The effect of water disinfection on the adaptive evolution of waterborne viruses

Submitted by

Andri Taruna Rachmadi

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Examination committee

Supervisor: Professor Satoshi Okabe (Hokkaido University)

Member: Professor Masahiro Takahashi (Hokkaido University)

Member: Associate Professor Daisuke Sano (Tohoku University)

Water Quality Control Engineering Laboratory

Division of Environmental Engineering

Graduate School of Engineering, Hokkaido University

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ABSTRACT

The scarcity of freshwater resources available for drinking has lead to the use of reclaimed wastewater for non-potable reuse, such as irrigation and recreational purposes. It is important to note that the reuse of reclaimed wastewater must associate with infectious disease risks. There are guideline values for $\log_{10}$ reduction ($\log_{10}$ reduction value: LRV) of pathogens (bacteria, protozoa, and viruses) in wastewater in order to achieve required/advised safety levels for the safe use of reclaimed wastewater, usually determined based on 10^{-6} disability adjusted life years (DALYs) loss per person per year (pppy) as tolerable additional disease burden. The guideline LRV of enteric virus is the highest among water pathogens, because of its relatively high infectivity and concentration in wastewater. Wastewater engineers need to design wastewater reclamation systems to achieve the target LRV of pathogens by summing the achievable LRV in each unit process of wastewater treatment. This means that the achievable LRV of water pathogens in each unit process needs to be determined in advance.

However, previous studies imply that the LRV of enteric virus in each unit process is dependent on the virus type. Since there are more than hundred types of enteric viruses in wastewater, it is necessary to characterize the achievable LRVs of each virus type in wastewater treatment processes. Particularly, the disinfection process needs a special attention, because it has been well known that the susceptibility to water disinfectants vary among virus types. A CT value, a product of disinfection concentration and contact time, shows a required disinfection stress to achieve an LRV of enteric virus, but a list of CT values is lacking for major enteric viruses, which makes it difficult to calculate the achievable LRV of enteric viruses in wastewater disinfection processes.

In order to characterize the disinfection susceptibility of some major enteric viruses, I conducted a meta-analysis of disinfection tests using free chlorine and monochloramine, followed by regression modeling to propose a list of CT values to achieve 2 $\log_{10}$ inactivation. Data were selected from peer-reviewed papers containing kinetics information on virus infectivity and residual chlorine in water. It was found that coxsackievirus and echovirus require higher CT values for 2 $\log_{10}$ inactivation than do adenovirus and human norovirus surrogates (Murine norovirus (MNV)). The influential factors for the required CT value are: virus type, pH, water temperature

and water matrix. The systematic review and meta-analysis reveals that enteroviruses are appropriate as representative among enteric viruses in water to calculate the required CT value to achieve a credit value in the disinfection process using free chlorine, because of their relatively lower susceptibility than that of norovirus and adenovirus.

It is also found in the systematic review and meta-analysis that the susceptibility to disinfectants is dependent on viral genotypes/strains, which let me establish a research question that disinfection-resistant strains can appear because of virus evolution. Enteric viruses, including the human norovirus, have a high genomic diversity due to their high mutation rates, and I tested whether free chlorine, one of the major water disinfectants, acts as selective pressure on norovirus evolution. Using MNV S7-PP3 as a human norovirus surrogate, two independent experiments were performed using ten-time repetitious cycle of free chlorine exposure and propagation in host cells (RAW264.7), followed by a capsid gene analysis using next generation sequencing. As a control, a cycle experiment with 10,000-fold dilution instead of free chlorine exposure, was conducted. The chlorine-treated populations showed lower susceptibility to free chlorine and a higher number of synonymous and nonsynonymous mutations compared to that of the control population. A nonsynonymous mutation at nucleotide (nt) position of 7280, was observed exclusively in the chlorine-treated population in both trials; thus, indicating that a relationship may exist to better survival during the free chlorine exposure. The principal coordinate analysis showed that the chlorine-treated populations at the fifth and tenth cycles were clustered separately from the control populations for the identical cycle numbers, and the original population as well as all of the populations at the first cycle were clustered together. The difference in evolutionary direction of the chlorine-treated populations suggest that the free chlorine treatment is the selection pressure on MNV. In addition, all clones isolated from the chlorine-treated populations with a mutation at nt T7280C[VP2:F200S], had lower susceptibility to free chlorine than the clones from the control population. Thus, suggesting that these mutations increase the stability of the viral capsid from disassembly due to chlorine exposure.

In order to further investigate the effect of free chlorine disinfection on the MNV evolution, I conducted a whole genome analysis of MNV treated with the ten-time repetitious cycle of free chlorine exposure and propagation in host cells. I found that the ORF3 coding the VP2 capsid protein had a highest genetic diversity in the MNV genome. The genetic diversity of ORF3 was

decreased along the ten-time cycle exposure to free chlorine, which implied that the free chlorine treatment was exerted as a selection pressure on this genetic region. The value of dN/dS o ORF3 also supported that the free chlorine exerted a positive selection toward MNV population. In addition to it, a lower evolutionary rate of chlorine-treated population than the control population was observed only in ORF3, which suggested that the positive selective pressure exerted by fee chlorine delayed the evolution process of MNV.

In conclusion, this study shows that the susceptibility to a disinfectant is dependent on viral type/strain, because the susceptibility is determined by evolutionary traits such as the stability of viral capsid proteins. Since it was proved that the free chlorine disinfection can delay the evolution of norovirus, the importance of intensive disinfection of norovirus in wastewater is emphasized to prevent the emergence of new genetic lineage with high pathogenicity.

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ABBREVIATION

AA: amino acid

Ancova: Analyis of covariance

AdV: adenovirus

BDF: buffered demand free

CB: coxsackievirus

CCL: contaminants candidate list

CDF: chlorine demand free

CI: confidence interval

ClO_2: chlorine dioxide

DALY: disability-adjusted life years

EPA: Environmental protection agency

EV: enterovirus

HPD: highest problability value

LRV: Log_{10} removal value

MNV: murine norovirus

NGS: Next generation sequencing

NoV: norovirus

NT: nucleotide

ORF: open reading frame

SD: standard deviation

WHO: World health organization

WWTP: Wastewater treatment plant

Chapter I: INTRODUCTION

1.1 RESEARCH BACKGROUND

Water management, on a global scale, is facing many problems due to the unbalanced distribution and limited resources of fresh water. According to World Health Organization (WHO) data from 2015, there are still 30% of people globally, who do not have access to basic drinking water services. Thus, universal and equitable access to safe and affordable drinking water for all, is prioritized as one of the United Nation (UN) Sustainable Development Goals (SDG). In addition, fresh water scarcity, mainly in climate-bound regional such as North Africa, the Middle East, Southern Europe, Australia and the South in the USA caused a deficit supply of drinking water resource[1,2]. To solve this problem, the desalinization of brackish water and seawater, as well as employing the reclaimed wastewater for other purpose beside drinking has been practiced in many places.

Contaminated drinking water, combined with unimproved and degrading sanitation levels, as well as poor hygiene practices, cause childhood diarrhea which leads to about 4.0% of total deaths, and 5.7% of all disability related and/or health issues in the world[3]. Furthermore, contaminated drinking water is estimated to cause more than 500,000 diarrheal deaths each year[3]. Contaminated water can transmit diseases included diarrhea, cholera, dysentery, typhoid and poliomyelitis which caused by pathogenic microorganisms such as bacteria, protozoa, and enteric viruses. There are possible harmful effects from enteric viruses, (i.e. adenovirus, calicivirus (including norovirus), and enterovirus (including poliovirus, coxsackievirus, and echovirus), if consumed at certain levels in drinking water.

Enteric viruses also pose an infection risk in wastewater reclamation and reuse. Infected individuals typically shed a high number of enteric viruses which can accumulate in the influent of the waste water treatment plant (WWTP); thus, the use of reclaimed wastewater for non-potable reuse must take additional, and necessary precautions. A multiple-barrier concept involving a combination of several steps, (such as: filtration, coagulation, flocculation, biological treatment,

and disinfection), is important to remove the enteric viruses from the WWTP influent[4].v As a guideline, the WHO requires a 6-7 $\log_{10}$ reduction of rotavirus to achieve a 10^{-6} DALY loss per person per year (pppy), as a tolerable additional disease burden for the safe use of wastewater, excreta, and greywater in unrestricted and localized irrigation [5].

Disinfection is typically conducted as the final barrier in the WWTP, before the release of treated wastewater back into the environment; with this process being vital for virus removal. A 3-4 $\log_{10}$ reduction of enteric viruses through disinfectants is necessary after previous removal steps, in order to fulfil the WHO guidelines for using wastewater, excreta, and greywater for water irrigation resources[4]. Sodium hypochlorite (NaClO), ozone (O_3), and peracetic acid (CH_3CO_3H) are used most commonly as disinfection agents for wastewater systems[2]. Of all the disinfection agents, free chlorine is still the most widely used disinfectant, due to the low cost and high efficacy for the inactivation of microorganisms; although, it is limited by the surface contact area if aggregation of pathogens were formed and other environmental inhibitors such as: organic matters, higher pH, and low temperature[6,7]. In addition to free chlorine, other chlorine species, such as monochloramine and chlorine dioxide, are also employed in WWTP. Although monochloramine has a lower biocide effect than free chlorine, it produces less bad odors and secondary byproducts. Comparatively speaking, chlorine dioxide has a higher biocide effect with lower concentrations, but it is more difficult to store and transport due to its gaseous form[6].

The successful inactivation of human enteric virus in a chlorination process depends upon several factors such as: the species of chlorine, contact time, concentration of disinfectant, pH, temperature, water matrix, and viral target [8–10]. The biocide effect of chlorine disinfectants is usually evaluated by comparing the product of chlorine concentration and exposure time (CT) value, for the inactivation of enteric virus in given level (e.g., 2 $\log_{10}$ reduction, or 99% inactivation). The CT concept based on an empiric logarithmic relationship was first reported by Watson (1908), and has been modified by Fair (1948), Hom (1972), Hoff (1986), and Charles Haas (1994). A higher resistance of an enteric virus to chlorine is showed by higher CT values for inactivation.

The governments and field engineers involved with wastewater treatment plants (WWTP) and wastewater reclamation, must take into consideration the chlorine species and CT values needed to achieve targeted $\log_{10}$ reduction values (LRVs) required for safe irrigation and recreational practices. Therefore, in this study, we investigate: the appropriate CT values for safe removal of enteric viruses in the wastewater treatment using free chlorine and monochloramine; the differences in sensitivity of enteric viruses to free chlorine and monochloramine; and how chlorine exposure acts as selective pressure in the norovirus, leading to changes in their evolution process.

1.2 OBJECTIVES

a. To determine the appropriate CT values necessary to obtain sufficient $\log_{10}$ reduction of enteric viruses using free chlorine and monochloramine in water.
b. To investigate the strain dependent free chlorine sensitivity in norovirus.
c. To investigate the impact of free chlorine disinfection on norovirus evolution.

1.3 STRUCTURE OF DISSERTATION

1.3.1 Chapter 1: Introduction

Chapter one summarizes the general background and the research questions addressed in this study. A summary related to each chapter content is provided.

1.3.2 Chapter 2: Required chlorination stress to fulfill the credit value for disinfection of enteric viruses in water

Chapter two provides the information related to CT values needed for appropriate enteric virus inactivation using free chlorine and monochloramine as the disinfectants. Adenovirus, norovirus, and enterovirus inactivation were highlighted as the representatives of enteric virus contaminants in water. A meta-analysis protocol followed by a regression analysis was used to generate the required chlorination stress to fulfill the appropriate value of inactivation of enteric viruses in water. The environmental and biological factors related to the efficacy of free chlorine and monochloramine in enteric virus inactivation are explained. In addition, the variance of CT values, due to the higher resistance of specific enteric viruses compared to others is described in this chapter.

1.3.3 Chapter 3: Strain-dependent susceptibility of norovirus to free chlorine

Strain-dependent susceptibility of enteric viruses to free chlorine and monochloramine was determined in this chapter, a deeper study related to distinct susceptibility among virus populations is discussed in chapter three. MNV as a surrogate of human norovirus is used to investigate the phenomenon of strain-dependent susceptibility to free chlorine. This chapter highlighted MNV populations in the same nonsynonymous mutation at nucleotide number 7280; which had significantly lower susceptibility to free chlorine than those from the control populations without the nonsynonymous mutation. Different evolutionary direction of MNV populations after free chlorine exposure were also demonstrated. Free chlorine disinfection acted as a selection pressure on MNV populations and its impact on evolutionary process is described in this chapter.

1.3.4 Chapter 4: Genetic diversity and rate of evolution for MNV after multiple chlorine exposure

In this chapter, evolution process of MNV due to free chlorine exposure was investigated by the single nucleotide polymorphisms (SNPs) generated after the whole genome of next generated sequencing (NGS), along with all cycles' experiments, and calculations of the genetic diversity and evolutionary rates. Additionally, this chapter highlighted the distinct genetic diversity displayed during repetitious chlorine exposure. In addition, a possibility of the delay of evolution process was highlighted by the lower number of evolutionary rates of chlorine treated MNV populations compared to control populations.

1.3.5 Chapter 5: Conclusions

This chapter summarizes the present study's conclusions and future perspective for the research field of water virology.

Chapter 2: REQUIRED CHLORINATION STRESS TO FULLFILL THE CREDIT VALUE FOR DISINFECTION OF ENTERIC VIRUSES IN WATER

2.1. INTRODUCTION

Fresh water is becoming scarce and unevenly distributed due to several factors such as the concentration of the human population into urbanized areas and global climate change. In response to this, the wastewater reclamation and reuse even for a drinking purpose has been regarded as a promising practice to mitigate the water stress [11,12], although waterborne disease risks caused by pathogens in wastewater need to be properly managed[12]. Particularly, enteric viruses deserve special attention as waterborne disease pathogens because of its high concentration in untreated wastewater [13]. Guidelines for drinking water need to be complied when reclaimed wastewater is used for a drinking water source, and some national and international guidelines urge water engineers to pay attentions to enteric viruses in water. For example, the United States Environmental Protection Agency (USEPA) has included adenovirus, calicivirus, enterovirus and hepatitis A virus in the contaminant candidate list 4 (CCL4) in 2016 as common drinking water microbial contaminants[14]. The guidelines of the World Health Organization (WHO) for drinking water quality (2011) classifies enterovirus, hepatitis A virus, norovirus, and rotavirus as important pathogens with some evidence for high health risks[5].

On the other hand, the WHO guidelines for the safe use of wastewater, excreta and greywater have recommended employ a multiple barrier system for the health risk management in the wastewater reclamation and reuse. In the multiple barrier system, unit processes of wastewater treatment, such as a primary settlement process, a biological treatment process and a disinfection process, are combined to obtain a sufficient total $\log_{10}$ reduction of pathogens/chemicals in water [15]. In order to achieve a health-based target based on the tolerable loss of disability-adjusted life year (DALY) per person per year, a virus reduction of 2-3 $\log_{10}$ and 6-7 $\log_{10}$ is required in the reclaimed water for restricted and unrestricted irrigation sources[16]. In another study, an additional 2-3 $\log_{10}$ reduction of viruses in water is recommended to ensure the safety of recycled water[17].

The final barrier in a wastewater treatment process is usually disinfection. Sodium hypochlorite, ozone, peracetic acid are used most commonly as disinfection agents for wastewater systems[2]. Among available disinfection agents and UV treatment, free chlorine (in sodium hypochlorite form) is still the most widely used disinfectant due to its low cost and efficacy for the inactivation of enteric virus, despite its limited surface contact area in the event that aggregation of enteric viruses were formed and other inhibitions from the environments such as the presence of organic matters[6,7]. The biocide effect of free chlorine is usually expressed with the product of chlorine concentration and exposure time (CT), which represents the amount of concentration and time required for the inactivation of enteric viruses to a given level (for example 2 $\log_{10}$ reduction or 99% inactivation). CT values are expected to differ among enteric viruses since the biocide effect of chlorine species is relative to the target enteric viruses, in which higher resistance strains requires higher CT values.

Until now, there has yet to be any detailed set of guidelines regarding the disinfection efficacy of chlorine species, specifically for each enteric virus listed as a water contaminant. Only partial and proxy information related to a CT value required to inactivate enteric viruses is available. For example, the American Water Works Association (AWWA) (1991) mentioned that CT values of 3, 4, and 6 were required to get 2 $\log_{10,}$ 3 $\log_{10,}$ and 4 $\log_{10}$ virus inactivation in water at 10°C and pH 6.0 – 9.0. WHO drinking water guidelines (2011) stated that a CT value range from 2-30 min.mg/L was needed to obtain 2 $\log_{10}$ reduction of viruses at 0-10°C and pH 7-9. In addition, USEPA guidelines (1986) determined the CT values needed for 2 $\log_{10}$ reduction of poliovirus and rotavirus using free chlorine and monochloramine. Due to various susceptibility of enteric viruses to chlorine and the limitation of available guidelines mentioning a detail CT values for enteric virus inactivation, an in-depth study is needed to determine the appropriate CT value needed for inactivation of all or specific enteric viruses.

This chapter aims to collect all the appropriate literature and furthermore provide an analysis for the appropriate CT value necessary to obtain sufficient $\log_{10}$ reduction of enteric viruses using free-chlorine and monochloramine. A proper CT value for 2 $\log_{10}$ reduction will be derived based on extracted data sets from various studies as calculated with censored and/or standard linear regression model.

2.1. METHODOLOGY

Data collection and selection is following the 2015 PRISMA-P meta-analysis protocols[18]. The primary inactivation data used in this chapter were selected within the criteria of: (1) peer reviewed articles written in English, excluding review papers, dissertations, online documents or reports, and proceedings of a conference or meeting; (2) published within 2000-2018; (3) experimental data obtained from water-related experiments and applicable to real water treatment; (4) containing $\log_{10}$ virus reduction data generated from infectivity test; and (5) including kinetics data, if available. Articles were eliminated if inactivation tests were conducted in food and hard/soft surface materials, different disinfectant agent, and when kinetics calculations of the available data were not possible due to unavailable or not enough information related to EFH model parameter.

Information related to $\log_{10}$ inactivation of enteric viruses by free chlorine and monochloramine were collected through NCBI and google scholar databases until January 2018. The keywords used were "virus type" + "chlorine" + "disinfectant" + "kinetics". Virus type included human norovirus (HuNoV), adenovirus (AdV), echovirus (EV), and coxsackievirus (CB). For human norovirus articles, murine norovirus (MNV) was included as a surrogate viruses due to the unavailability of robust cell lines for human norovirus infectivity assay [18]. Data were extracted by a single reviewer (ATR) and verified by another reviewer (DS). Data extraction included CT value, inactivation data ($\log_{10}$ reduction), virus strain, and environmental parameters (pH, temperature, turbidity) of water matrices used in the experiments. After getting the initial searching results, each paper's title and abstract was cross-checked to ensure it fulfills all the requirements mentioned above. Duplicates found in both databases were also eliminated in the final results.

