showed that thymidine or thymidine 5' -monophosphate was least reacted with free chlorine. Excess amounts of free chlorine (200, 400 ppm) made thymidine to degrade at LOQ of HPLC. Hexon segments (1~7) showed a little bit different degradation plot. All viral gene

digestion experiments showed that thymidine was the most resistant to free chlorine.

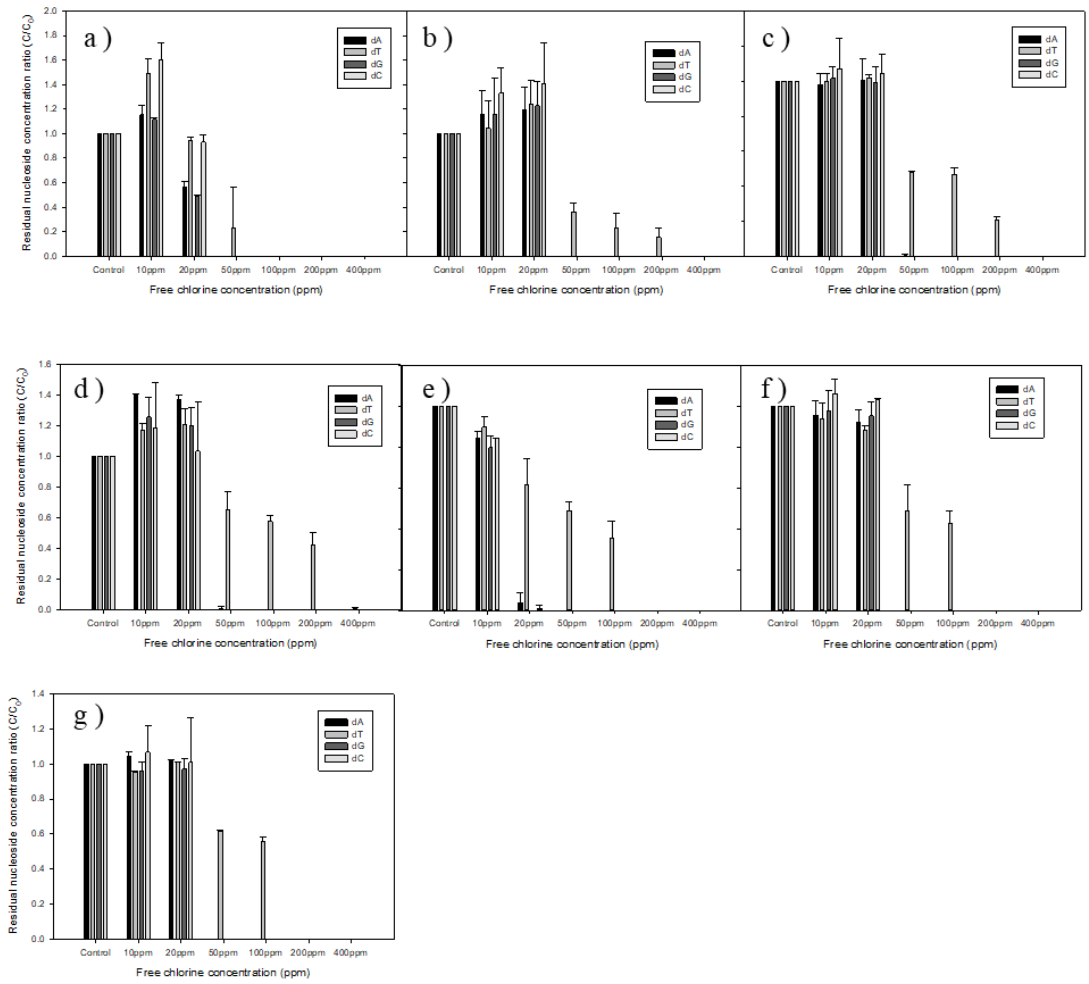

Figure 17. Degradation of nucleotide in hexon segment gene a) Hex_segment 1, b) Hex_segment 2, c) Hex_segment 3, d) Hex_segment 4, e) Hex_segment 5, f) Hex_segment 6, g) Hex_segment. $[\text{Viral gene}]_0$ = 10 ng / μL.