Three methods were employed to extract the CT and $\log_{10}$ reduction values. First, when the CT values were already calculated using EFH model provided in the selected paper, and no EFH constant was available, then those value from such reference were directly used. Second, when all the constants in the kinetics model were available in a paper or supplemental information,

then a manual calculation to obtain the CT values needed for certain $\log_{10}$ reduction was conducted using EFH model described in the following formula [19]:

$$ln\,(S) = -k'\,C_0^n t^m \left[\frac{1-exp\left(\frac{-n\,k^*\,t}{m}\right)}{\left(\frac{n\,k^*\,t}{m}\right)}\right]^m \quad \text{(eq 2.1)}$$

Where C_0 is the initial concentration of chlorine, k* is the chlorine decay rate (min^{-1}), m is the unity constant, n is the coefficient of dilution (dimensionless), and k' is the viral inactivation rate (dimensionless). Third, for all data available only in graphs, we extracted the CT and $\log_{10}$ points using imageJ software prior to regression modelling.

Prior to regression model construction, extracted CT values and $\log_{10}$ reduction plots from selected literature were combined together within the same virus species. Different genotypes/serotypes of a virus were gathered in the same group and calculated together in the construction of the model, where there were no statistical differences between the constant parameters in the regression model. A simple linear regression was applied as the prediction model. In the presence of limit detection calculation in $\log_{10}$ reduction data, the data were treated as censored data and Tobit regression model was applied. Tobit regression model was used to demonstrate the regression correlation between CT value and $\log_{10}$ reduction of specific viruses. The model was chosen because it is designed to estimate the linear relationship between variables when there is either left or right censoring in the dependent variable [20,21].

In the Tobit and simple regression models, $\log_{10}$ reduction was used as a dependent variable, while CT value, pH, and temperature were the independent variables. As the output, correlation parameters included slope, intercept, and 95% confidence interval range were generated by STATA and R software. For model evaluations, an adjusted R^2 value higher than 0.40 and P-value lower than 0.05 of coefficient slope was set as the minimum requirement. In addition, residuals to fitted values were compared and the adjusted confidence of determination (R^2) was calculated. After the modelling, the CT value for 2 $\log_{10}$ reduction was calculated based on a slope coefficient value in each data set. Calculated CT values were put together with available guidelines in one table for comparison.

Analysis of a covariance (Ancova) test was employed to determine whether regression slope coefficients were significantly different among different strains, pH, and temperature. When no statistical differences were shown, the data sets were combined. All modelling and statistical analysis were conducted using Stata (v.15.0, Tobit command) and R software version 3.3 (http://www.r-project.org).

2.2. RESULTS

2.2.1 Literature selection process.

Articles were obtained up to January 20, 2018. In total, there were 5,958 partly duplicated articles and based on the title, 344 articles were verified for further screening through the employed criteria (Fig. 2.1). Ninety-two articles were chosen after the initial screening (Fig 2.1). Furthermore, 23 papers were selected following full-text screening. In the end, we narrowed down the references to three articles on murine norovirus [22–24], three on echovirus and coxsackievirus each [23,25,26], and six on adenovirus [23,27–31]. Papers selected in this study include chlorine demand free buffer (CDF), and buffered demand free (BDF) as matrices buffers, although some of them are using real treated water to compare the effect of different water sources in CT value [25,32]. In addition, temperature and pH values selected from the literature ranged from 1-22 °C and 6-9, respectively.

2.2.2 Free chlorine and monochloramine inactivation of human norovirus surrogate.

I used murine norovirus as human norovirus surrogates. For murine norovirus free chlorine inactivation, I separated the data into 2 datasets based on different matrices used in the selected literatures (Fig. 2.2). All literatures applied MNV-1 in the experiment. First data set utilized three different treated waters which have TOC content of 1.9 – 18 (mg/L as C) and has residual free chlorine. Based on the matrix condition of the first data set, I speculated that other chlorine species, such as chloramine, were formed due to reaction between free chlorine and ammonia in the treated water [9].

The slope coefficient derived from the first data set was higher (45.25 ± 6.14) compared to that from the second data set (10.62 ± 2.95) applied oxidant demand free buffer as their matrix. Both slope coefficient values were statistically different (Ancova, $Pr(F) = 3.04 \times 10^{-5}$). A slope coefficient of 45.25 and 10.62 means that if there is 0.01 value increase of CT value, there will be a 0.45 and 0.10 increase in $\log_{10}$ reduction, respectively. The coefficient of determination (R^2) and P values in the first and second data sets were 0.41 and 0.54, 0.021 and lower than 0.000 respectively. This means the model is acceptable.

Data for murine norovirus inactivation by monochloramine was separated based on temperatures (5 and 15°C) with a pH range of 7-8 (Fig. 2.2B). Data derived from water samples exposed to UV pretreatment to remove residual chlorine was used for water matrix. CT values were extracted directly from selected literatures that included calculated CT values using EFH model for 2,3, and 4 $\log_{10}$ reduction. Linear regression modelling showed a higher slope coefficient among data collected from experiments conducted at 15ºC (0.028 ± 0.003) compared to those at 5°C (0.012 ± 0.001). Both slope coefficients are statistically different (Ancova, $Pr(>F) = 1.25 \times 10^{-5}$) hence higher temperature accelerates the inactivation process. The adjusted correlation coefficients were 0.69 and 0.68 for the first (15°C) and second (5°C) data sets.

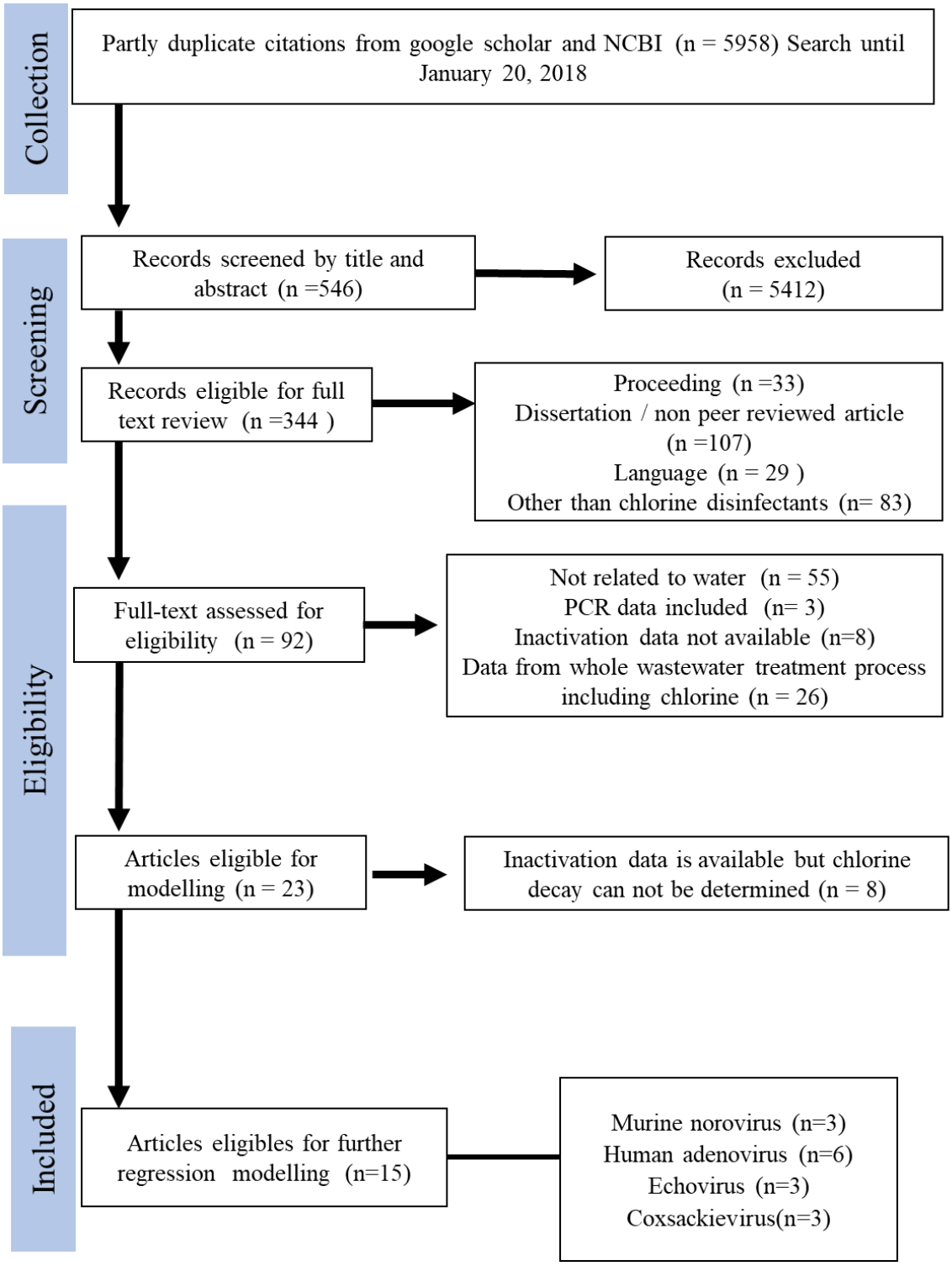

Figure 2.1. Flowchart of the systematic review process used to select the appropriate studies

Residual plots were close to zero, signifying that the slope coefficients of regression models for the first and second data sets were acceptable. The slope coefficient value in monochloramine inactivation is much lower than that in free chlorine treatment, indicating that a longer time of inactivation was needed to achieve the needed $\log_{10}$ reduction.

2.2.3 Free chlorine and monochloramine inactivation of adenovirus.

Experimental conditions applied in the data set for adenovirus (AdV) inactivation with free chlorine consists of four different serotypes (AdV2, AdV5, AdV40, and AdV41), conducted in pH ranges of 6.0-8.5, temperature of 1 and 5°C, and varieties of buffers (demand-free borate buffer, buffered demand free, and chlorine-demand free) as matrices (Fig. 2.3). CT value and $\log_{10}$ reduction were extracted directly from the figures describing those values in the selected literatures. Ancova analysis showed a statistical difference between slope coefficient of AdV40 and AdV2 (pH 6.8-8 and temperature of 1 and 5°C) yet no statistical difference between AdV41 and AdV1 (pH 8.5 in 5°C) with Pr (>F) of 0.0005 and 0.51, respectively. Combined data sets consisting of all strains exhibited slope coefficient of 10.41± 1.22 and an adjusted R^2 of 0.58 (Fig. 2.3).

AdV serotype 5 and 41 was used in the monochloramine inactivation data set. At pH 8.5 and 5 °C, there was no statistical difference between the slope coefficients for both serotypes (Ancova P(>F) = 0.73). The combined inactivation data set for AdV 5 and 41 showed slope coefficients of 0.007 ± 0.0008 with an adjusted R^2 of 0.73 (Fig. 2.3B). Overall, AdV has a lower slope coefficient compared to human norovirus surrogate viruses for both free chlorine and monochloramine inactivation, which indicates that AdV has higher resistance to free chlorine and monochloramine compared to MNV.

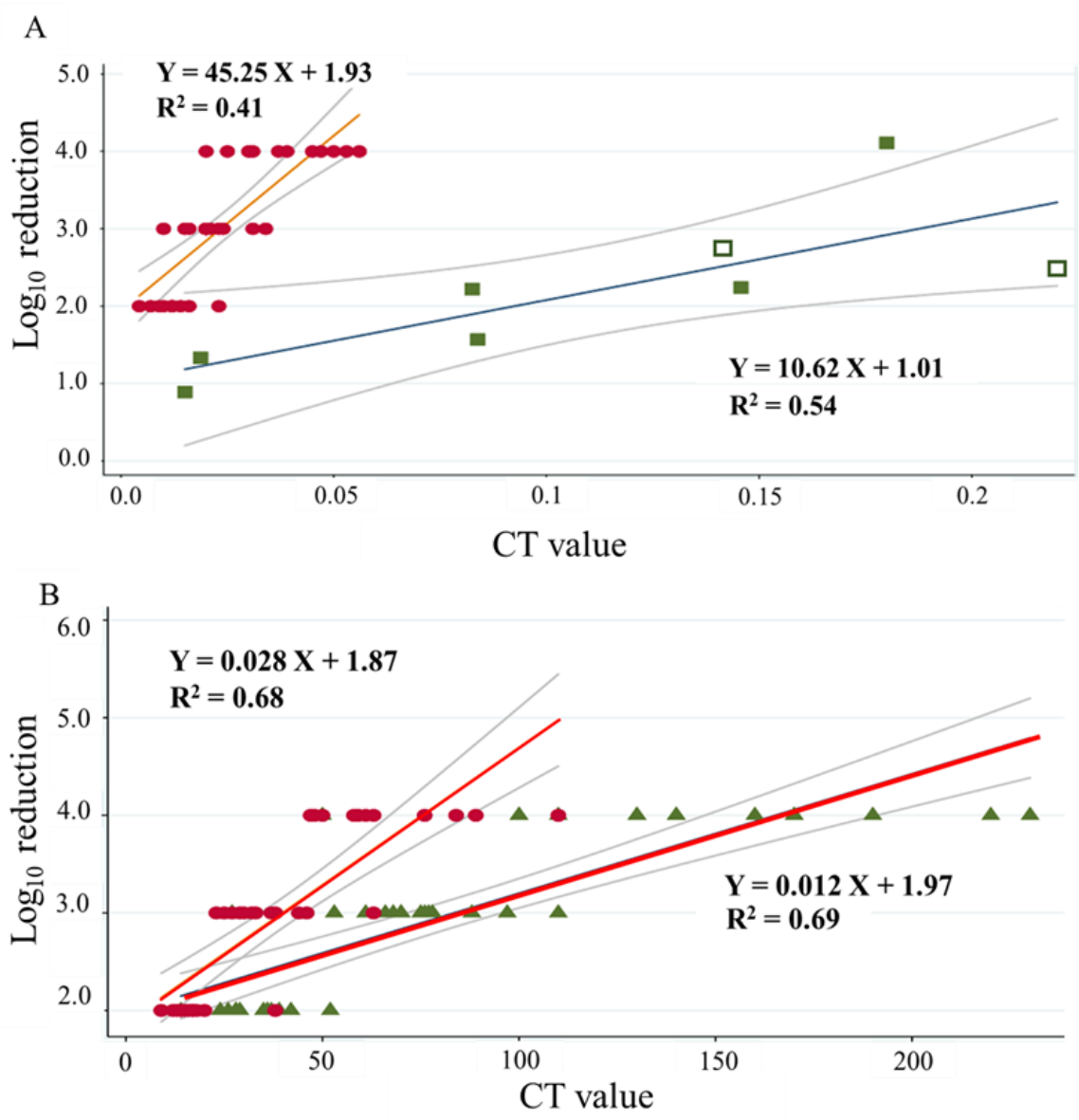

Figure 2.2. (A) Tobit and linear regression model for free chlorine inactivation of murine norovirus. (B) Linear regression model for monochloramine inactivation of murine norovirus.

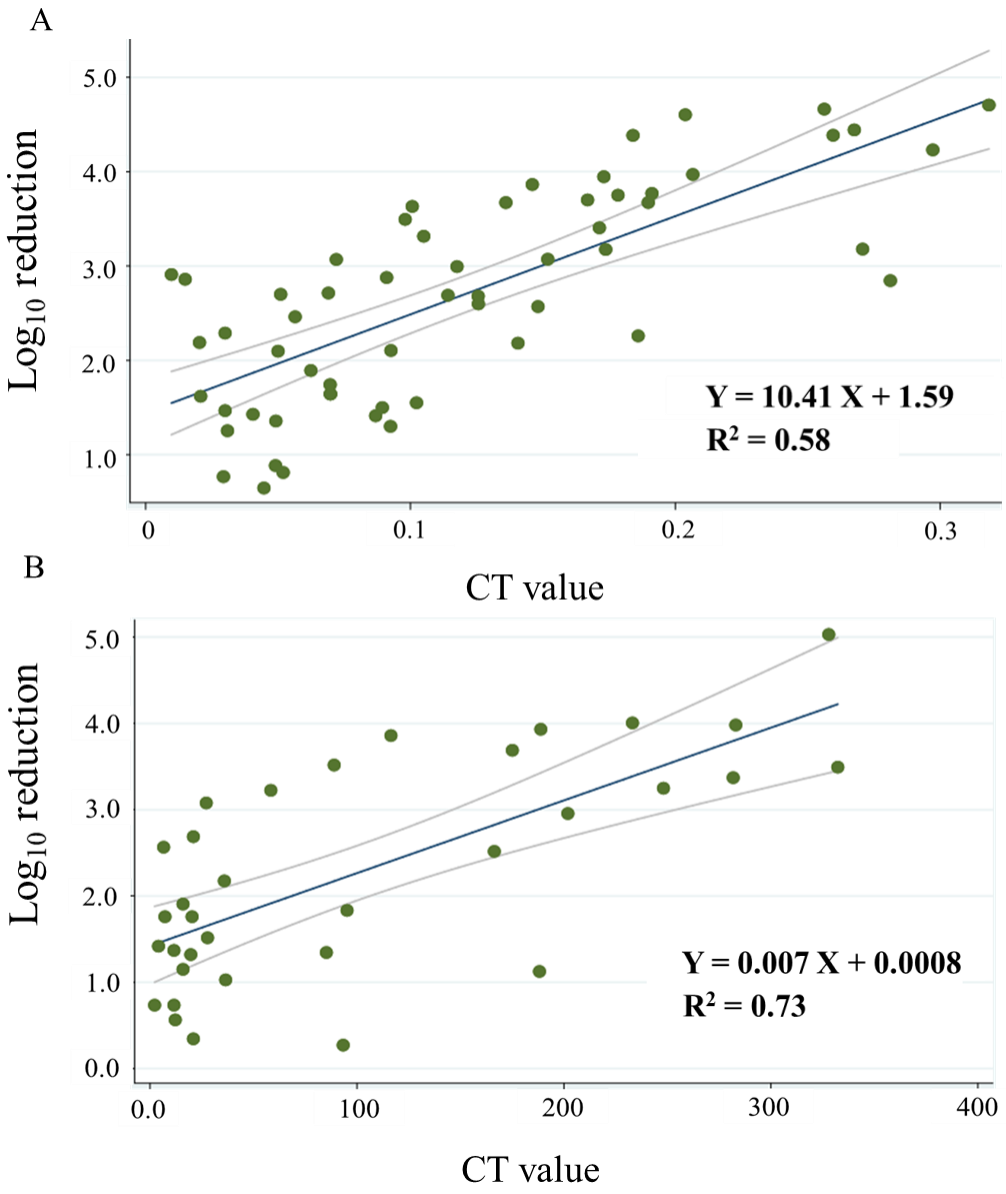

Figure 2.3. Linear regression model for (A) free chlorine and (B) monochloramine inactivation of adenovirus.