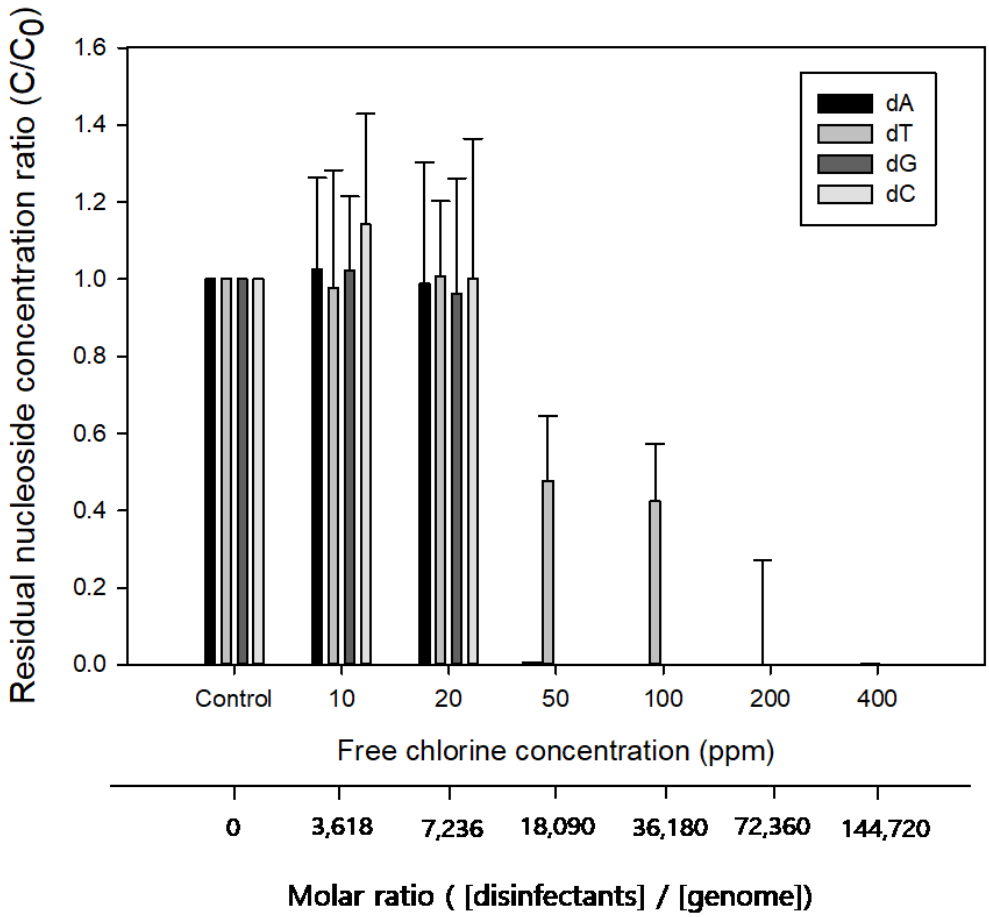

Figure 18. Average degradation of nucleotide in chlorine disinfected hexon gene. $[\text{Viral gene}]_0 = 10$ ng / μL

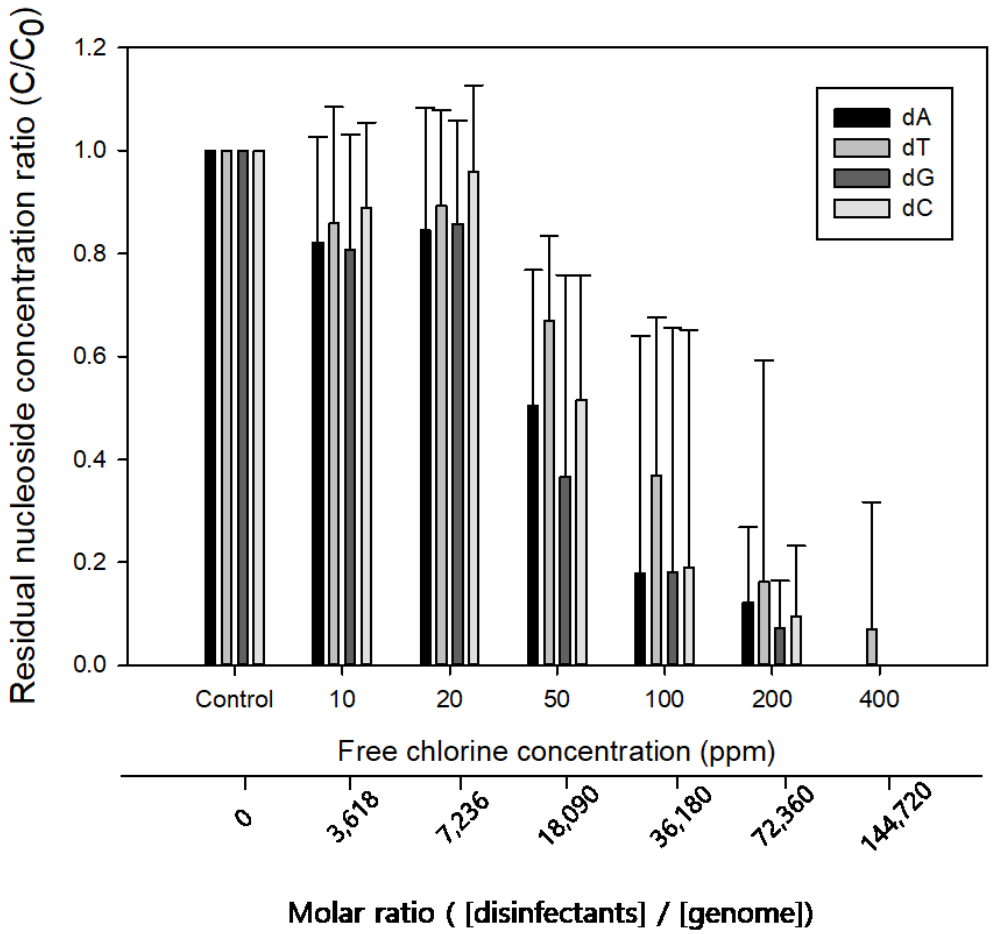

Figure 19. Average degradation of nucleotide in chlorine disinfected fiber gene. $[\text{Viral gene}]_0$ = 10 ng / μL

Because of the limitations of making ozone in ozone generator, ozone concentration was 0.5 ppm to 10 ppm. Molar ratio of viral gene to ozone aqueous solution ([ozone] / [viral gene]) was 280.5 to 5,610. Until 10 ppm of ozone solution reacted with viral gene, there was no significant degradation of average nucleoside in viral genes (hexon, fiber).

The third finding of digestion experiment was that there was small genome damage in low dose of ozone. As Ito, et al., 2005,

ozone caused cleavage of double strand bond of DNA and phosphodiester bond.[12] In the early reactions of ozone with viral gene, double strand bond dissociation or phosphodiester cleavage was occurred before base modifications.