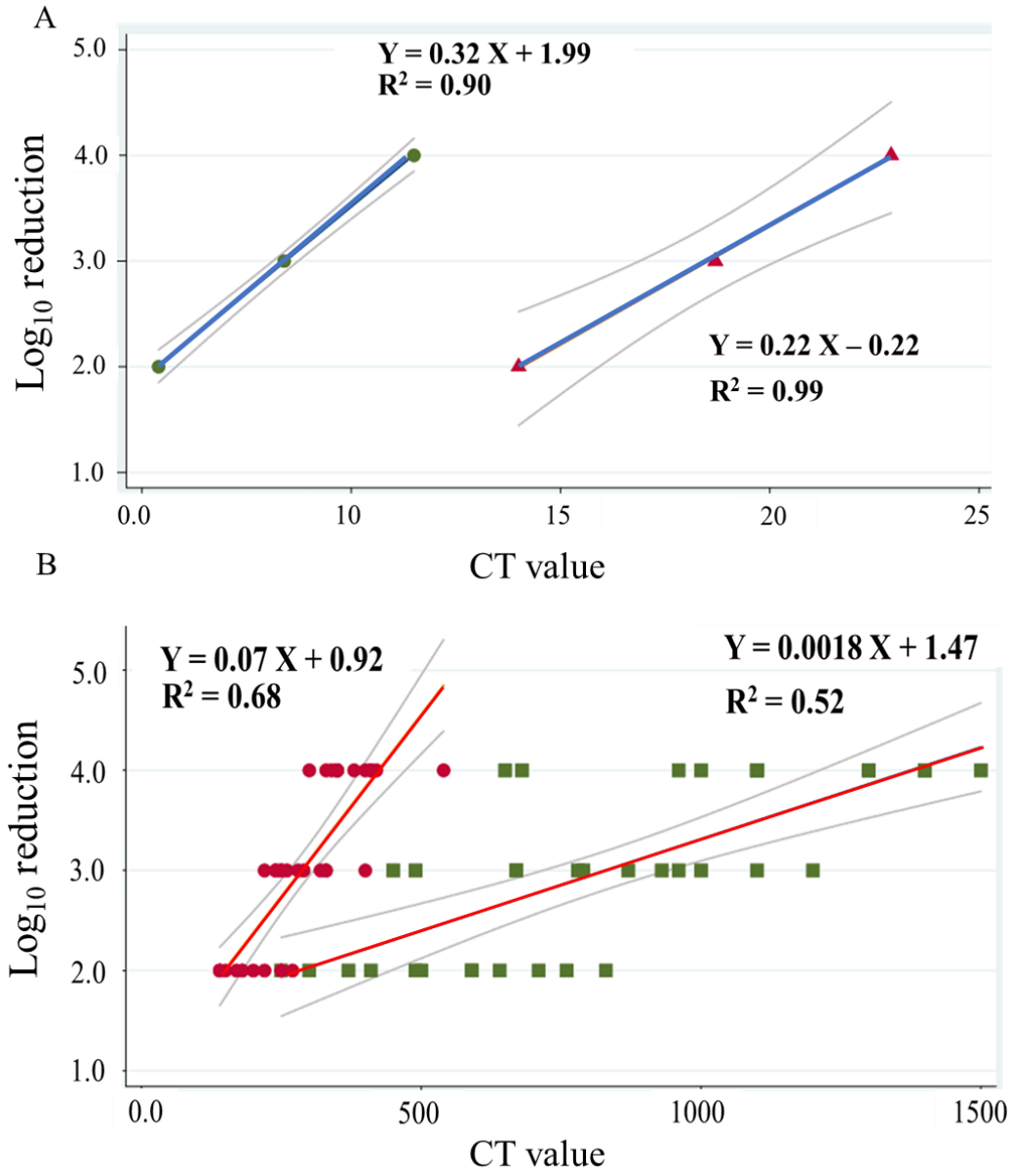

Figure 2.4. Linear regression model for (A) free chlorine and (B) monochloramine inactivation of coxsackievirus.

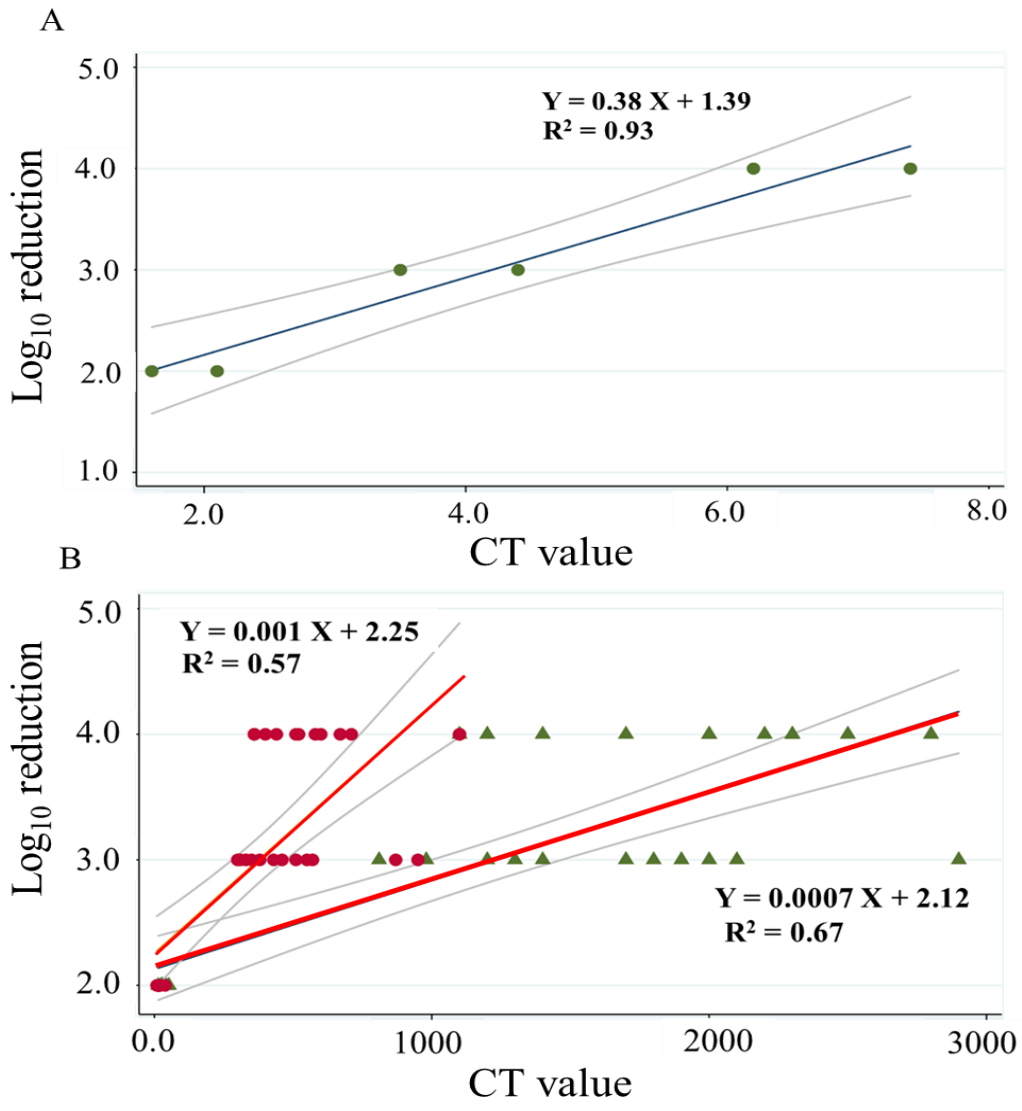

Figure 2.5. Linear regression model for (A) free chlorine and (B) monochloramine inactivation of echovirus.

2.2.4 Free chlorine and monochloramine inactivation of enterovirus.

There was a statistical difference between slope coefficients generated by the linear regression model for echovirus inactivation data set with free chlorine, which applied 2 different pH of 7.5 and 9 (ancova, Pr(>F) = 0.008), temperature of 5°C and BDF buffer as experimental matrix. The slope coefficients were 0.38 ± 0.003 (Fig. 2.5) and 0.22 ± 0.007 for pH of 7.5 and 9, respectively (data of pH 9 were not included in Figure 4 as their P-value was higher than 0.05). The inactivation data set of coxsackievirus exhibited similar slope coefficient value to that of echovirus (0.32 ± 0.003) in a pH of 7.5 in free chlorine inactivation in the same experimental BDF buffer and temperature of 5°C, although a lower coefficient value was shown at pH 9 (0.22 ± 0.007) (Fig. 2.4). Both slopes are statistically different (Ancova, Pr(Pr>F) = 0.008).

Linear regression models for coxsackievirus and echovirus inactivation with monochloramine were created based on two water samples pretreated with UV to remove residual chlorine at 15 and 5°C (Fig. 2.4 and 2.5). In the echovirus inactivation data set (Fig. 2.4), linear regression model implied a higher slope coefficient at 15°C (0.001 ± 0.0002) compared to at 5°C (0.0007 ± 0.00008). The R^2 values of both slope coefficients were 0.57 and 0.67 and they were statistically different (Ancova, P(>F) = 2.43 x 10^{-5}). Linear regression model in coxsackievirus (Fig. 5) showed a slope coefficient value that was higher at 15°C (0.07 ± 0.0008) than at 5°C (0.0018 ± 0.0002), which indicated that the CT value needed for $\log_{10}$ reduction was lower at a higher temperature (Table 2.2). The adjusted R^2 displayed for both linear regression model at 15 and 5°C were 0.68 and 0.52, respectively.

2.2.5 CT value proposed for 2 $\log_{10}$ reduction of enteric viruses.

CT value for 2 log_{10} reduction was calculated by dividing the log_{10} reduction with the slope coefficient values generated by the linear regression model. Two log_{10} reduction was chosen so that the value could be compared to the EPA project summary (Table 2.1). CT values were expressed as values with 95% confidence interval. For 2-log reduction using free chlorine, murine norovirus has the lowest CT value of 0.04 (0.03-0.06), followed by adenovirus 0.20 (0.17-0.21). The highest CT value at pH 6-7.5 and a temperature of 5°C was exhibited by coxsackievirus 6.25 (5.75-6.89), followed by echovirus 5.26 (4.76-5.88) (Table 2.1). Similar trends were observed in the CT value needed for 2 log_{10} reductions using monochloramine, where lowest CT value was shown by murine norovirus with 100 (86-117), followed by adenovirus with 262 (237-293) (Table 2.2). The highest CT value was needed by coxsackievirus, with 1090 (956-1325) and echovirus with 1011 (952-1176) (Table 2.2).

Table 2.1 Proposed CT value for 2 log_{10} reduction of enteric viruses with free chlorine. CT values were calculated based on the slope of Tobit and linear regression models.

Microorganism	Strain	CT value needed for 2-log_{10} reduction (mg.min/L)				Reference
		Free chlorine mean [95% Confidence Interval]	CT Values range from the selected literature	pH	Temp (°C)	
Murine norovirus	MNV-1	**0.19 [0.11-0.56]**	**0.01-0.22**	7-8	5-20	This study
Adenovirus	AdV2,5, 40,41	**0.20 [0.17-0.21]**	**0.02-0.29**	6-8.5	5	This study
Coxsackievirus data 1	CVB5	**6.25 [5.71-6.89]**	**5.41-11.5**	7.5	5	This study
Coxsackievirus data 2	CVB5	**9.09 [8.81-9.38]**	**14.0-22.9**	9	5	This study
Echovirus data 1	E1, E12	**5.26 [4.69-5.98]**	**1.6-17.41**	7.5	5	This study
Escherichia coli	NI**	**0.034-0.05**	**NA***	6-7	5	EPA 1986
Poliovirus 1		**1.1-2.5**	**NA***	6-7	5	EPA 1986
Rotavirus		**0.01-0.05**	**NA***	6-7	5	EPA 1986

*Range of CT values calculated from the selected literatures
**NI = Not indicated

Table 2.2 Proposed CT values for 2 log_{10} reduction of enteric viruses with monochloramine. CT values were calculated based on the slope of Tobit and linear regression models.

Microorganism	Strain	CT value needed for 2-log_{10} reduction (mg.min/L)				Reference
		Monochloramine mean [95% Confidence Interval]	Range of CT values calculated from selected literature	pH	Temp (°C)	
Murine norovirus	MNV-1	**100 [86-117]**	**23-110**	7-8	15	This study
Murine norovirus	MNV-1	**166 [151-185]**	**26-230**	7-8	5	This study
Adenovirus	AdV5,41	**262 [237-293]**	**2-332**	8.5	5	This study
Coxsackievirus data 1	CVB3 CVB5	**277 [249-313]**	**170-540**	7-8	15	This study
Coxsackievirus data 2	CVB3 CVB5	**1090 [956-1325]**	**140-1500**	7-8	5	This study
Echovirus	E1	**1011 [952-1176]**	**170-1500**	7-8	5-15	This study
Escherichia coli	NI**	**95-180**	**NA***	3-9	5	EPA 1986
Polio 1		**768-3740**	**NA***	3-9	5	EPA 1986
Rotavirus		**3806-6476**	**NA***	3-9	5	EPA 1986

* Range of CT values calculated from the selected literatures
**NI = Not indicated

2.3. DISCUSSION

I calculated CT values needed for 2 log_{10} reduction of MNV, AdV, Coxsackievirus, and Echovirus with free chlorine and monochloramine in water. CT values for 2 log_{10} reduction (mean 95% of confidence interval) for free chlorine inactivation are ranging from 0.19 [0.11-0.56] to 9.09 [8.81-9.38] mg x min/L (Table 2.1). As a comparison, EPA project summary of the inactivation of microbial agents by chemical disinfectant in 1986 mentioned CT values ranging from 0.01-0.05 and 1.1-2.5 mg x min/L for 2 log_{10} reduction by free chlorine for rotavirus and poliovirus 1, respectively (Table 2.1). EPA's guidance manual for compliance with the filtration and disinfection requirements for public water sources (AWWA, 1991) suggested that CT values ranging from 1.0 at 25°C to 6.0 mg x min/L at 0.5°C for 2 log_{10} reduction of viruses by free chlorine. On the other hand, WHO guidelines (2008) for drinking water required CT values of 12 and 8 mg x min/L for 2 log_{10} virus inactivation using free chlorine at pH 7-7.5, and temperatures of 0-5 °C and 10°C, respectively. More recent WHO guideline (2011) required 2-30 min x mg/L of CT value to obtain minimum removal of 2-log_{10} reduction for enteric viruses at temperatures of 0-10°C and

pH of 7-9. Our results provided more comprehensive data for higher resistance enteric virus inactivation, which was not included in the EPA project summary and other guidelines but listed in the CCL list. Such information may be added to the existing guidelines.

CT values (mean [95% of confidence interval]) for 2 $\log_{10}$ reduction of enteric viruses using monochloramine are ranging from 100 [86-117] to 1011 [952-1176] (Table 2.2). As a comparison, EPA (1986) required CT values of 768-3740 and 3806-6476 by preformed chloramine for 2 $\log_{10}$ reduction of poliovirus 1 and rotavirus, respectively. Higher values of CT mentioned in the USEPA (1986) guidelines are sufficient to be applied in a real WWTP. In regards to the chlorine species biocide effect in reducing the number of enteric viruses, CT values for virus inactivation using free chlorine was a lot lower compared to monochloramine, indicating that longer time was needed for the same $\log_{10}$ inactivation of enteric viruses. Monochloramine is not as potent as free chlorine in terms of disinfection efficacy although it provides more stable disinfection residual and does not react to form trihalomethanes [33]. WHO guidelines for drinking water (2008) required CT values of monochloramine ranging from 430 to 1240 mg x min/L for 2 $\log_{10}$ reduction of viruses at a pH of 6-9, and a temperature of 1°C and 15°C, respectively. AWWA (1991) showed that CT values for 2 $\log_{10}$ reduction range from 214 at 25 °C to 1,243 at 1°C, which was covered in our calculations.

Environmental factors such as temperature, pH, and matrices also have an impact in higher or lower rates of virus inactivation. CT values for free chlorine inactivation were higher at pH ≥ 8 compared to pH ≤ 7.5 in coxsackievirus. This phenomenon was caused by the speciation of free chlorine at higher pH (≥ 8), which leads to higher percentage of hypochlorite-ion (ClO^-) species compared to hypochlorous acid (HOCl) species[9]. Hypochlorous acid (HOCl) is more reactive by 50-fold than hypochlorite ion ClO^-, so higher CT values for enteric virus inactivation at pH ≥ 8 were expected. CT values at a higher temperature ($\Delta T = 10°C$) were lower compared to at lower temperatures, which suggested that chlorination process was more efficient. Similar results in a previous study also showed chlorine inactivation in other microorganisms[34]. At a higher temperature, the activation energy (the minimum quantity of energy a reacting species should possess to undergo a specific reaction) was higher, which led to an increase of diffusion rate of chlorine in water and more contact with virus particles occurred [8].

Not all virus can be grown in the cell host; thus, inactivation studies using infectivity assay would not be available for all enteric viruses. Although PCR data can be applied, underestimation due to low viral RNA/DNA recovery or overestimation caused by indiscrimination of damage and undamaged viral particle after chlorine inactivation remains a difficult problem to be solved [35]. For that reason, a surrogate is employed as a proxy for the real target. For human norovirus, MNV is commonly used due to its closely related genomes and the availability of the cell hosts for in vitro study[36]. In addition, MNV is more resistant to low pH compared to other surrogates, although it is more sensitive to alcohol[37]. Based on those reasons, MNV was employed as a surrogate for human norovirus in this study.

The linear and Tobit censored regression analyses showed that human norovirus surrogates (MNV) have higher sensitivity to free chlorine and monochloramine compared to adenovirus, coxsackievirus, and echovirus. Enteroviruses, such as coxsackievirus and echovirus, had the highest CT value due to their resistance to chlorine species. The varieties of CT values needed for 2 $\log_{10}$ reduction indicated that free chlorine and monochloramine susceptibility differs among enteric virus types. The reason for various susceptibility of enteric virus to chlorine is still unknown although some propose that the kinetics of the inactivation were relevant to the targeted subcellular components, such as viral DNA, RNA, capsid, and non-structural protein. Therefore, chlorine may disrupt distinct proteins and lead to different mechanisms of viral inactivation [38–40]. The slope coefficients derived from linear regression analyses for AdV2 (serotype C) and AdV40 (serotype F) were statistically different, which stipulated distinct susceptibility among adenovirus serotypes. Previous studies revealed different free chorine susceptibility among laboratory-grown polioviruses strains, which were inactivated more rapidly compared to naturally indigenous polioviruses [41]. In the genogroup level of human norovirus (NoV), NoV GII had higher $\log_{10}$ reduction values to chlorine disinfection in WWTP compared to NoV GIV and GI [42,43]. Another possibility of variance in susceptibility among viral genogroup/serotype/quasispecies is the evolutionary process of viruses with high mutation rates, which may lead to the acquisition of resistance to chlorine. Previous study mentioned that echovirus 11 and bacteriophage MS2 gained resistance to chlorine dioxide following serial exposure and regrowth in the cell hosts [44–46]. A study

related to the evolution process of other enteric viruses after free chlorine exposure is needed in the future.