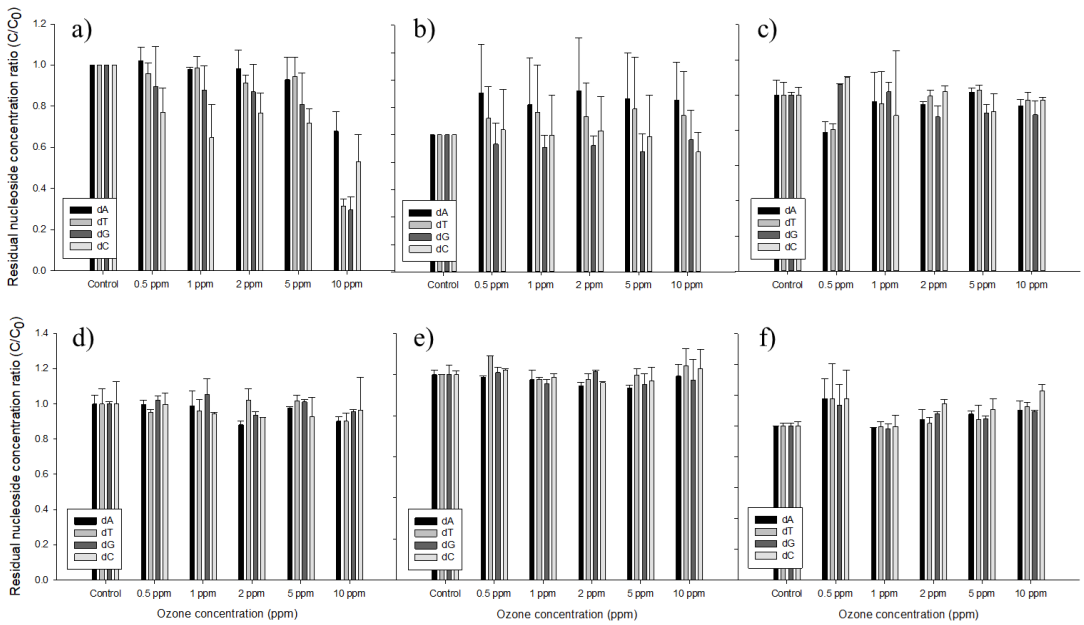

Figure 20. Degradation of nucleotide in hexon segment with ozone disinfection: a) segment 1, b) segment 2, c) segment 3, d) segment 5, e) segment 6, f) segment 7. [Viral gene] = 10 ng / μL.

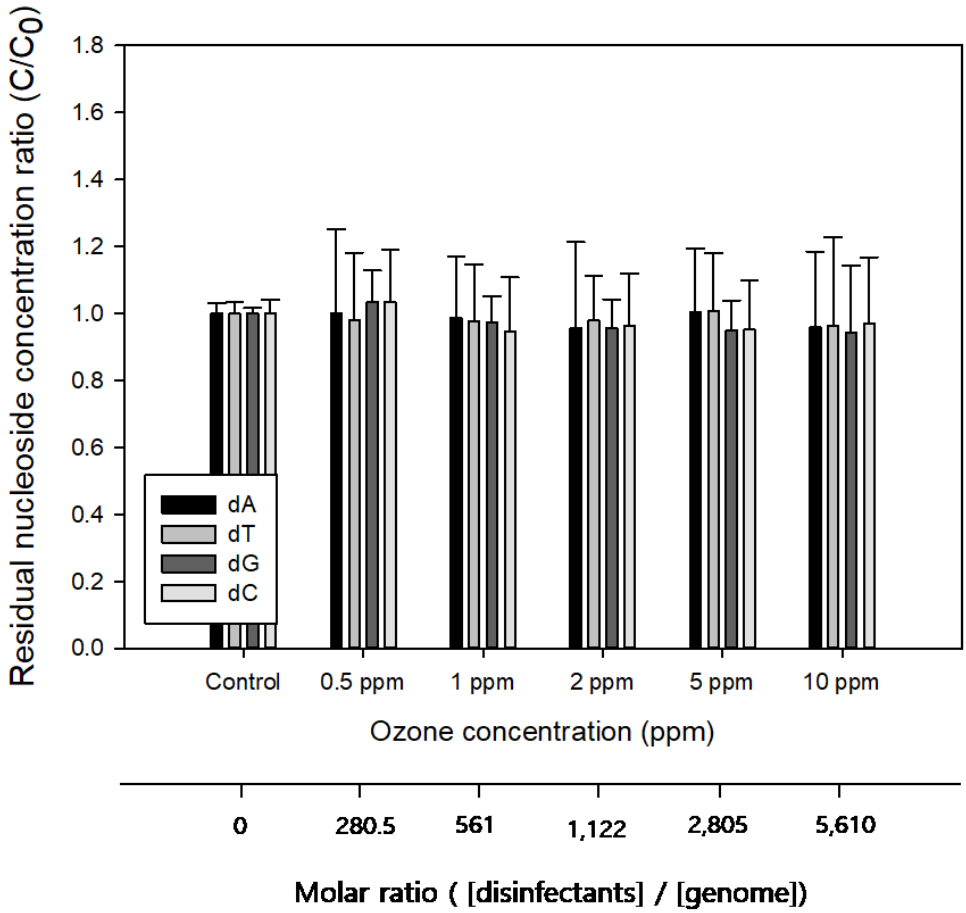

Figure 21. Average degradation of nucleotide in ozone disinfected hexon gene.