This study demonstrated the range of CT values required for 2 $\log_{10}$ reduction as generated by Tobit and linear regression models for adenovirus, coxsackievirus, echovirus, and human norovirus surrogates like murine norovirus using free chlorine and monochloramine. It is important for policy makers and engineers to consider the most appropriate disinfection agents and CT values to be applied in the WWTP to keep the public health safety level since enteric viruses are well-known to cause gastroenteritis and some respiratory diseases [47–49]. To obtain 6-7 $\log_{10}$ reduction of enteric viruses as required for the reuse of WWTP effluent as reclaimed water, a disinfection process needs to achieve a performance of 3-4 $\log_{10}$ enteric virus removal if I assume 3 $\log_{10}$ reduction of enteric virus were achieved by the primary and secondary treatments prior to disinfection [4]. In that case, a two magnitude higher of CT values provided in table 2.1 and 2.2 are recommended to attain 4 $\log_{10}$ reduction in disinfection process. Enteric viruses including coxsackievirus and echovirus, should be used as representatives of enteric viruses for chlorination in water due to their higher resistance to chlorine. It is important to employ less susceptible strains in the disinfection tests, as the disinfection rate values obtained from more susceptible strains would be less useful in predicting virus inactivation efficiency of circulating strains under practical disinfection conditions. One last important point, in case of a new findings related to emerging enteric viruses and strains with higher resistance to free chlorine, the efficacy of chlorine species as a disinfection agent has to be renewed and inspected.

Chapter 3: STRAIN- DEPENDENT SUSCEPTIBILITY OF NOROVIRUS TO FREE CHLORINE AND COMBINED CHLORINE

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with the title of "Free-chlorine disinfection as a selection pressure on norovirus"

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ABSTRACT

Human noroviruses are excreted in feces from infected individuals and included in wastewater. It is critical to remove/inactivate them in wastewater treatment processes, particularly in the disinfection step, before release to aquatic environments. However, the high mutation rates of human noroviruses raise concerns about the emergence of strains that are less susceptible to disinfectants and can survive even after wastewater treatment. This study aimed to demonstrate the strain-dependent susceptibility of norovirus to free chlorine. A population originated from the murine norovirus strain S7-PP3, a surrogate for human noroviruses in environmental testing, was exposed to free chlorine and then propagated in a host cell. This cycle of free chlorine exposure followed by propagation in cells was repeated 10 times, and populations with lower susceptibility to free chlorine were obtained from two independent trials of chlorine exposure cycles. Open reading frames 2 (ORF2) and ORF3 of the murine norovirus genome were analyzed by next-generation sequencing, and a unique nonsynonymous mutation (from phenylalanine to serine) at nucleotide (nt) 7280 in ORF3, which encodes the minor capsid protein VP2, was found in chlorine-exposed populations from both trials. It was confirmed that all of the clones from the chlorine-treated population had lower susceptibility to free chlorine than those from the control population. These results indicate that exposure to free chlorine and dilution exert different driving forces to form murine norovirus (MNV) quasispecies, and that there is a selective force to form MNV quasispecies under free chlorine exposure.

3.1. INTRODUCTION

Norovirus, from the family *Caliciviridae,* is a nonenveloped virus with an icosahedral shape and a diameter of 38 nm. The norovirus genome is single-stranded RNA ~7.5 kb in length, with three open reading frames (ORFs) [50]. Norovirus has been divided into five major genogroups, of which genogroups I(GI), GII, and GIV infect humans [51], and GIII and GV infect bovine and mice, respectively [52,53]. Human noroviruses are a major cause of acute nonbacterial gastroenteritis outbreaks worldwide [54,55]. In the United States, outbreaks caused by human norovirus increased from 1% in 1991 to 12% in 2000 [56]. The Centers for Disease Control and Prevention (CDC) reported that approximately 1,753 outbreaks of human norovirus occurred from 2015 to 2016 [57]. In Japan, the National Institute of Infectious Diseases (NIID) reported more than 2,400 outbreaks of human norovirus from 2010 to 2017 [58]. In England and the Netherlands, there were 4.1 and 4.6 cases, respectively, per 100 person-years of human norovirus incidences in the general population during 1993-1996 [59,60]. Human norovirus has accounted for more than 70% of all food-borne pathogens associated with illness and hospitalization in France every year [61]. Furthermore, in African and Asian countries, human norovirus is responsible for 22% of diarrhea-related diseases among infants [62].

Human noroviruses are shed in high numbers (more than 10^8 viruses per gram) from the feces of infected individuals [13] and are relatively stable in the environment for more than two weeks [63]. The presence of human noroviruses in effluent from wastewater treatment plants (WWTPs) poses a health hazard for humans, and thus the removal/inactivation of human noroviruses at WWTPs is critical to reduce the load of this virus to water environments [42,43]. The final barrier in a wastewater treatment process is generally disinfection, and chlorine-based disinfectants are widely used due to the low cost and efficacy for the inactivation of pathogenic microbes [64,65]. From the viewpoint of a multiple-barrier, which is the basic concept for the management of human health risks in water usage, it is necessary to determine a representative value of norovirus inactivation efficiency in each treatment process as a form of the $\log_{10}$ reduction value (LRV) [4]. However, the difference in LRVs among norovirus genogroups has been reported. For example, Hata *et al.* reported mean (± standard deviation (SD)) LRVs of 0.15 (± 0.37), 0.49 (± 0.14), and 0.38 (± 0.72) in a free chlorine treatment for human noroviruses GIV, GII, and GI, respectively [42]. Another study reported mean (± SD) LRVs of 1.65 (± 1.16) and 2.14 (± 0.83) at a

WWTP and 2.57 (± 1.01) and 2.85 (± 0.83) of mean (± SD) LRVs at another WWTP, both of which employ chlorine disinfection as the final treatment process, for noroviruses GI and GII, respectively [43]. These results indicate that the susceptibility of human noroviruses to wastewater treatment processes, including free chlorine treatment, varies among genogroups.

The difference in the removal/inactivation efficiency among genotypes/strains of human noroviruses is expected, because genetically diverse strains can emerge due to a high mutation rate. RNA viruses, including human norovirus, tend to undergo higher genome mutation than do DNA-based microorganisms [66–69], which facilitates the diversification of an RNA viral population consisting of mutant spectra, called quasispecies [70]. The evolution of viral quasispecies may provide human noroviruses greater opportunities to acquire resistance to disinfectants, because high resistance or less susceptible strains can be selected through disinfection treatment and survive in the progeny generation. However, the strain-dependent susceptibility of norovirus to disinfection has not been proved so far.

This study thus aims to obtain the lower susceptibility of norovirus to free chlorine and determine the genetic changes after free chlorine exposure. In order to achieve this goal, a population originating from murine norovirus (MNV) S7-PP3, a surrogate for human noroviruses based on genome similarity and persistency in various conditions (pH, temperature, and disinfectant) compared to other surrogates [71], was exposed to free chlorine and then propagated in cell culture. This cycle of free chlorine exposure followed by propagation in cells was repeated ten times. Then, ORF2 and ORF3 of the murine norovirus genome were analyzed by next-generation sequencing (NGS), and mutations observed in the chlorine-exposed population were compared with the corresponding control (non-chlorine-exposed) populations. Plaque-purified clones containing the unique mutation, observed only in the chlorine-exposed population, were isolated, and the susceptibility to free chlorine, growth rate, and cell binding capability was compared between the mutants and the control clones.

3.2 METHODOLOGY

3.2.1 MNV isolates and cell lines for chlorine cycle experiment.

The MNV S7-PP3 strain, which is genetically close to MNV3 [72], was obtained from Prof. Yukinobu Tohya, Nihon University, Japan. Both MNV S7 and a prototype MNV-1 have been used as foodborne pathogen models for human noroviruses. MNV S7 strains have been widely used Japanese and US researchers as a free chlorine inactivation model [73], in a test for a plant-based antiviral substance [74], and in an investigation of functional receptor for MNV [75]. MNV S7-PP3 was propagated, enumerated, and purified according to the published protocols [76]. Viral stocks were stored in -81 ºC before use. RAW 264.7 cells (ATCC TIB-71) were used as host cells and cultured in modified Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal bovine serum (FBS), 0.075% NaHCO3, 2mM L-glutamine, 10 mM nonessential amino acids, 100 mg/ml penicillin, and 100 U/ml streptomycin. Cells were cultured at 37 ºC and 5% CO_2.

3.2.2 Chlorine cycle experiment.

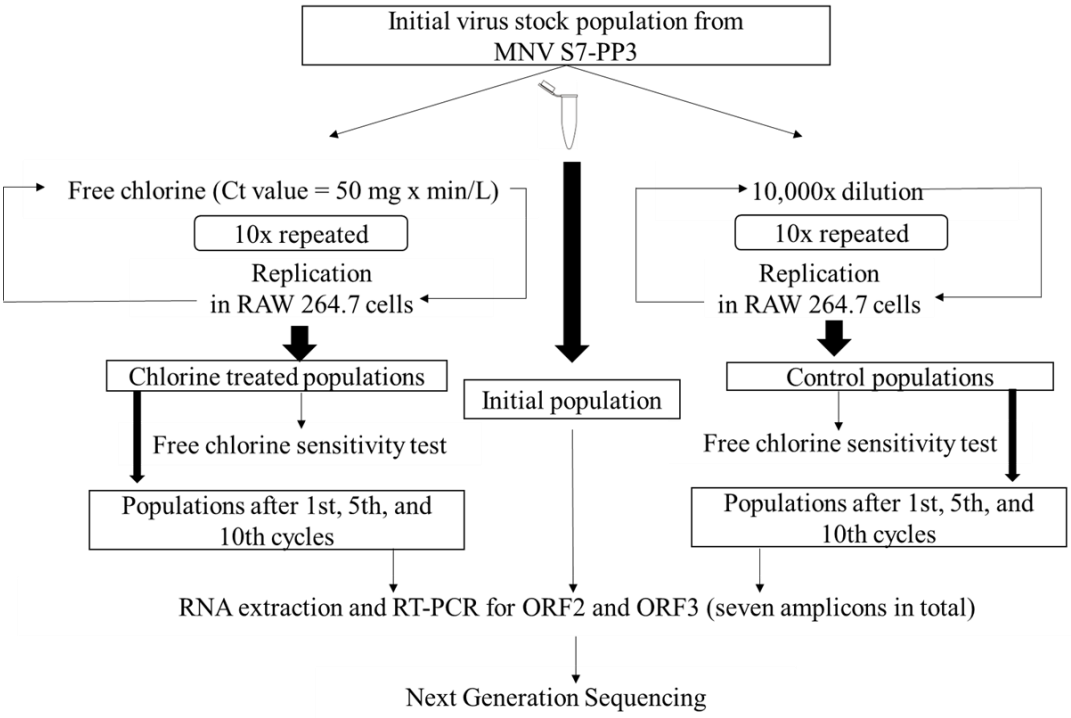

Figure 3.1. Outline of the cycle experiment

To investigate the lowering of susceptibility of norovirus to free chlorine, MNV S7-PP3 was treated by free chlorine repeatedly ten times (Fig. 3.1). This cycle experiment was conducted twice. The free chlorine solution was prepared by mixing sodium hypochlorite solution (Wako, Osaka, Japan) with PBS solution with an initial chlorine concentration of 50 ppm measured using a

chlorine comparator DPD method (Shibata, Saitama, Japan). Surviving virus from the first chlorine cycle was regrown in RAW 264.7 cells and repeated for another nine cycles. As a control treatment, the MNV S7-PP3 population was subjected to repetitious growth in RAW 264.7 cells for ten cycles without chlorine exposure. As a control, the initial MNV population identical with that used for the free chlorine disinfection was diluted 10^4-fold and propagated in RAW 264.7 cells, and this dilution-propagation cycle was repeated ten times to obtain a control population (control population). Chlorine susceptibility for both control and chlorine-treated populations in each cycle was determined by measuring $\log_{10}$ reduction exposure to free chlorine. Due to present of amide (NH_2) compound in DMEM medium, there are a possibility that combine chlorine was formed and involve in murine norovirus inactivation process. Combined chlorine concentration was not measured in this experiment.

3.2.3 Next-generation sequencing (NGS).

To examine genetic changes in MNV S7-PP3 populations due to the free chlorine intervention, next-generation sequencing (NGS) was employed for both the first and second trials of the cycle experiment. The sequencing target was the capsid region of MNV since it is known that free chlorine cause damages to the viral capsid [77]. The MNV S7-PP3 capsid gene (2,252 bp in length) proteins was divided into seven regions (Fig. 3.2). Primers were designed to cover all seven regions with an overlapping sequence (Table 3.1). The original population, the fifth and tenth cycles of the control, and the free chlorine-treated populations were picked for NGS. The process of amplicon preparation involves RNA extraction, reverse transcription (RT) PCR, PCR targeting ORF2 and ORF3, gel electrophoresis to check the length of the PCR products, and PCR product purification from the gel. Reverse transcript (RT) PCR, PCR, and PCR product purification was performed following the PrimeScript Perfect Real-Time RT Reagent (Takara Bio, Shiga, Japan), the Phusion High Fidelity PCR Kit (New England Biolabs, Ipswich, MA), and the Fast Gene Gel/PCR Purification Kit (Nippon Genetics, Tokyo, Japan) manufacturing conditions, respectively. The PCR temperature profile is shown in Table 3.2.

In order to prepare MiSeq libraries for the first trial of the cycle experiment, each PCR product was ligated to different Y type adapters, which consist of i5 (D501 - 507) and i7 (D701 - 707) oligomer [78]. First, 49 types of the Y adapters were prepared by annealing each i5 and i7 oligomer. Annealing mixtures contained one of the i5 oligomers (50uM) 6 µl, one of the i7

oligomers phosphorylated in 5'-end (50uM) 6µl, and an annealing buffer (0.06M Tris-HCl, 0.01M EDTA, 0.025M NaCl) 1.35 µl. The annealing mixture was incubated on a T100TM Thermal Cycler (BioRad, Hercules, CA) with the following steps: 5 min at 95°C, 140 times of 30 s at the degree decreased 0.5°C per each cycle, and 40 min at 25°C, and storage at 12°C.

Table 3.1 List of primers used for sequencing of MNV ORF2 and ORF3

Primer	Sequence (5' – 3')	Location
MNV-ORF2-AN1-F-Pho	[PHO] CACTCCCAGGACATGCTCAG	4831-4850
MNV-ORF2-AN1-R-Pho	[PHO] CCGAGGGCCAGATCAAACAA	5290-5390
MNV-ORF2-AN2-F-Pho	[PHO] CGCCGGGCAAATCAATCAAA	5187-5206
MNV-ORF2-AN2-R-Pho	[PHO] CGGCCAGAGACCACAAAAGA	5647-5666
MNV-ORF2-AN3-F-Pho	[PHO] CTGGTTTGCATGCTGTACACG	5596-5616
MNV-ORF2-AN3-R-Pho	[PHO] GCACCTCGATCTCTAGTTGTCC	6052-6073
MNV-ORF2-AN4-F-Pho	[PHO] GCTTACGAGTTCCAGTCCGG	5935-5954
MNV-ORF2-AN4-R-Pho	[PHO] GTGGAAGGGCACAGTCGATG	6399-6418
MNV-ORF2-AN5-F-Pho	[PHO] TCAGATTGACAGCACTGACGC	6366-6386
MNV-ORF2-AN5-R-Pho	[PHO] CGTTGCAAGCAGGGAAGAATTG	6800-6821
MNV-ORF3-AN1-F-Pho	[PHO] ACTCACCTTCCCGACTGATG	6573-6592
MNV-ORF3-AN1-R-Pho	[PHO] TGTTGATGGCATTCTCCTGGG	6943-6963
MNV-ORF3-AN2-F-Pho	[PHO] TCCAAACCAACTCTTTCAAGCA	6877-6898
MNV-ORF3-AN2-R-Pho	[PHO] CACAAAAGGTTTCTCTTCCAAC	7308-7329

[PHO]: Phosphorylated primer
Location of nucleotide was determined using MNV S7-PP3 references (GenBank access number: AB435515.1

The PCR products were adenylated in 3'-end in 50 µl reaction volume with PCR products 0.15-1.5 pmol, Ex Taq buffer (TaKaRa) 5µl, 25mM $MgCl_2$ (TaKaRa) 3 µl, 10mM dATP (Promega, Madison, WI) 1µl, Ex Taq (TaKaRa) 2.5U, and PCR grade water up to 50µl. An adenylation reaction was performed at 72°C for one hr by a T100TM Thermal Cycler (BioRad). Adenylated products were purified by the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), and DNA concentration was measured by the Quantus™ Fluorometer using the QuantiFluor dsDNA System (Promega). These adenylated products were ligated to a different Y adapter as soon as possible after adenylation. The reaction mixture consisted of 50µl with ligation buffer (TaKaRa)

5μl, T4 DNA ligase (TaKaRa) 1μl, each adenylated product with 40μl, one of the Y-type adapters 100 times the mol amount of the adenylated product, and PCR grade water up to 50μl.

Table 3.2 Temperature profile of PCR for each seven regions from MNV ORF2 and ORF3

Region	Temperature (°C)	Time	Cycle number
1	94	2 min	1
	94 66 72	30 s 45 s 45 s	20
	72	7 min	1
	4	∞	1
2	94	2 min	1
	94 68 72	30 s 45 s 45 s	20
	72	7 min	1
	4	∞	1
3	94	2 min	1
	94 67 72	30 s 45 s 45 s	20
	72	7 min	1
	4	∞	1
4	94	2 min	1
	94 66 72	30 s 45 s 45 s	20
	72	7 min	1
	4	∞	1
5	94	2 min	1
	94 70 72	30 s 45 s 45 s	20
	72	7 min	1
	4	∞	1
6	94	2 min	1
	94 64 72	30 s 45 s 45 s	20
	72	7 min	1
	4	∞	1
7	94	2 min	1
	94 64 72	30 s 45 s 45 s	20
	72	7 min	1
	4	∞	1

Ligation reactions were performed 19 hrs at 16°C and 65°C 2 min by a T100TM Thermal Cycler (BioRad). Ligation products were purified by the Agencourt AmpureXP (Beckman Coulter, Brea, CA), and DNA concentration was measured by the Quantus™ Fluorometer using the QuantiFluor dsDNA System (Promega).

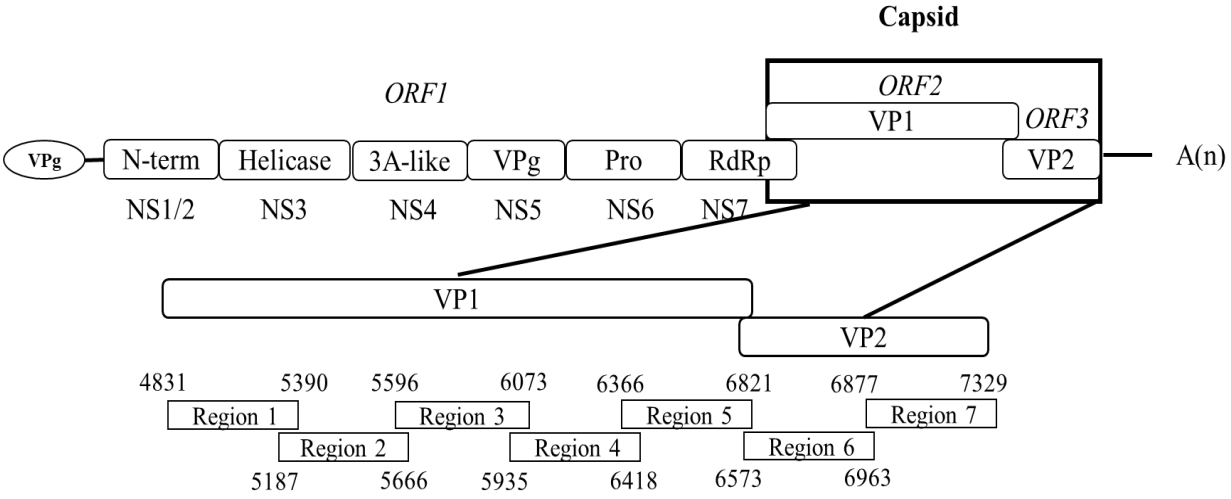

Figure 3.2. Next generation sequencing targets in murine norovirus (MNV S7-PP3, accession number: AB435515.1) capsid gen, which consists of ORF2 and ORF3 encoding VP1 and VP2, respectively

To enrich the DNA libraries, a second PCR was performed in a 10 µl reaction mixture with Protocol Phusion® High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs) 5 µl, 10µM KAPA illumina primer P1 (5'-AAT GAT ACG GCG ACC ACC GA-3') 0.5 µl, 10µM illumina primer P2 (5'-CAA GCA GAA GAC GGC ATA CGA-3') 0.5 µl, each ligated DNA 3 µl, and PCR-grade water up to 10 µl. PCR was performed under the following temperature profile: 30 s at 94°C, 15 cycles of 10 s at 94°C, 30 s at 60°C, 1 min at 72°C, a final extension step of 5 min at 72°C, and storage at 4°C. PCR products were purified using the Agencourt AmpureXP (Beckman Coulter).

A quality check for all libraries was performed by the Bioanalyzer (Agilent) using the Agilent High Sensitivity DNA Kit (Agilent). To quantitate the concentration of the library DNA, real-time PCR was performed in a 10 µl reaction volume with KAPA SYBR FAST q PCR Master Mix (KAPA) 5µl, 10µM illumina P1 primer 0.2µl, 10µM illumina P2 primer 0.2µl, 1000 times diluted library DNA or DNA quantitative standards (KAPA) 2µl, and PCR-grade water 2.6µl.

Each quantification was triplicated. PCR cycle conditions followed the protocol of the KAPA Library Quantification Kits. Forty-nine libraries were pooled in equal molar amounts. Because the concentration of the pooled library was too low to run MiSeq, this pooled library was concentrated using the Agencourt AmpureXP (Beckman Coulter). The pooled library was again quantified according to the same conditions of the real-time PCR and was adjusted to 2nM. Thirty percent Phix control (illumina) was spiked into the library. Finally, 600 µl of 6 pM denatured library and 300bp pared-end sequencing was carried out by the Advanced Research Support Center at Ehime University using the MiSeq (illumina).

I used a library preparation kit in the second trial, which was not used in the 1st trial. The kit saved a lot of times in the library preparation. The amplicon library was prepared using the TruSeq DNA PCR-Free LT Library Preparation Kit (Illumina, Inc., Catalog No. FC-121-3001 and FC-121-3003) on PCR products amplified with phosphorylated primers. Steps from the kit's protocol guide were followed from the adenylation of the 3' ends to the adapter ligation, with some modifications. The 49 amplicon libraries were then qualified to verify fragment size by checking the library size distribution via the Agilent 2100 BioAnalyzer using the High Sensitivity DNA chip. Quantification of the libraries was performed via qPCR using the KAPA Library Quantification Kits for Illumina sequencing platforms (Kapa Biosystems). Prior to sequencing, final amplicon libraries were normalized to 2 nM and pooled. Finally, 600 µl of a 6 pM denatured pooled library with PhiX (30% final concentration) (Illumina, Inc.) was prepared, and a 300 bp paired-end sequencing was performed using the MiSeq Reagent Kit v3 (600-cycle) (Illumina, Inc.) according to the manufacturer's instructions. Sequencing was carried out at the Division of Analytical Bio-Medicine, Advanced Research Support Center (ADRES), Ehime University (Toon, Ehime Prefecture, Japan). Read data of the pooled libraries were demultiplexed using the on-board MiSEq Reporter software of the sequencing platform.

NGS data were analyzed using CLC Genomics Workbench and Galaxy (https://usegalaxy.org/) platform. The analysis included quality control of contigs, forward and reverse contigs merging, adapter trimming, mapping into a reference (MNV S7-PP3), and creating the consensus sequences. Reads with lower than 100 sequences were trimmed. The median of the score of reads of any base quality should be more than 20, measured using FastQC software in Galaxy platform.

After the consensus sequences were generated, alignment between the consensus sequences in the same region was conducted using MEGA 7 software. In addition to the observation of the other SNPs including minority sequences and their percentage in NGS reads, IGV (Integrative Genomics Viewer) software were used. A heat map representing the ratio of the percentage of SNPs to the percentage of non-mutated nucleotide in original population sequences in each cycle was determined using R software with a gene scale command available in the bioconductor package (https://www.bioconductor.org/install/). The Z score for the heat map was calculated by the following formula:

$$Z = \frac{(x - mean)}{sd} \quad \text{(equation 2)}$$

where x is ratio of SNP's percentages compared to the reference sequence in a certain cycle (cycles 1, 5 and 10). The mean value and standard deviation (SD) were calculated from x in all cycles separately per region. In addition, to characterize the evolutionary pattern of genetic divergence among populations with different treatments and treatment cycles, principal coordinate analysis (PCoA) was conducted using CLC software [79]. As the input distance matrix, Bray-Curtis dissimilarity was calculated on the relative abundance (i.e., reads) of haplotypes in each population [80].

3.2.4 Free chlorine susceptibility of plaque purified clones.

In order to examine whether SNPs found in the capsid region are associated with the lower susceptibility of chlorine, a chlorine sensitivity test was carried out for clones isolated from both the control and chlorine-treated populations. The chlorine-treated and control populations of MNV S7-PP3 were inoculated to a confluent RAW 264.7 cells in a 6- well plate with a multiplicity of infection (MOI) of 0.1. After 2-3 days of incubation, only clean and dispersed plaques were chosen for isolation. Plaques were picked using 1,000 µL of barrier tips cut by a sterilized cutter to obtain a clear, round shape. Six plaques were picked from each chlorine-treated and control population, and mixed with DMEM medium, and incubated in 37ºC for 90 min before being regrown in a new confluent of RAW 264.7 cells in 75cm^2 flasks containing 15 ml of DMEM. The first plaque purified MNV clones was subjected to a second round of plaque purification. Each clone (12 in total, six each from the chlorine-treated and control populations) was confirmed to have the mutation at nt 7280. After the clones were isolated, they were stored in a -80 ºC freezer before

conducting a free chlorine sensitivity test. The free chlorine sensitivity test was conducted using chlorine demand-free glassware, which was prepared by soaking overnight in a high concentration of free chlorine solution (>75 mg/L). After soaking, the glassware was rinsed and baked at 120 ºC overnight and then thoroughly washed and sterilized by autoclave. The chlorine sensitivity test was conducted using 9:1 of composition of PBS and DMEM with FBS. Free chlorine concentration was measured three times with the USEPA DPD method using HACH DR 900 equipment at time 0 and after 5 min contact time. The chlorine decay time was determined according to the following formula:

$C_t = C_0 \exp(-k^* t)$ (equation 3),

where *Ct* is the chlorine concentration at specific sampling times, C_0 is the initial concentration of chlorine, k^* is the chlorine decay rate (min^{-1}), and *t* is the contact time (0, 0.5, 1, 2, 3, 5 min). A preliminary chlorine decay experiment showed that the free chlorine (initial concentration: 2 ppm) in the PBS solution was fully consumed in 1 min. The chlorine decay rate (k^*) calculated using the equation 3 was 1.18 (min^{-1}). In the chlorine sensitivity assay, an X clone (from the control population) was paired with an A clone (from the chlorine treated population), and nine pairs were selected randomly. Then, the $\log_{10}$ reduction ratio after free chlorine exposure with an initial concentration of 2 ppm and 1 min contact time was obtained three times for each pair. The free chlorine solution was made by mixing 50 µL of sodium hypochlorite stock solution (Wako, Osaka, Japan) with 100 ml PBS. After mixing by shaking for 10-15 sec, 9 ml of the mixture was transferred to small tube and 1 ml of clone suspension was added and mixed for 1 min. After 1 min, 10 µL of sodium thiosulphate solution 1% (v/v) was added to neutralize the chlorine. The original concentration of plaque-purified clones before and after chlorine treatment was measured by plaque assay.

3.2.5 Replicative fitness of plaque-purified clones.

Replicative fitness was determined by comparing the growth rate between plaque-purified clones isolated from control and chlorine-treated populations. Low MOI (0.002-0.005) was applied for infection to the RAW 264.7 cells. Confluent RAW 264.7 cells in a $75cm^2$ flask were rinsed with PBS twice and then infected with the targeting plaque-purified clones. After 90-min incubation at 37°C, the flasks were rinsed with DMEM to remove the unattached virus particles.

Virus samples were taken at 6, 12, 24, 36, and 48 hr to obtain the growth curve [81]. The growth rate was fitted from the lag phase of virus growth, and an exponential fitting model was used [82]:

$Y = e^{\mu t}$, so $\ln(Y) = \mu t$ (equation 4),

where Y is the virus yield, μ is the growth rate, and t is the cultivation time. To compare the relative replicative fitness of the different clones, selection coefficient (s) was calculated using the following formula [44]:

$$s = 1 - \mu/\mu_{\_WT} \quad \text{(equation 5)},$$

where μ and μ_WT are the log phase growth rates of the evolved clones (plaque- purified clones isolated from the chlorine-treated population) and wild-type clones (plaque- purified clones isolated from the control population), respectively. The s value less than zero means that the evolved clones has a superior replicative fitness.

3.2.6 Binding efficiency to cell surface.

A binding test was carried out according to a previous study with modifications [74]. Briefly, RAW 264.7 cells were rinsed twice using PBS (-) to remove residual DMEM before the infection process. Virus was inoculated to the cells with MOI of 0.1 and incubated at 4°C for 90 min to allow the attachment of virus particles while preventing initiation of the replication cycle at low temperatures. Gentle rocking every 10-15 min was employed during the incubation period to prevent the cells from drying. RNA extraction was conducted as soon as incubation was complete. To remove unbound virus particles, the plates were rinsed three times with PBS (-). After rinsing, the cells were scraped using a cell scraper, and then a 150 µl of mixture of cells and attached virus was subjected to viral RNA extraction with the QIAmp VIRAL RNA Kita (QIAGEN). qPCR for MNV was employed to determine the copy numbers of viral RNA before and after the cell binding experiment [83].

3.2.7 Statistical analyses.

All statistical analyses were performed using R software version 3.3 (http://www.r-project.org). All data sets were assumed as not normally distributed. To examine if the ratio of $\log_{10}$ reduction between clones isolated from the control population (X1, X2, X3) and the chlorine-

treated population (A1, A2, A3) is lower than 1 (null hypothesis), the Wilcoxon signed rank test was employed. To determine if there was no statistical difference between the growth rate and the doubling time of each chlorine-treated and control clone, the Kruskal-Wallis test was conducted with a significance level of $\alpha = 0.05$. To check the statistical difference between the selection coefficient (s) among clone combinations (A1 vs X1, A1 vs X2, A1 vs X3, A2 vs X1, A2 vs X2, A2 vs X3, A3 vs X1, A3 vs X2, A3 vs X3), the Kruskal-Wallis test was performed. This test examined whether replicative fitness is statistically higher than 0. The ratio of bound viral particle per initial spiked virus between isolated clones (A1, A2, A3, X1, X2, X3) was tested using the Wilcoxon rank sum test.

3.3 RESULTS

3.3.1 Acquisition of less susceptible MNV populations to free chlorine.

MNV populations derived from the S7-PP3 strain were repeatedly exposed to free chlorine as shown in Fig. 3.1. The cycle of free chlorine exposure followed by propagation in RAW 264.7 cells was repeated ten times (chlorine-treated population). The repeated exposure experiment was conducted twice independently to examine the reproducibility. As a control, the initial MNV population identical with that used for the free chlorine disinfection was diluted 10^4-fold and propagated in RAW 264.7 cells, and this dilution-propagation cycle was repeated ten times (control population). The strength of free chlorine disinfection in each cycle was expressed as the product of an initial free chlorine concentration of 50 ppm. This initial concentration level reduced the infectious titer of MNV by 4.11 (± 0.14)-log (n = 3) and 3.21 (± 0.15)-log (n = 3) at the beginning of the first and second trials, respectively (Fig. 3.3). At each cycle after the growth in RAW 264.7 cells, the susceptibility to free chlorine was tested for both the chlorine-treated and control populations (Fig. 3.3). There was no significant difference in the $\log_{10}$ reduction value (LRV) in the first four cycles in the first trial of cycle experiments (Fig. 3.3(A)) and two cycles in the second trial (Fig. 3.3(B)). However, the susceptibility of chlorine-treated populations to free chlorine became gradually lower compared to the control populations, and the difference in LRVs between chlorine-treated and control populations reached to 0.71 log in the first trial and 2.74 log in the second trial. These results demonstrate that MNV populations with relatively lower susceptibility to free chlorine were reproducibly obtained by the repeated exposure to free chlorine.

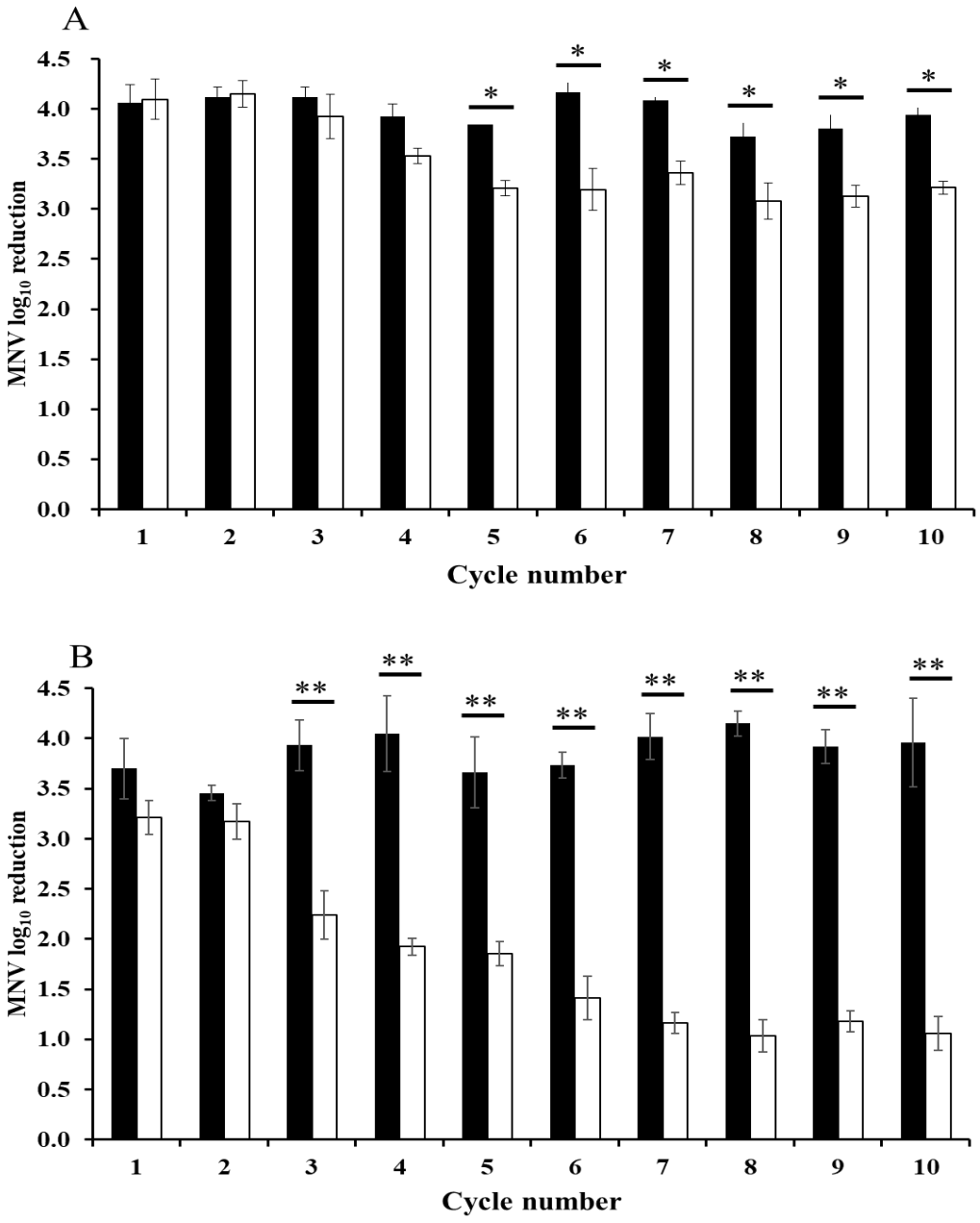

Figure 3.3. Log10 reduction of chlorine-treated population (white bar) and control population (black bar) during 1st trial (A) and 2nd (B) trial of cycle experiments. *, statistically different at $\alpha < 0.05$ (Wilcoxon signed-rank test); **, statistically different at $\alpha < 0.01$ (Wilcoxon signed-rank test).

3.3.2 Single nucleotide polymorphisms (SNPs) in MNV populations.

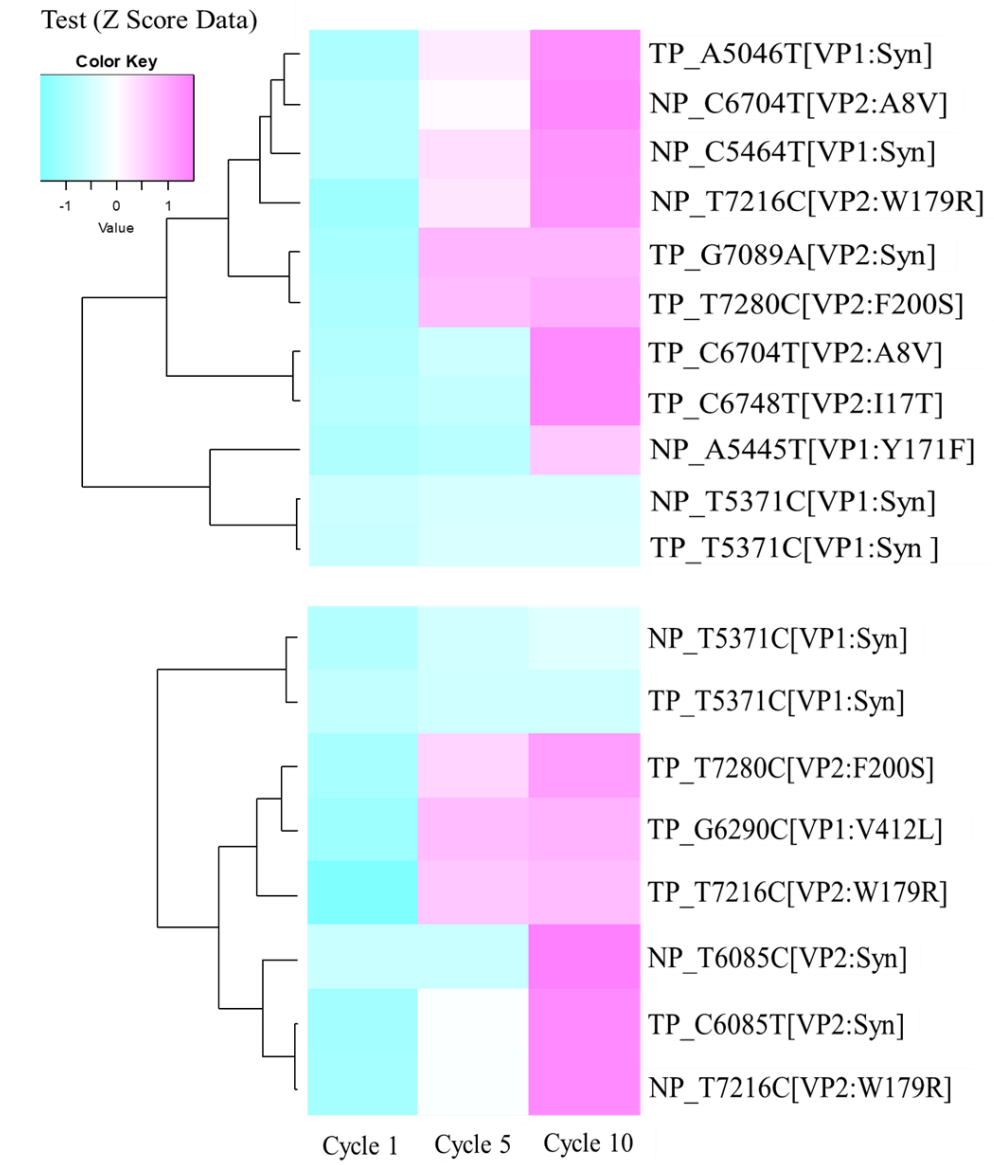

Figure 3.4. Changes in nucleotides in the 1st, 5th, and 10th cycles in the chlorine-treated and control populations in the first (upper) and second trials (lower). The Z score represents the standardized percentage value of a mutation in each cycle.