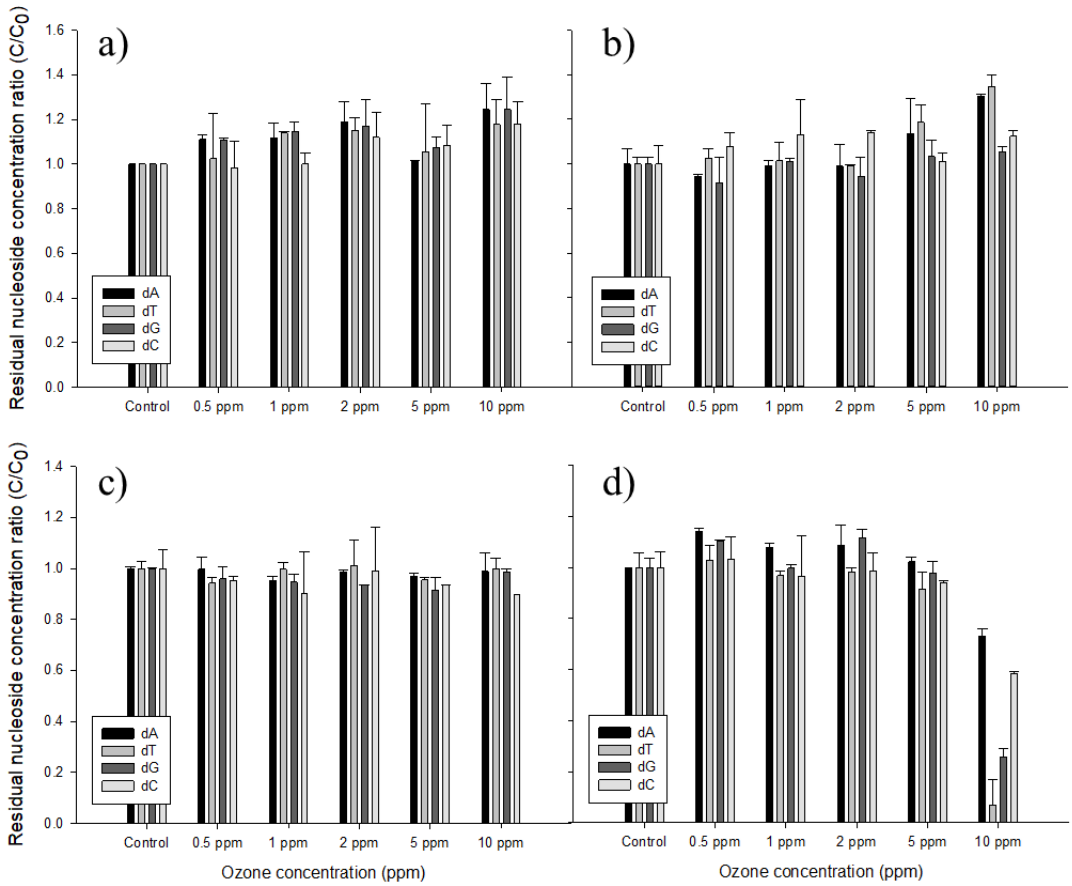

Figure 22. Degradation of nucleotide in fiber segment with ozone disinfection: a) segment 1, b) segment 2, c) segment 3, d) segment 4.

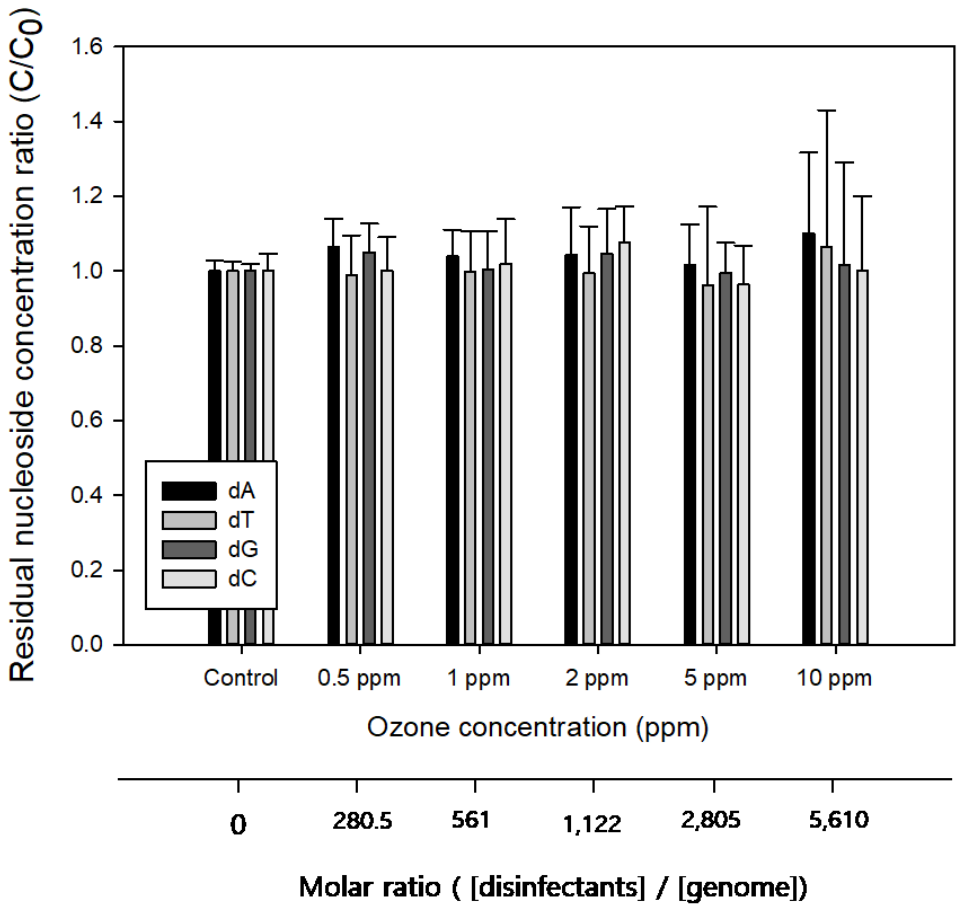

Figure 23. Average degradation of nucleotide in ozone disinfected fiber gene digestion results