A next-generation sequencing (NGS) technique was used to investigate the genomic basis for lower chlorine susceptibility of MNV. PCR products of ORF2 and ORF3, divided into seven

amplicons (Fig. 3.2), were obtained from the MNV populations (chlorine-treated and control populations from the first and second trials) in the first, fifth, and tenth cycles, and analyzed by NGS. In total, six nonsynonymous and five synonymous mutations in the first trial and four nonsynonymous and four synonymous mutations in the second trial were found from a 2,252-bp sequence of (Fig. 3.4). Mutations were more frequently observed in region 7 (nucleotide position of 6877-7329, reference strain: MNV S7-PP3, accession number: AB435515.1) in both trials. Among them, two nonsynonymous and one synonymous mutations were shared by both trials. The shared synonymous mutation was observed at nucleotide (nt) 5371 in ORF2 in both control and chlorine-treated population in both trial. The relative quantity of this mutant among total reads gradually increased from 61% to 98% in the 10 cycles. One of the two shared nonsynonymous mutations was located at nt 7216 in ORF3, in which tryptophan was replaced by arginine in control population from the first trial and in both populations from the second trial. The relative quantity of this nonsynonymous mutation gradually increased from 9% to 99% over the 10 cycles.

One of the two shared nonsynonymous mutations was located at nt 5371 in ORF2, in which tyrosine was replaced by histidine, in the chlorine-treated population from the first trial and in both populations from the second trial. The relative quantity of this nonsynonymous mutation gradually increased from the 61% to 98% over the ten cycles. The other shared nonsynonymous mutation was located at nt 7280 in ORF3, in which phenylalanine was substituted for serine, and appeared only in the chlorine-treated populations from both trials. The relative quantity for this mutation gradually increased from 1%, 90%, and 99% along cycle 1, 5, and 10, respectively. These results indicate that the MNV population with the nonsynonymous mutation at nt 7280 survived better than the wild-type under the repeated free chlorine exposure.

3.3.3 Principal coordinate analysis (PCoA).

PCoA was employed to examine the similarities and differences in the substitution rates among the populations using SNPs as distance matrices in the region 7 from the original population (before cycle experiment) and the chlorine-treated and control populations in the first, fifth, and tenth cycles (Fig. 3.5). The chlorine-treated and control populations in the first cycle were clustered together close to the original populations. Meanwhile, the chlorine-treated populations in the fifth and tenth cycles were clustered separately from the original and control populations. These results suggest that there was a different driving force to form MNV quasispecies between the exposure

to free chlorine and dilution, and that there is a selective force to form MNV quasispecies under the free chlorine exposure. On the other hand, genetic drift caused by dilution may cause different MNV quasispecies in control populations.

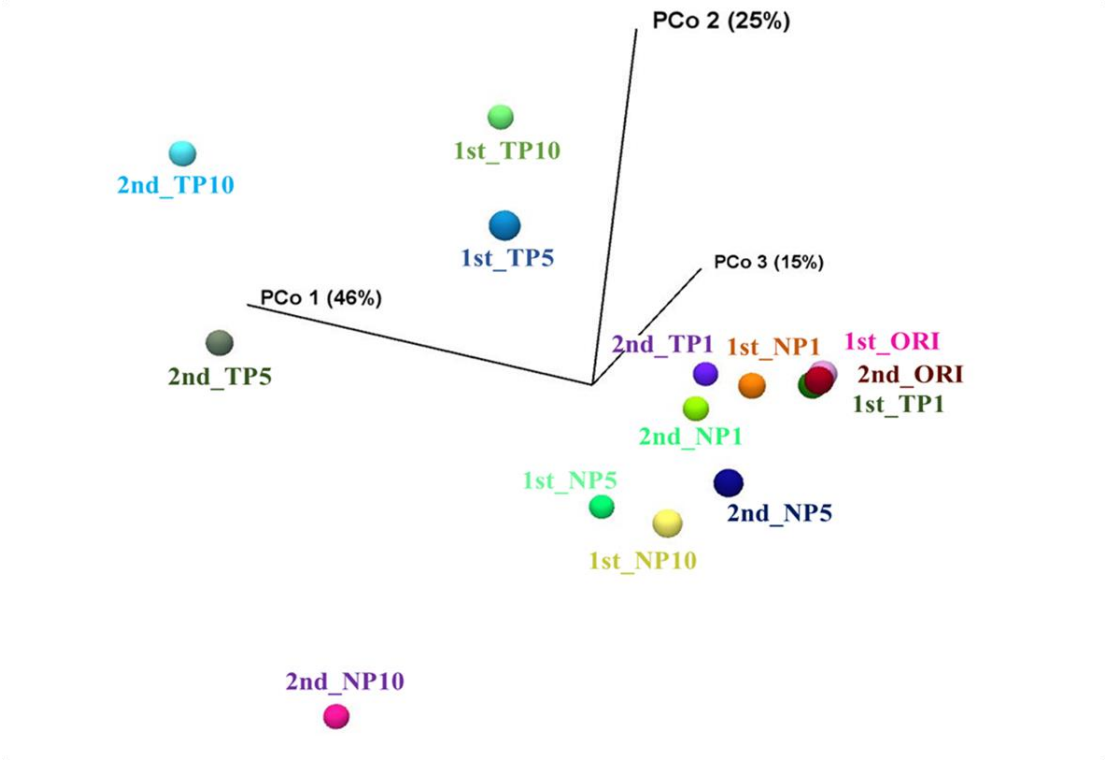

Figure 3.5. Principal coordinate analysis used to illustrate similarities and difference in the substitution rate using single nucleotide polymorphisms composition as distance matrices of two populations in the first, fifth, and tenth cycles in chlorine-treated (TP) and control populations (NP) in the first and second trials. TP1, TP5, and TP10 are chlorine-treated population in the first, fifth, and tenth cycles, respectively. Ori, NP1, NP5, and NP10 are the original population and control population in the 1st, 5th, and 10th cycles, respectively.

3.3.4 Free chlorine susceptibility of plaque-purified clones.

In order to examine whether SNPs found in the capsid region are associated with the lower susceptibility of chlorine, a chlorine sensitivity test was carried out. Six plaque-purified clones were acquired from each population from the first trial. Clones X1, X2, X3, X4, X5, and X6 were obtained from the control population in the tenth cycle, and clones A1, A2, A3, A4, A5, and A6 were obtained from the chlorine-treated population in the tenth cycle. The genome sequence of the region 7 was also determined by the Sanger sequencing method, and it was found that all clones from the chlorine-treated population (A1-A6) had the nonsynonymous mutation at nt 7280 (Fig.

3.7 in the Supplementary Information). From twelve clones, six clones (three clones each isolated from control and chlorine-treated population) were used for free chlorine susceptibility, growth rate, replicative fitness, and binding efficiency to cell surface test.

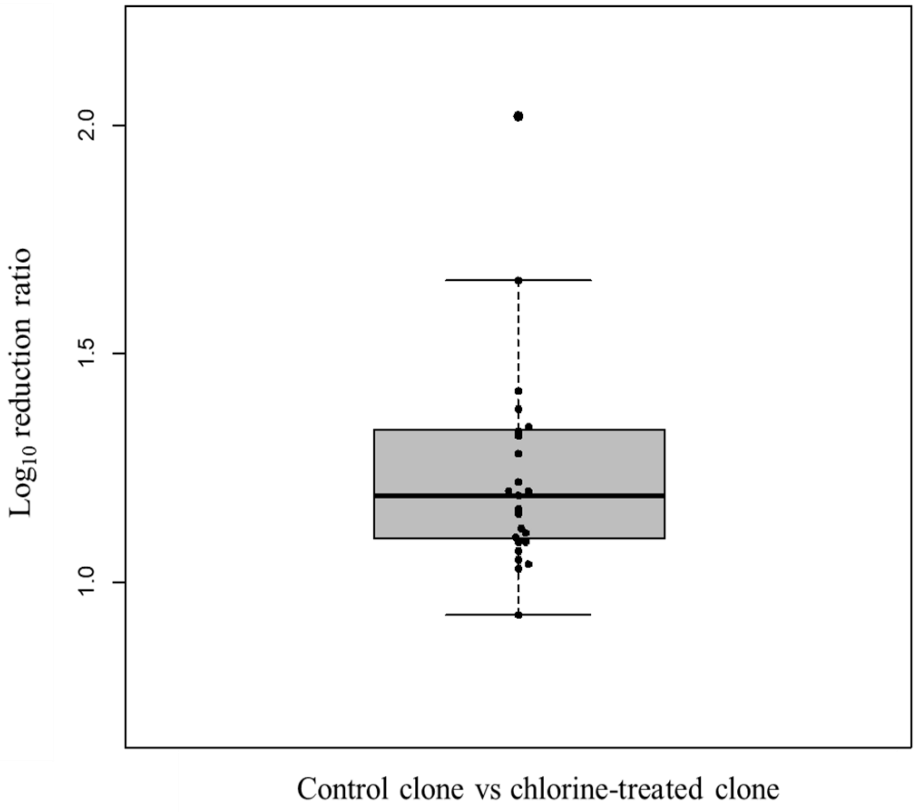

Figure 3.6. Log10 reduction ratio of clones from the control population (X clones) to those from the chlorine-treated population (A clones). The chlorine sensitivity test was conducted for nine clone combinations (X1/A1, X1/A2, X1/A3, X2/A1, X2/A2, X2/A3, X3/A1, X3/A2, X3/A3) with initial concentration of 2 ppm, and 1 min incubation. The test was repeated three times for each combination, and there are 27 dots in total.

The free chlorine sensitivity of the plaque-purified clones was evaluated to investigate whether the nonsynonymous mutation (T7280C[VP2:F200S]) would affect the susceptibility of MNV to free chlorine. An X clone was paired with an A clone, and nine pairs were selected randomly (A1 versus X1, A1 versus X2, A1 versus X3, A2 versus X1, A2 versus X2, A2 versus X3, A3 versus X1, A3 versus X2, and A3 versus X3). Then, the $\log_{10}$ reduction ratio after free

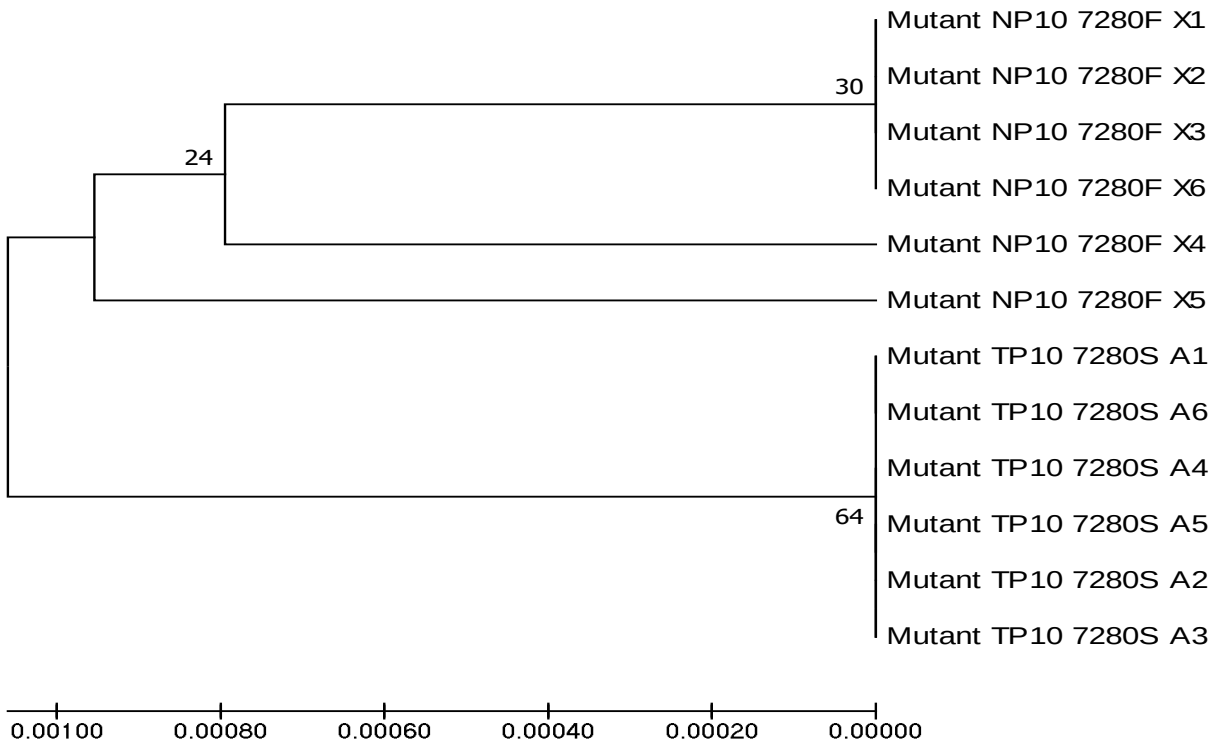

Figure 3.7. A phylogenic tree of the chlorine-tretaed clones (A1, A2,A3, A4, A5, A6) and the control clones (X1, X2, X3, X4, X5, X6) derived from a murine norovirus S7 lineage. Forward and reverse SANGER sequences for each strains was merged using ExPasy website. Merged sequences were aligned by MUSCLE and a phylogenetic tree was generated using bootsrap method with 5000 replications in MEGA 7 software. Evolutionary distances were compute using Tamura-Nei model, and evolutionary history was inferred usingUPGMA method.

chlorine exposure with an initial concentration of 2 ppm and 1 min contact time was obtained three times for each pair, providing the results represented by the 27 dots in Fig. 3.6. The average of ratio values was 1.24, and the minimum and maximum ratio values were 0.93 and 2.02, respectively. These ratio values were significantly greater than 1.0 (one-sample Wilcoxon signed rank test, $p = 5.22 \times 10^{-8}$), which means that the plaque-purified clones from the chlorine-treated population had significantly lower susceptibility to free chlorine than those from control populations.

3.3.5 Replicative fitness of plaque-purified clones.

A one-step growth curve experiment was conducted to investigate whether clones isolated from the chlorine-treated population after the tenth cycle had higher replicative fitness compared to clones from the control population. The growth rate and doubling time, calculated from a one-step growth curve of each plaque-purified clone from the control and chlorine-treated populations (six clones in total), ranged from 0.76 - 0.83 infectious virus particle/hour and 0.84-0.91 hour respectively (Table 3.3).

Table 3.3 Growth rate and doubling time for each clone isolated from the chlorine-treated population (A1, A2, A3) and the control population (X1, X2, X3)

Clone(s)	Growth Rate (μ)/h	Doubling Time (H)
A1	0.76 ± 0.02	0.91 ± 0.02
A2	0.77 ± 0.02	0.90 ± 0.02
A3	0.80 ± 0.01	0.87 ± 0.01
X1	0.83 ± 0.01	0.86 ± 0.05
X2	0.82 ± 0.02	0.84 ± 0.03
X3	0.81 ± 0.01	0.85 ± 0.01

There were no statistically significant differences in the growth rate and doubling time between the clones from the chlorine-treated and control populations, which were tested using the Kruskal-Wallis test ($p = 0.07$ for growth rate and $p = 0.19$ for doubling time). The selection coefficient (s) (equation 5) of plaque-purified clones from the chlorine-treated and control populations was calculated to compare the relative replication fitness of the clones. There was no statistically significant difference in the selection coefficient (s) among tested clones, as determined by the Kruskal-Wallis test (Table 3.4, $p = 0.06$). The positive value of the selection coefficient (>0) suggested that the clones from the chlorine-treated population had a slightly lower fitness value compared to the clones from the control population. However, statistical tests showed

that s values were not higher than 0 (Wilcoxon signed rank test, P = 0.002, α = 0.05), indicating no significance difference in the replicative fitness among the clones.

Table 3.4 Relative replicative fitness between clones isolated from the chlorine-treated population (A1, A3, A2) and the control population (X1, X2, X3)

Selection coefficient (s) value			
Clone(s)	X1	X2	X3
A1	0.07	0.07	0.06
A2	0.06	0.07	0.05
A3	0.03	0.04	0.02

3.3.6 Binding efficiency to cell surface.

The binding efficiency of plaque-purified clones with and without the nonsynonymous mutation (T7280C[VP2:F200S]) to RAW 264.7 cell surface was evaluated. The number of viral particles bound to the host cell surface was determined by RT-qPCR, and we found that the average values of the bound MNV count divided by the spiked MNV count were -2.24 and -1.69 $\log_{10}$ for clones from the chlorine-treated (with T7280C[VP2:F200S]) and control populations, respectively (Fig. 3.7). Statistical tests showed that the ratio of bound viral gene copy number to added copy number was not significantly different between mutants (T7280C[VP2:F200S]) and the control clones (Wilcoxon rank sum test, p = 0.98), which indicated that all clones exhibited a similar ability to bind to the host cells.