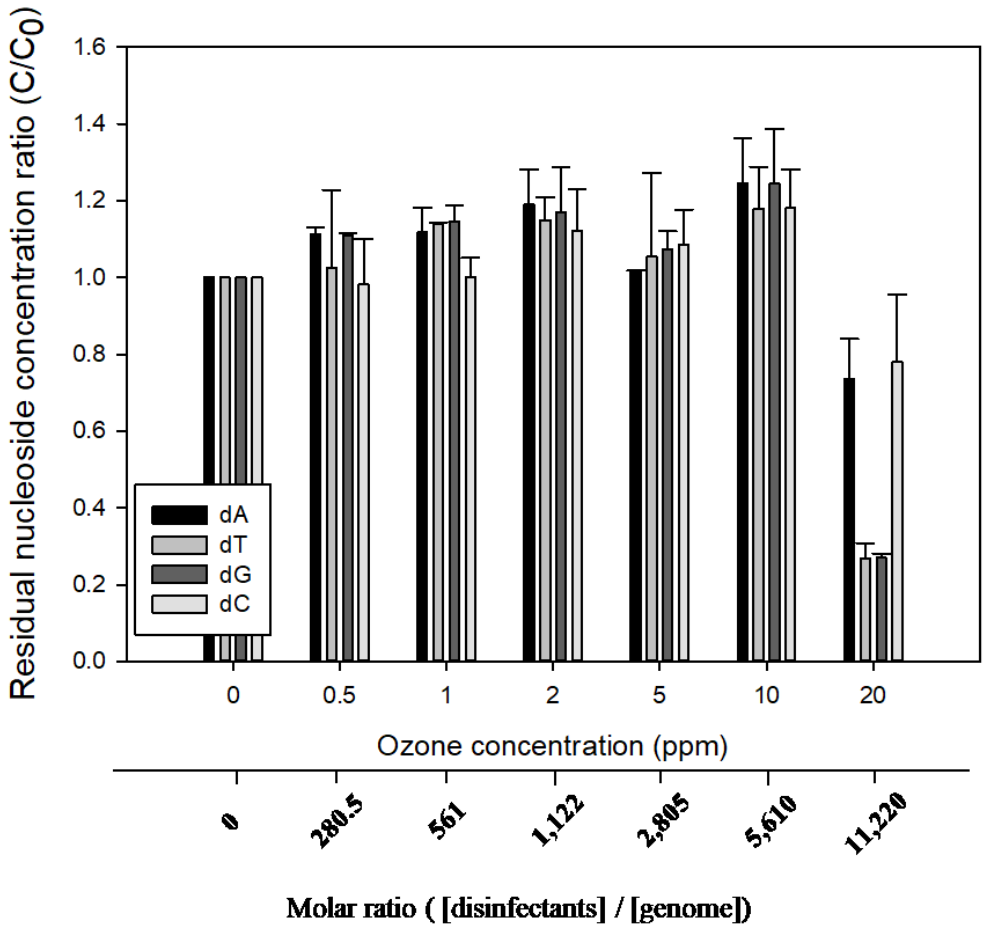

Figure 24. Degradation of nucleotide ozone disinfected fiber gene segment 1

Hexon gene segment 1 and fiber gene segment 1 which were reacted with 20 ppm ozone aqueous solution seemed all nucleosides degraded. Nucleosides in both hexon gene segment 1 and fiber gene segment 1 were degraded. Nucleosides degradation in gene segment was deoxyadenosine $\leq$ deoxycytidine $\ll$ thymidine $\leq$ deoxyguanosine in both hexon gene segment 1 and fiber gene segment 1. These results followed the literature ozone disinfection kinetics with nucleotides.[10]

Figure 25, 26 showed the relationship between genome damage and nucleotide degradation during free chlorine disinfection. As nucleotides distinctly degraded at 50 ppm of free chlorine, genome damage which was quantified by qPCR increased over 2 log degradation in figure 25. Fiber gene segments reacted with free chlorine showed the similar to hexon gene segments. Distinct degradation of nucleotides was shown over 20 ppm of free chlorine. More than 50 ppm of free chlorine damaged fiber gene roughly 3 log degradation.

Fiber gene segment was not reacted with ozone until 10 ppm of ozone disinfection. Both nucleotides degradation and genome damage were not observed until 10 ppm of ozone disinfection except hexon segment 1. However, 20 ppm of ozone disinfection degraded nucleotides specially thymidine and deoxyguanosine. These degradation rates were seemed to correlate with kinetics of ozone with nucleotides shown on Table 2. Genome damage also increased in 20 ppm of ozone disinfection. Genome damage in 20 ppm of ozone showed 1 log degradation.

To sum up the result of viral genome digestion experiments, low

dose of disinfectants(free chlorine, O_3) could not make base modification effectively. Free chlorine over 50 ppm could effectively degraded nucleoside in viral genome but, thymidine was detected even more than 100 ppm of free chlorine reactions. Because of limitation of making high dose of ozone aqueous solutions, only low dose of ozone was reacted with viral genome in this study. There was no significant degradation of nucleoside in viral genome after ozone disinfection.