3.3.7 The calculation of dN/dS ratio.

To determine if either positive selection, purifying effect, and/or genetic drift occurred in the virus population, the ratio of synonymous to non-synonymous mutation (dN/dS) was calculated for each open reading frame. Prior to dN/dS calculation, the expected number of synonymous (*si*) and non-synonymous (*ni*) sites were calculated. Then, for each read *j* covering entire codon *i*, the number of observed synonymous (s*ij*) and non-synonymous (n*ij*) substitutions with respect to the

consensus sequence in the population. Based on all codons, where si>0 and $\sum_{jSij}^{0} > 0$, the estimation for the number of synonymous substitution per synonymous site (Ps) was calculated with the following equation:

$Ps = \frac{1}{Nod}\sum_{i=1}^{Ncod}\frac{1}{ri}\sum_{j=1}^{ri} Sij/si$ (equation 7) [84]

Where Ncod is the number of codons when the condition mentioned above are met and ri is the number of reads spanning entirely codon *i*. The number of non-synonymous substitution per site (*Pn*) was calculated analogously of equation 7. Lastly, the number of non-synonymous substitution (dN) and synonymous substitution (dS) per site was calculated using the following formula:

$dN = -\frac{3}{4}\ln\left(1-\frac{4Pn}{3}\right)$; $dS = -\frac{3}{4}\ln\left(1-\frac{4Ps}{3}\right)$ (equation 8) [85]. The dN/dS ratios were calculated at the VP2 region from both of first and second trial of chlorine treated population with 50 ppm as initial chlorine concentration. Other populations did not fulfill the requirement for sij>0 and nij>0 (equation 8). In the VP2 site of the first trial of the chlorine treated population, the dN/dS values were 0.36 and 0.85 for cycle 5 and 10, respectively. On the other hand, the dN/dS values of 0.84 and 0.83 were shown at VP2 site of the second trial of chlorine treated population in cycle 5 and 10, respectively.

3.4 DISCUSSION

I have demonstrated the viral strain-dependency of the free chlorine susceptibility. The NGS results showed that the ratio of a nonsynonymous mutation in ORF3, which encodes the minor capsid protein VP2, gradually increased when the MNV population was repeatedly treated with free chlorine. The plaque-purified clones from the chlorine-treated population, which shared the same nonsynonymous mutation at the nucleotide number (nt) of 7280, had significantly lower susceptibility to free chlorine than those from the control populations without the nonsynonymous mutation. The growth rate and binding efficiency to the host cell surface of plaque-purified clones from the chlorine-treated population were not significantly different from those from the control population. Combined chlorine may be generated during cycle experiment and involve in inactivation process, although lower concentration of amide compound in DMEM medium used as the virus stock, and lower biocidal effect of combined chlorine compared to free chlorine, the

effect of combined chlorine in virus evolution may be low although it can not be completely ignored.

Viral quasispecies evolution is influenced by population genetics factors, including mutation, selection, and genetic drift [86]. The MNV S7 strain was known to exhibit higher genetic diversity compared to MNV-1, which indicates the coexistence of multiple haplotypes in a viral isolate [87]. In both the chlorine-treated and control populations, a larger number of nucleotide changes was observed in ORF3 than in ORF2 (Fig. 3.4). A previous study investigating in vitro replication of MNV in RAW 264.7 cells also reported a higher mutation rate in ORF3 (53.13×10^{-5} mutation/site/day) compared to ORF2 (12.89×10^{-5} mutation/site/day) [88]. The reason why the mutation rate in ORF3 of MNV is higher than that in ORF2 remains unclear, but VP2 may have a lower structural constraint than VP1, which could explain the accumulation of mutations in ORF3. The three-dimensional (3D) structure and localization of VP2 in a virion need to be determined to understand the mechanism of the higher mutation rate in ORF3 than in ORF2.

Larger numbers of synonymous and nonsynonymous mutations were observed in the chlorine-treated populations than in the control populations (Fig. 3.4), and the evolution direction of the chlorine-treated populations was different from that of the control populations (Fig. 3.5). These results indicate that the free chlorine treatment has a distinct effect on norovirus evolution from genetic drift. Through free chlorine selection, strains less susceptible to free chlorine may be selected. The frequencies of these strains increased with the numbers of treatment cycles. These less susceptible strains may have already existed in the original population in a lower proportion. However, the larger number of mutations found in the treated populations underpin another possibility that the strains were newly developed via mutation during the treatments.

Some synonymous and nonsynonymous mutations were observed in the control populations from both trials (Fig. 3.4). The control population was not exposed to free chlorine, but it was diluted 10,000-fold before replication in host cells at each cycle. This dilution at each cycle introduces the bottleneck effect to the MNV population, in which only a small subset of the population is allowed to produce progeny in the host cells. In other words, the control population reflects only the evolutionary forces of mutation and genetic drift, eliminating the effect of selection under chorine treatment. The PCoA pattern found in control populations showed a smaller evolutionary shift from the original population as compared to the chlorine-treated

populations (except for the population at the tenth cycles of the second trial (Fig. 3.5). Mutations of control populations may be balanced by genetic drift, which tends to remove mutants and constrain population divergence. A previous study also showed that the repetitious passage of MNV S7 at low MOI, in which a genetic bottleneck is emphasized, resulted in a convergence in the frequency of haplotypes [87].

The acquisition of less susceptible populations after repeated exposure to chlorine-based disinfectants was also observed in different species of RNA virus. For example, a chlorine dioxide-exposed population of bacteriophage MS2 with nonsynonymous mutations of A467T and A1443G showed lower susceptibility to chlorine dioxide [44]. In the case of echovirus 11, a chlorine dioxide-treated population included nonsynonymous mutations of G1373C, A2397T, and C3101A, and this population showed less sensitivity to chlorine dioxide compared to the original population [45]. These previous studies suggest that nonsynonymous mutations appearing after the repeated exposure to the chlorine disinfectant were linked to the improvement of RNA viruses to bind with the host cell surface due to the amino acid substitution of chlorine-labile amino acids for chlorine-stabile ones [44,45]. The echovirus study concluded that the lower susceptibility to chlorine dioxide was caused by the acquisition ability of the virus to utilize alternative cell receptors to attach to the host cell surface due to nonsynonymous mutations in the capsid region [45].

The ability of MNV to bind to glycolipid and glycoprotein attachment receptors on the host cell surface may be strain dependent, since a mutant with only one amino acid change from a wild type was able to exhibit distinct changes in tissue tropism [89]. Some MNV lineages such as MNV-1, WU11, and S99 bind to microphages through a terminal sialic acid on the ganglioside GD1a ex vivo [90], whereas other strains such as CR3 can bind to another glycan [89], which means that the lower susceptibility of MNV populations observed in the present study can also be explained by the change in the binding efficiency to the host cell surface. However, the nonsynonymous mutation that appeared only in the chlorine-treated populations (T7280C) was located in ORF3, encoding VP2, which does not have a role for binding to the host cell surface, in contrast to the VP1 protein [75,91]. Our results showed that all plaque-purified strains with T7280C[VP2:F200S] exhibited lower susceptibility to free chlorine compared to control strains, but there were no statistical differences in the replicative fitness (Table 3.4) and binding ability (Fig. 3.7) between

them, which suggests that the lower susceptibility to free chlorine was not an apparent outcome caused by higher binding and replication efficiencies.

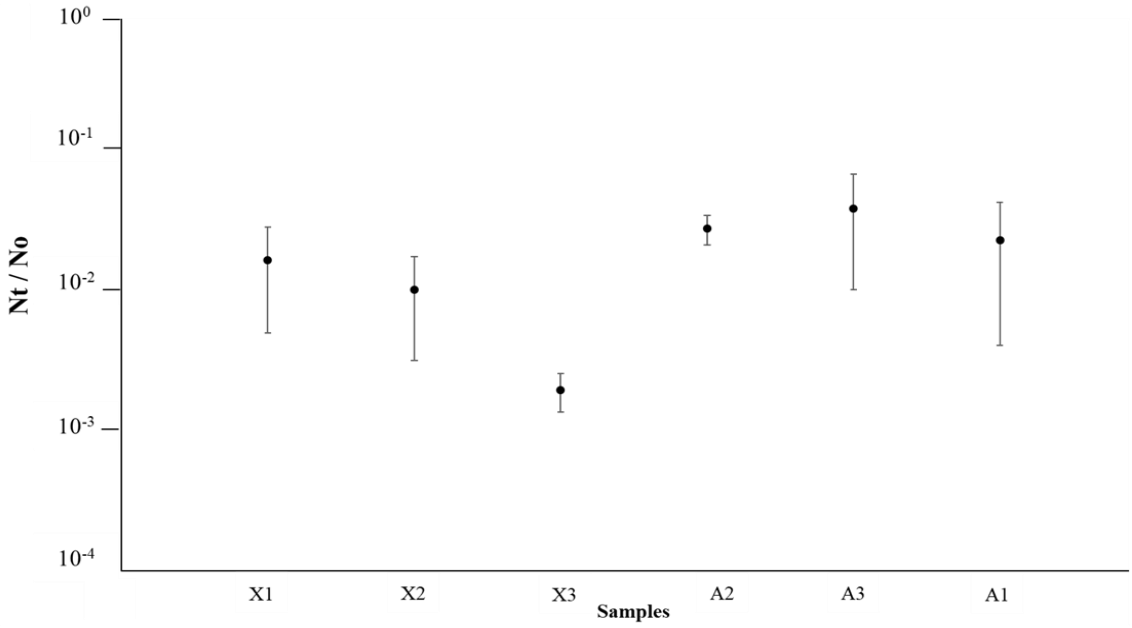

Figure 3.8 The ratio of MNV concentration in the supernatant after incubation at 4 ˚C for 90 minutes (N_t) to the initial MNV concentration (N_0).

One possible explanation for the lower susceptibility to free chlorine is that the VP2 protein increases the stability of the capsid by protecting VP1 proteins from disassembly due to free chlorine exposure [92–94]. A previous study reported that the yield of human norovirus-like particles (VLP) was low if the ORF3 was not included in the recombinant genome, which suggests that VP2 has a pivotal role in forming a virion of human norovirus [95]. Although an analogical inference between the capsid structure of human and murine noroviruses is not easy because the localization of VP2 has been analyzed only for Norwalk virus so far [96], the contribution of T7280C[VP2:F200S] to the structural stability of MNV virion may be proved by the information about the 3D location of MNV VP2 in the future.

Both population showed dN/dS ratio close to one, which indicated that positive selection has occurred [97]. In addition, the positive selection seems to have occurred faster for the second

trial compared to the first trial of population showed by higher dN/dS ratio. This finding explains why the less susceptible population appeared earlier in the second trial compared to first trial (Fig. 3.3).

It must be noted that there was a clear difference in the susceptibility to free chlorine between the first and second trials of the chlorine-treated population (Fig. 3.3). This difference cannot be explained by the mutation at nt 7280, because clones from both populations share this mutation. A possible explanation is that the other mutations also play roles for the chlorine susceptibility. For example, a nonsynonymous mutation (T6290C[VP1:V352L]) was observed only for the chlorine-treated population from the 2nd trial. There is also a possibility that mutations in ORF1, which encodes non-structural proteins, affect the free chlorine susceptibility, although this region was not analyzed in the present study. The large variation in the $\log_{10}$ reduction ratio in Fig. 3.6 can be also explained by the presence of multiple mutations responsible for the free chlorine susceptibility. A synergistic effect of multiple mutations in the MNV genome on the free chlorine susceptibility needs to be investigated in the further study.

3.5 CONCLUSION

This study demonstrated that free chlorine disinfection acted as a selection pressure on an MNV population. If this is also the case for human noroviruses, it is important to use less susceptible (stronger) strains in a water disinfection test for estimating the virus inactivation rate; otherwise the disinfection efficiency in an actual setting is not reaching the expected level and an infection risk is posed even with disinfected water. The strain-dependent susceptibility of RNA viruses to a variety of disinfection technologies, including ozonation and ultraviolet light irradiation, and the possibilities of cross-resistance with other disinfectant and or antiviral needs which has been described previously [46,98] to be further investigated to achieve safer usage of water possibly contaminated by pathogenic viruses.

Chapter 4: GENETIC DIVERSITY AND RATE OF EVOLUTION FOR MNV AFTER MULTIPLE CHLORINE EXPOSURES

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with the title of "Disinfection as a selection pressure on RNA virus evolution"

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4.1. INTRODUCTION

Emerging infectious diseases, including severe acute respiratory syndrome, filovirus disease, and Middle East respiratory syndrome, during the last two decades has led the researchers to put more awareness into RNA virus evolution studies. Changes in land use and increase in urbanization are also raise some alerts in emergence of viral pathogens jumping into new host mainly from animal to human. The understanding of virus evolution mechanisms is thus pivotal to obtain knowledge about how to predict and control viral infectious diseases.

The RNA virus evolution is characterized by multiple features, including a small genome size, explosive replication rate, and high mutation rate caused by the lack of proofreading function of the RNA-dependent RNA polymerase, which result in forming quasispecies in a viral population composed of mutant spectra [99] [86]. The formation of quasispecies is a key in the population dynamics theory of RNA viruses, in which mutation, natural selection, genetic drift, and recombination are mainly working (Figure 1) [100], and this is also the case for human norovirus, an icosahedral single-stranded RNA virus [101]. The mutation is caused by the error-prone replication of viral genome in a host cell, which increases the genetic diversity of human norovirus populations. Meanwhile, the natural selection is mainly caused by a host immune pressure, and decreases the genetic diversity. For example, the evolution of norovirus GII.4 strains, in which 69-97% nucleotide similarity is observed among strains, is explained by the emergence of escape mutants from immune pressure [102,103]. The genetic drift is caused by the bottleneck effect in a life cycle of RNA virus, which is exerted by the random attainment to the next host after released to outside the host body. Recombination of human noroviruses has been also reported, which

decreases the genetic diversity by eliminating detrimental genetic sequences for efficient replication [104].

Among these driving forces in the norovirus evolution, the natural selection process may be also exerted by another factor that has not been fully discussed so far, which is social infrastructure, including water and wastewater treatment practices. Water and Wastewater treatment processes, one of the important infrastructure mainly in developed countries, usually include a disinfection step (Figure 2). For example, United States Environmental Protection Agency demands 99.99% of enteric virus removal with appropriate disinfection technologies such as ultraviolet (UV) light or using chemical oxidants like free chlorine, chloramines, ozone, and chlorine dioxide for drinking water resources [105]. However, the genogroup/genotype/serotype-dependent inactivation efficiency of RNA viruses has been reported. For example, the log reduction values of norovirus GI, GII and GIV with free chlorine are different where GI strains have higher resistance compared to the other genogroups [42]. Coxsackievirus B4 isolates from drinking water treated by free chlorine showed higher resistance to free chlorine compared to a laboratory isolate [106]. The quasispecies-level difference in the susceptibility to disinfectants/UV has been also reported. After repetitious chlorine dioxide exposure, each mutants in bacteriophage MS2 population exhibits different sensitivity to chlorine dioxide and tends to obtain lower susceptibility to chlorine compared to initial population [44]. The same research group reported similar phenomena in echovirus 11 (EV11) where an initial population exhibited higher susceptibility compared to a population which were exposed to chlorine dioxide repeatedly [107]. In quasispecies level, a natural selection caused by chlorine dioxide decreased the number of higher susceptibility haplotypes among MS2 and EV11 populations and resulting in survival of lower susceptible population which can be proliferated in the subsequent replication. In phenotypic level, lower susceptibility was caused by nonsynonymous mutations to more stable amino acids against chlorine dioxide treatment, and these mutation led to more stable host binding of mutants compared to wild type [44]. These responses of RNA viruses to disinfection may result in the release of survived lower susceptible strains to the environment. This means that the disinfection processes may work as a natural selection pressure in the life cycle of RNA viruses.

The finding of genogroup/genotype/serotype/strain-dependent inactivation efficiency of RNA viruses indicated above holds our attention to the impact of the social infrastructure-driven

pressure on norovirus evolution. In the previous chapter, I described the selective pressure of chlorine to murine norovirus capsid, which had exerted a different evolutional direction compared to the control population group with a serial dilution. This chapter aims to examine the selective pressure effect on MNV evolution at the whole genome level by measuring the genetic diversity and evolutionary rates of MNV following the cycle experiments described in the chapter three.

4.2 METHODOLOGY

4.2.1. MNV whole genome next generation sequencing

RNA samples were extracted from MNV populations in the previous cycle experiments, including chlorine treated population and control population at cycle 1, 3, and 10. In addition, chlorine treated population with 25 ppm of initial concentration was also included. RNA extractions were conducted using QIA cube with QIAamp viral kits following the manufacturer's instructions (QIAGEN, QIAamp Viral RNA Kit). Viral RNAs with a final volume of 60 µl were generated as final products in this step.

The NEBNext® Ultra™ RNA Library prep kit for Illumina (New England Biolabs, Cat No E7530L) was used for library preparation following the manual protocol with some modifications. The library preparation consists several steps including RNA fragmentation, cDNA synthesis, dA-tailing process, adapter ligation, and PCR reaction to amplify the amplicons. For RNA fragmentation and priming, a mixture of 13.5 µl of mRNAs (minimum concentration of 30 ng/µl), NEB first strand synthesis reaction buffer (5X), and 10 pmol random primers were incubated at 94°C for 10 minutes, followed by cooling on ice. After RNA fragmentation, the first strand cDNA synthesis was conducted by mixing the fragmented RNA with 0.002 units of RNase inhibitor and 2 units ProtoScript II reverse transcriptase provided in the kit with total volume of 20µl . Reverse transcription were conducted as the following: 25 °C for 10 minutes, 42 °C for 50 minutes, and 70°C for 15 minutes. The first strand cDNAs (8 µl) were added to to a mixture of second strand synthesis reaction buffer (10 X) and 2 units of second strand synthesis enzyme mix and nuclease-free water up to 80 µl, followed by incubation at 16 °C to generate double-stranded cDNAs. Sample purification was done using 1.8X agencourt AMPure XP beads and 80% ethanol solution, and the purified products were eluted in 10 mM Tris-HCl..

Prior to performing NGS adapter ligation, end repair/dA-tail of cDNA library was performed using NEBNext end repair buffer (10X) and NEBNext end repair enzyme (1.5 units). The mixture was incubated at 20 °C for 30 minutes, followed by 65°C for 30 minutesm and hold at 4 °C. Prior to adapter ligation, 15 µl of the Blunt/TA ligase master mix and 2.5 µl nuclease free water was added to 65 µl dA-Tailed cDNA. Adapter ligation was performed using the NEBNext adapter for illumina at 10-fold dilution to obtain 1.5 µM concentration, . one µL of which was added to the repaired-double stranded cDNA followed by incubation at 37°C for 15 minutes in an 83.5 µl total volume. Ligated products were purified using the Agencourt AmpureXP (Beckman Coulter). To enrich the DNA libraries, a PCR was performed in a 50 µl reaction mixture with NEBNext Q5 hot start Hifi PCR master mix (5X) , 1 pmol of i5 and i7 primers for every 20 µl purified and ligated cDNA. PCR was performed under the following temperature profiles: 30s at 98°C for initial denaturation, 10s at 98°C for denaturation, 75s at 65°C for annealing/extension. Denaturation and annealing step was repeated for 17 cycles, followed by a final extension step of 5 min at 65°C, and storage at 4°C. PCR products were purified using the Agencourt AmpureXP (Beckman Coulter). The quality of all libraries was confirmed by Bioanalyzer (Agilent) using the Agilent High Sensitivity DNA Kit (Agilent) and DNA concentration was measured using Quibit fluorescent quantification. Next generation sequencing was performed by Genewiz Japan Corporation.