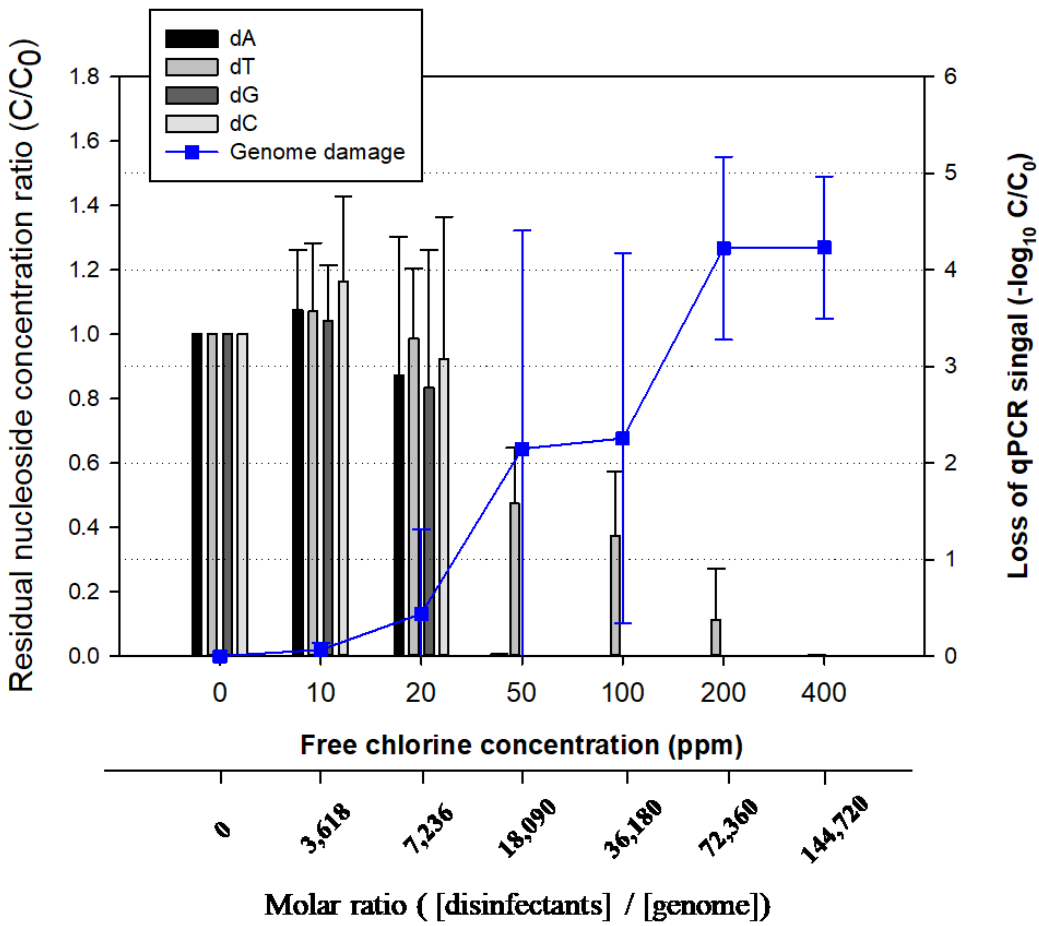

Figure 25. Comparison of hexon gene damage and nucleotide degradation during free chlorine disinfection.

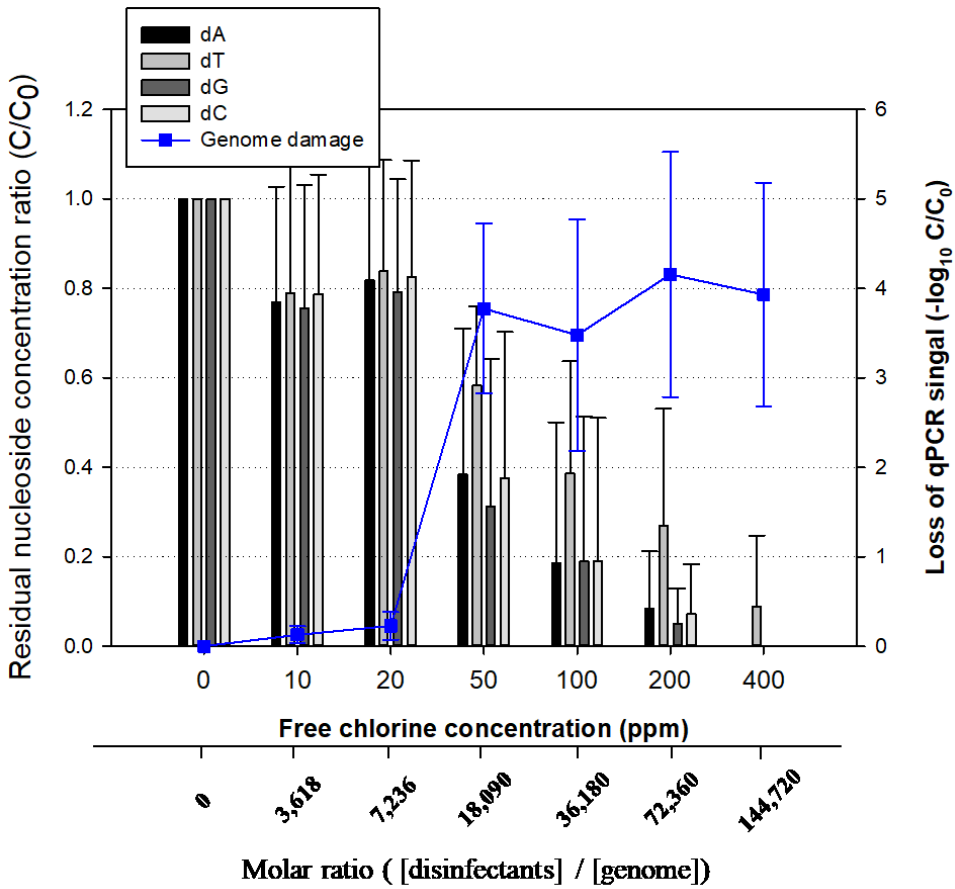

Figure 26. Comparison of fiber gene damage and nucleotide degradation during free chlorine disinfection.

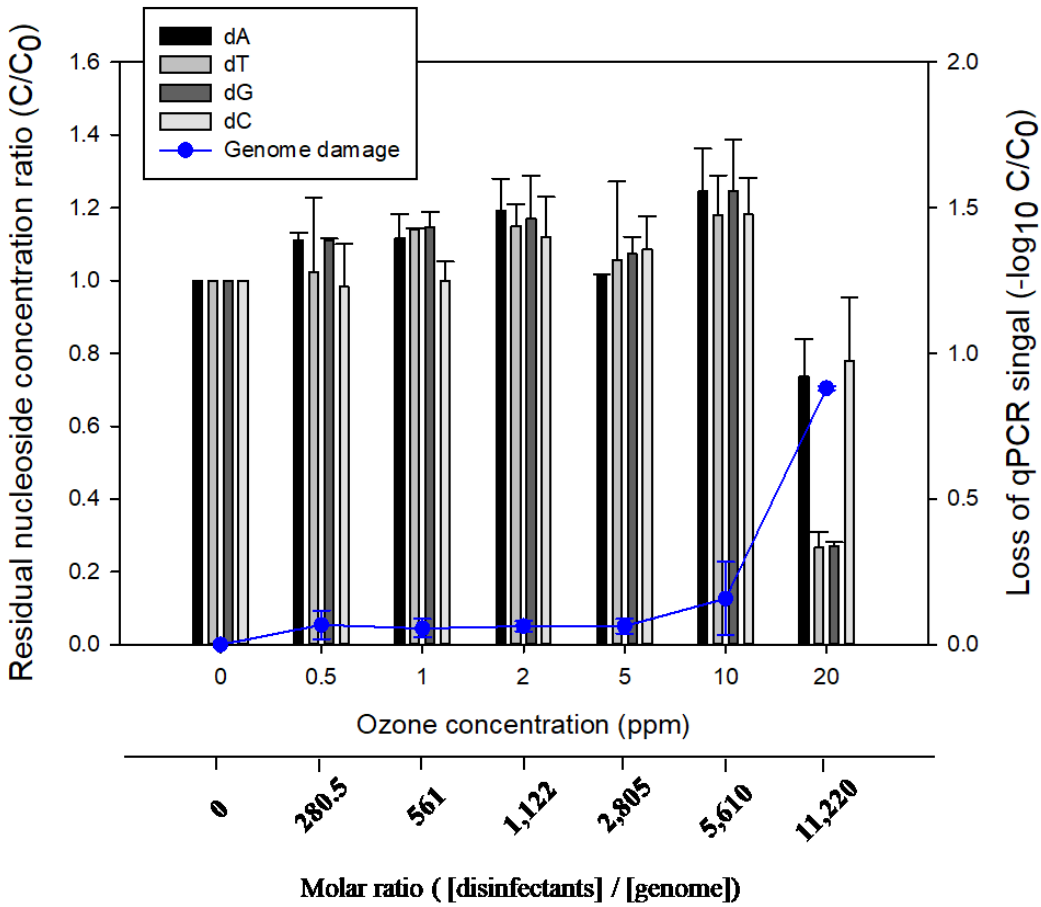

Figure 27. Comparison of fiber gene segment 1 genome damage and nucleotide degradation during ozone disinfection.

Chapter 4. Conclusions

4.1. Conclusions

Uncultured viruses or emerging viruses were not elucidated its genome sequence and properties. As viruses transmitted by many pathways, viruses could be changed by mutation in its genome. Vaccination of viruses was hard work because of these virus mutations. Viral genome inactivation activities should be elucidated to solve virus problem in water treatment. Formal studies (Dennis, et al., 1979, Nuanualsuwan, et al., 2003, Wigginton, et al., 2010)[13-15] elucidated that viral protein was the major target of disinfectants. Impact of modified viral nucleic acids during disinfection was not fully elucidated.

Results from CT values of nucleotides with disinfectants experiment, viral genome disinfection experiment and viral genome digestion experiment suggest several environmental implications.

Firstly, this study focused on reactivity of viral genome to common disinfectants (free chlorine, ozone) from nucleotide to whole viral genome. Kinetics of nucleotide with nucleotides were calculated in this study. Our results were similar to that of other researchers (Prütz, 1996, ISHIZAKI, et al., 1984). However, nucleoside and nucleotide containing thymine base were not reacted with free chlorine. Rate constants of ozone with nucleotides were bigger than rate constants of free chlorine with nucleotides.

Secondly, direct reaction viral genome with disinfectants free chlorine and ozone was done in this research. Viral capsid protein was damaged during disinfection step in water treatment process. But, they also reacted with nucleic acid in virus. In this research, two sites of gene were selected. Hexon protein and fiber protein

were made by transcription and translation in L4, L5 genes. Regions of L4 gene and L5 gene where translated to hexon protein and fiber protein were damaged by disinfectants (free chlorine, O_3). Due to the technical limitations, genome loss signal was not linearly increased as dose of disinfectants increased.

Viral genome had stability for the phosphate backbone and hydrogen bond between complementary bases. Because of the stability of viral DNA, DNA has resistance to disinfectants in low concentrations. As dose increased, phosphate backbone and double strand bond cleavage were occurred by disinfectants and base modifications also occurred. Nucleosides in viral genome were degraded after reaction with disinfectants. Nucleosides were not degraded in early reactions with low dose of disinfectants (FC: 10–20 ppm, O_3: 0.5–10 ppm). As dose increased, nucleosides were degraded until LOQ of HPLC–UV. Thymidine was the most resistant nucleoside in viral genome to free chlorine.

Lastly, this research focused on the nucleotide degradation during genome disinfection. Nucleotide containing thymine base had lowest reactivity with free chlorine. Otherwise, Ozone reacted well with nucleotides including thymidine. Reactivity of nucleotide with disinfectants (FC, O_3) was fairly following the rate constants of single nucleotide with disinfectants. Viral genome had regions that making gene products. Hexon protein and fiber protein were made from L4 gene and L5 gene each. These gene regions had different sequence and properties of nucleotide. Viral genome may have weak regions to disinfectants because of the different nucleotide properties. This work could help to find weak regions or resistant regions to disinfectants of emerging viruses.

In this research, CT values of single nucleotides with free

chlorine were calculated. Viral genome damage by disinfectants (free chlorine, O_3) was measured by qPCR analysis. Damages in nucleotides in viral gene were measured using restriction enzyme. However there were several limitations in this research.

Firstly, kinetics of single nucleotide with ozone disinfection were not measured in this research. It is because reaction volume (2 mL) was limited to measure residual ozone concentration. Also, concentration of ozone aqueous solution was limited due to ozone generator ability to make ozone.

Secondly, there were subtle limitation in qPCR analysis. Viral genome damage analysis has limitation because of SYBR Green fluorescence. SYBR Green fluorescence analysis couldn't discrete double strand break by disinfectants and denaturation in PCR process. Because SYBR green fluorescence was attached between DNA double strand. SYBR green fluorescence also was attached in nonspecific double strand DNA. Sequence specific analysis needed more detailed analysis tool. TaqMan probe analysis could be used for more sequence specific analysis. SYBR Green fluorescence was used because focus on this research was elucidation of the overall viral DNA damage during disinfectants.

In the further research, mechanisms of viral DNA break by disinfectants would require elucidation. In this research, there was limitation to discrete types of DNA damage (double strand break, phosphodiester bond cleavage, base modification). Using ^{15}P isotope analysis in phosphate backbone could be used to monitor phosphodiester bond cleavage. In this research, we identify the single nucleoside degradation by HPLC-UV analysis. However, kinds of disinfected byproducts (modified nucleotides) were not elucidated in this research.

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국문초록

아데노바이러스를 포함한 수인성바이러스는 인간에게 전염되어 폐렴 등의 호흡기 질환을 일으키기도 한다. 바이러스는 적은 개체수에서도 빠르게 전파할 수 있다. 수처리되지 않은 물은 수인성 바이러스 발병의 원인이 되기도 한다. 물 속에 존재하는 바이러스는 수처리 중 소독 과정을 통해 비활성되어 처리된다. 바이러스는 여러 항원형이 존재하기 때문에, 바이러스들의 CT 값은 상이하며 이러한 값은 같은 종의 바이러스에서도 다른 값을 가지기도 한다. 바이러스 소독 기작에 대한 선행연구는 많은 연구자들에 의해 수행되었다. 선행 연구에서는 소독에 의해 바이러스 단백질의 변형이 바이러스 비활성화에 크게 기여했음을 밝혀냈다. 하지만, 소독과정에서 바이러스 유전자의 반응에 대한 연구는 충분히 밝혀지지 않았으며, 그 기작에 대한 연구도 부족하다.

본 연구는 단일 뉴클레오티드와 유리 염소 및 오존과의 반응성 실험을 통해 유리 염소에 대한 CT 값을 구하였다. 유전자의 구성물질인 뉴클레오티드(Adenosine 5'-monophosphate, Thymidine 5'-monophosphate, Guanosine 5'-monophosphate, Cytidine 5'-monophosphate)는 유리 염소에 대해서 각각 다른 CT 값을 가지고 있었다(0.6 to 8.175 $M^{-1}s^{-1}$). 하지만 Thymidine 5'-monophosphate는 유리 염소와 거의 반응하지 않았다. 바이러스 유전자는 종과 항원형에 따라 다른 유전자 서열과 뉴클레오티드 함량을 가지고 있다. 이러한 특성을 통해 바이러스 유전자는 유전 서열 속 특정 지점에서 소독에 취약한 점이 있을 것으로 기대된다. 아데노바이러스 타입2 DNA를 이용하여 유전체 속 특정 유전자(L4, L5)를 선정하여 유전서열 및 뉴클레오티드 함량의 영향에 대해 알아보고자 한다.

본 연구에서 소독을 통한 바이러스 유전자의 손상은 qPCR 분석기법을 통해 정량 하였다. 또한, 소독된 바이러스 유전자는 제한효소를 통해 단일 뉴클레오사이드로 분리되어 HPLC-UV를 통해 각각의 염기를 정량 하였다. 소독에 의한 감소한 유전자 속 염기들은 대체로 뉴클레오티드와 소독제의 반응 속도 상수 값을 따랐다.

본 연구를 통해 소독처리과정에서 사용되는 유리 염소와 오존이 바이러스 유전자를 손상시킬 수 있음을 알 수 있었으며, 염기의 손상 또한 관찰하였다. 바이러스 유전자 손상과 유전자 속 염기의 감소

비교를 통해 유전자 속 염기의 손상이 전체 유전자 손상의 주된 원인이 될 수 있음을 알 수 있었다.

본 연구를 통해 신종 바이러스에 대한 소독 연구에서 서열을 모르는 바이러스 유전자와 소독물질과의 반응성에 대한 예측에 도움이 될 수 있을 것으로 기대한다. 바이러스 유전자 손상과 뉴클레오티드에 대한 손상 기작에 대한 데이터가 충분히 모인다면, 데이터 기반의 미래형 스마트 도시에서의 수처리에도 사용될 것으로 기대한다. 하지만 데이터를 수집하는 데 필요한 비용이 비싸고 유전자 데이터 수집이 실시간으로 이루어지기 어렵다는 한계점이 있다. 또한, 유전자 손상의 기작에 대한 전반적인 연구는 향후 더 필요하며, 유전자 손상과 염기 손상의 상관관계에 대한 연구가 더 필요할 것으로 보인다.

주요어 : 소독처리;염소;오존;DNA;뉴클레오티드

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