4.2.2. Single nucleotide polymorphism (SNPs) determination

NGS data were analyzed using CLC Genomics Workbench and Galaxy (https://usegalaxy.org/) platform. The analysis included quality control of contigs, forward and reverse contigs merging, adapter trimming, and mapping into a reference (MNV S7-PP3, accession number: AB435515.1). Reads with lower than 30 sequences were trimmed. The median of the score of reads for any base quality should be no more than 20 when measured by the FastQC software in Galaxy platform. In addition to the observation of the other SNPs, including minority sequences and their percentage in NGS reads, CLC variant measurement was used. Depth coverage for each nucleotide in MNV was measured using BAM alignment file using SAMtools software (http://samtools.sourceforge.net/)

4.2.3. Genetic diversity and Evolutionary rate

Genetic diversity was measured using Shannon entropy (SE). This index measured the diversity within a viral population at a single nucleotide position or a whole sequence level and defined as:

$$E = -\frac{1}{N}\sum_{i=1}^{N}(fiAlnfiA + fiGlnfiG + fiTlnfiT + fiClbfiC) \quad \text{(equation 6)} \ [108]$$

Where fi is the frequency of nucleotide A, C, G or T at position I and N is the total length of the targeted open reading frame. The highest value was obtained when there was an equal percentage of 25% of all nucleotides in a specific site and zero value when generated where only one nucleotide was present at a frequency of 100%.

For the estimation of evolutionary rates, three different regions were used for the calculation including region A (nt of 8-2081) which covered NS1/2 and NS3 protein, region B (nt 4831-6681) which covered VP1 protein, and region C (nt 6877-7329) which cover parts of VP2 protein (Fig. 4.1). The rates of nucleotide mutation/site/day within different chlorine-treated populations (25 ppm and 50 ppm of initial concentration for both the first and second trials) and control population for both the first and second trials were inferred using the Bayesian Markov chain Monte Carlo method implemented in the BEAST software package (BEAUti, BEAST) [109]. The GTR+ɣ+ invariant sites model was selected as the substitution model. The BEAST files were run assuming either a strict molecular clock and lognormal relaxed clock under the Yule plot model. The output of BEAST files was then analyzed using TRACER software (http://beast.community/tracer).

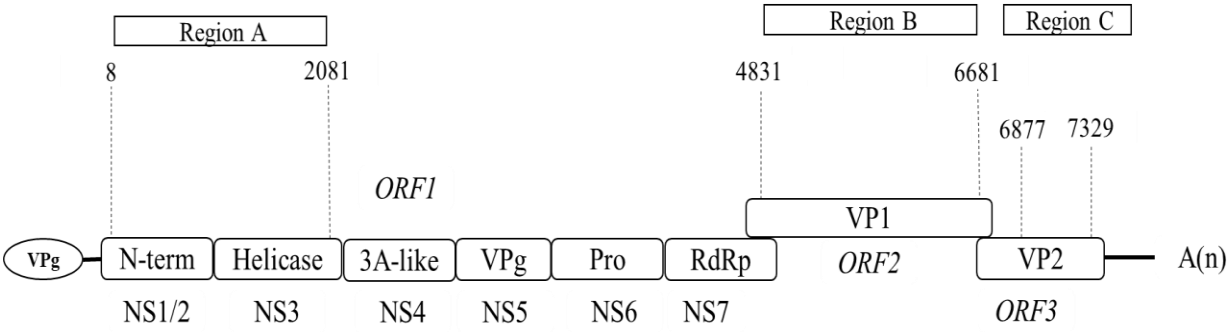

Figure 4.1. Region-based evolutionary rates region in murine norovirus (MNV S7-PP3, accession number: AB435515.1).

4.3 RESULTS AND DISCUSSION

A total of 576,164 reads were generated from all samples, with1,328 - 79,426 reads for each population. The depth coverage for nucleotide sequences were varied between 0 – 4467 (Figure 4.2). The depth coverage in all population is enough to conduct further downstream analysis included SNPs calling for genetic diversity and evolutionary rate calculation. Unfortunately, the sample cycle 10 of chlorine treated populations from the first experimental trial did not showed any reads. It may be caused by imperfect ligation process lead to unattachment of adapter and the library was not readable in the NGS process or lower initial viral titer[110]. All sequences showed a match with the references (MNV S7-PP3, accession number: AB435515.1).

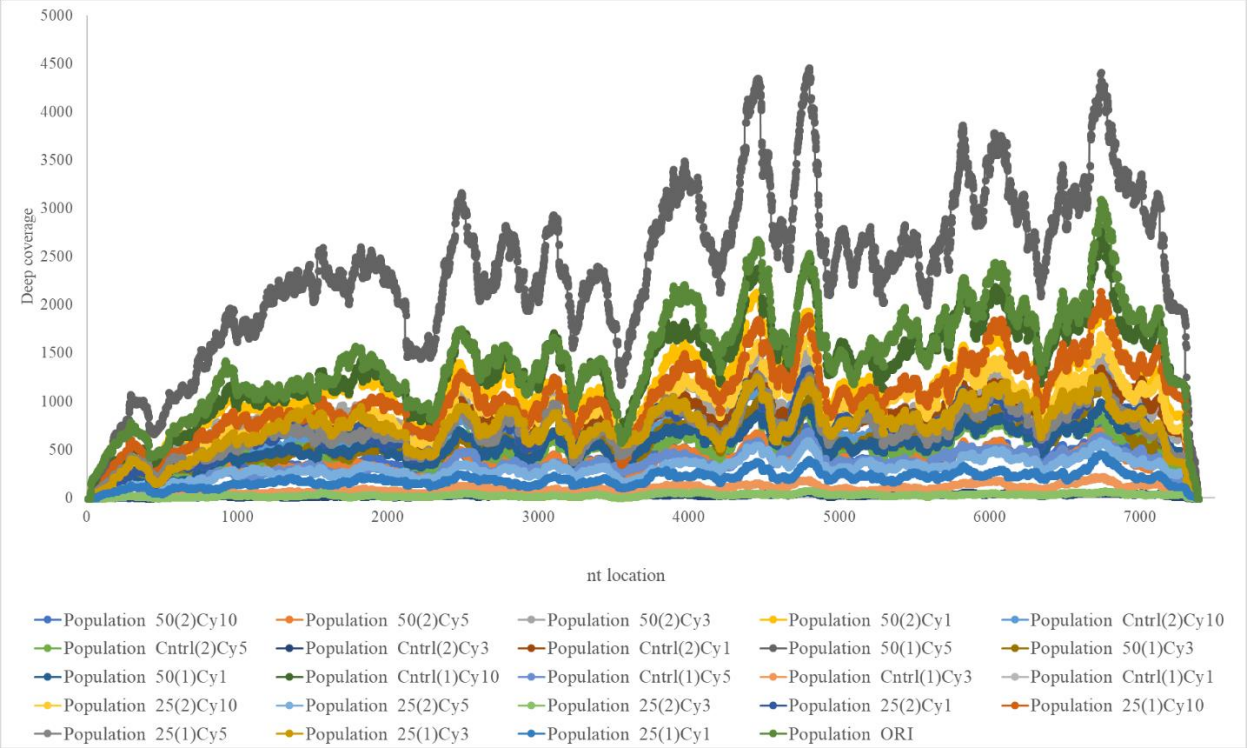

Figure 4.2. Depth coverage generated at each nucleotide of MNV population through whole genome sequencing.

As represented by the Shannon index, higher genetic diversity was observed in the capsid region (VP1 and VP2), compared to non-structure protein region. In VP2 region, the viral genetic diversity in chlorine-treated populations increased from cycle one to cycle 5 and decreased in cycle 10 (excluded data at chlorine treated population with 50 ppm of initial concentration due to no sequence reads were available) (Fig. 4.3). On the other hand, in control populations, genetic diversity increased from cycle 1 to 3 and remained constant up to cycle 10. These differences in genetic diversity fluctuation may be caused by distinct evolution forces at work in the MNV population, where selective pressure by chlorine and genetic drift worked in chlorine treated populations and control populations, respectively [111]. In VP1 region, the genetic diversity in the second trial of chlorine treated population are increased from cycle one to ten but no significant changes occurred in the others population suggesting different evolution process is worked in this region. Similar condition happened in control population where there are no significant changes in genetic diversity except for the tenth cycle of the first trial experiment (Fig. 4.4).

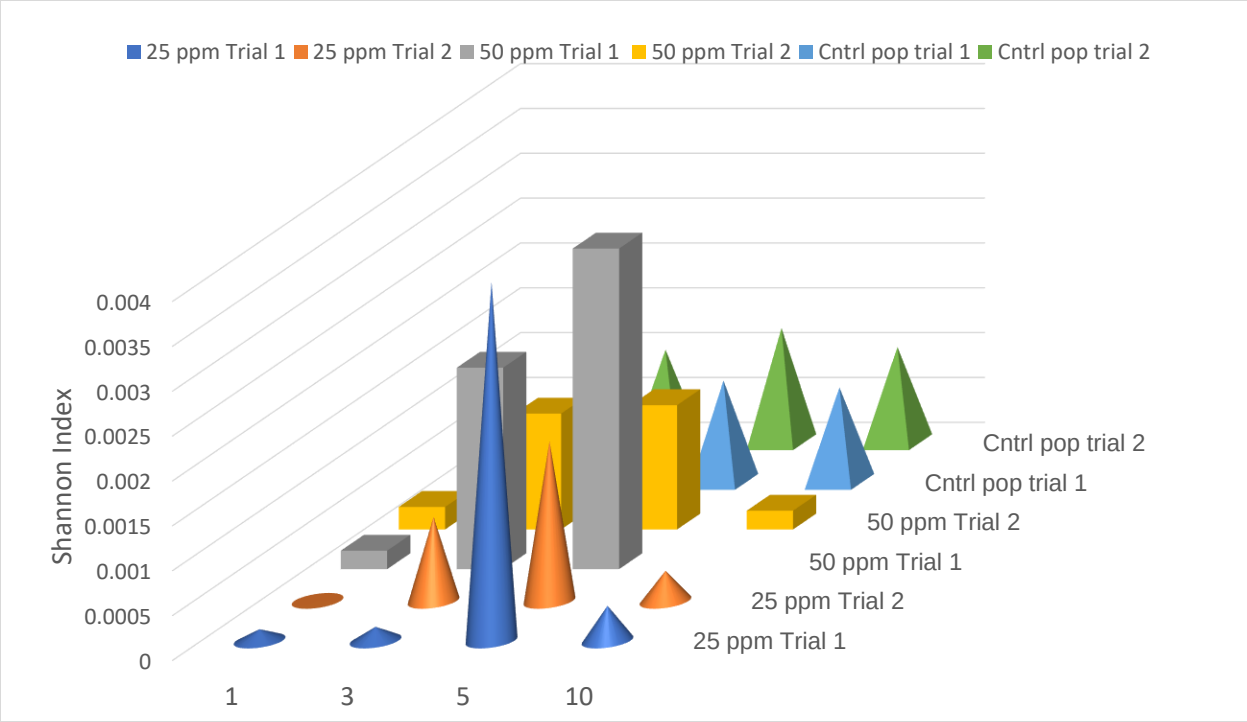

Figure 4.3. Genetic diversity in ORF3 (VP2) of all population as measured by the Shannon index.

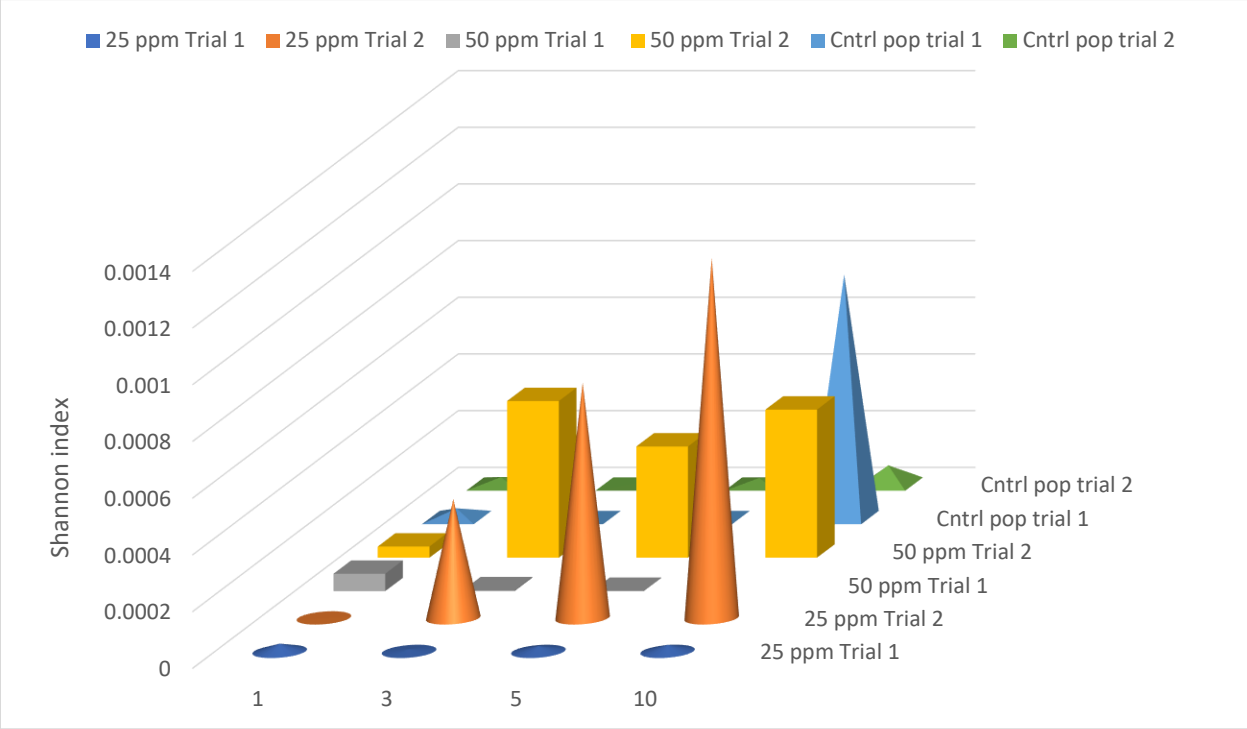

Figure 4.4. Genetic diversity in ORF2 (VP1) of all population as measured by the Shannon index.

Genetic diversity has a fitness benefit for MNV population, such as increased viral replication, antigenic variability, and enhanced virulence [110]. It is possible that the increase of diversity in the first five cycles was aimed to increase the probability for the virus to persist in chlorine pressure. Another study mentioned an increase of quasispecies diversity due to a statistically significant increase in the number of unique haplotypes following serial passaging of MNV 1 in RAW 264.7 cells for twenty generations in the presence of Ribavirin [112]. In the same study, Ribavirin's impact on significantly reducing the relative proportion of the dominant sequence within the quasispecies was demonstrated [112].

The results showed that the evolutionary rate calculated using strict and relaxed molecular clocks in region C located in the VP2 region is higher than that in region A and B (Fig 4.4). The evolutionary rates with a strict molecular clock (expressed as mutation/site/cycle in 10^{-5} [95% of highest probability density values]) ranged from 2.13[0.51-5.53] – 58.8[0.11-15.4], 8.89[0.04-18.9] – 55.3[0.63-149], and 30.4 [1.41-77.1] - 70.2 [1.68-188] at region A, B, and C respectively (Figure 4.4). On the other hand, lower evolutionary rates were generated from the relaxed molecular clock calculation with a range from 1.32[0.29-3.25] – 9.81[0.11-25.1], 5.89[0.29-14.3] – 54.2[0.61-131], and 30.1[0.24-76.8] – 88.3 [1.51-233] clock (expressed as mutation/site/cycle in 10^{-5} [95% of highest probability density values]) at region A, B, and C respectively (Figure 4.5).

A strict clock model assumes that every branch in a phylogenetic tree evolves according to the same evolutionary rate, while on the other hand, an uncorrelated relaxed clock allows each branch of a phylogenetic tree to have its own evolutionary rate[109]. A similar trend was described in a previous study where the evolutionary rates were found to be two-fold higher in value compared to other regions in MNV during the vitro replication in the VP2 region compared to VP1 and NSP. The evolutionary rate in the control population revealed higher rates compared to the chlorine-treated population which suggested that selective pressure of free chlorine might have caused a slower evolution process in chlorine-treated population compared to the genetic drift resulted by a dilution in the control population.

	Evolutionary Rate (95% HPD*) Expressed as mutation/site/cycle in 10^{-5}		
Population	Region A	Region B	Region C
50 ppm Trial 1	10.1[0.07-28.2]	16.4[2.74-201]	30.4[1.41-77.1]
50 ppm Trial 2	58.8[0.11-15.4]	22.1 [0.34-54.1]	31.3[0.23-83.2]
25 ppm Trial1	17.4[0.31-44.1]	22.1[0.42-56.8]	32.7[1.01-83.1]
25 ppm Trial 2	2.49[0.31-6.23]	8.89[0.04-18.9]	34.6 [0.46-91.2]
Control Trial 1	2.13[0.51-5.53]	24.1 [1.87-23.4]	70.2[1.68-188]
Control Trial 2	2.44[0.63-6.26]	55.3[0.63-149]	68.7[0.85-103]

Figure 4.5. Nucleotide evolutionary rates, estimated by the Bayesian Markov chain Monte Carlo method with the software BEAST, assuming restricted molecular clock models under the Yule model.

	Evolutionary Rate (95% HPD*) Expressed as mutation/site/cycle in 10^{-5}		
Population	Region A	Region B	Region C
50 ppm Trial 1	6.46[0.07-17.6]	23.0[0.23-56.9]	30.1[0.24-76.8]
50 ppm Trial 2	9.58[0.11-25.1]	28.2[0.89-98.2]	38.5[1.12-104]
25 ppm Trial1	9.81[0.21-25.4]	46.1[2.63-101]	49.1[0.71-122]
25 ppm Trial 2	1.32[0.29-3.25]	5.89[0.29-14.3]	47.4[0.54-124]
Control Trial 1	1.89[0.09-4.96]	42.3[0.25-85.7]	60.2[1.21-163]
Control Trial 2	2.56[0.02-6.84]	54.2[0.61-131]	88.3[1.51-233]

Figure 4.6. Nucleotide evolutionary rates as estimated by the Bayesian Markov chain Monte Carlo method with the software BEAST, assuming the relaxed molecular clock models under the Yule model.

In total, 11 mutations were found, three of them are synonymous and the other eight are non-synonymous mutations. In the non-structural protein region, only NS1/2 and NS3 protein showed nucleotide changes. From 11 mutations, 6 synonymous mutations were observed at NS1/2 and NS3 and 5 of them were non-synonymous mutations. Non-structural proteins are essential for virus replication [113]. The NS1/2 protein is unique to noroviruses and contains a highly disordered proline-rich N-terminus, a putative C-terminal transmembrane domain, and caspase cleavage sites [114,115]. Another study mentioned that NS1/2 region in norovirus was slightly conserved, although one amino acid change (D94E) was sufficient enough to generate a persistent phenotype on the virus. This suggeststhat the NS1/2 region determines viral tropism necessary for persistence[116].

Non-synonymous mutations in ORF1 were also observed in the NS3 protein. Until now, NS3 function in MNV is not clearly described, although some studies proposed that NS3 was intimately associated with the viral replication complex and dsRNA [117]. Nucleotide changes for NS3 may be related to obtain more efficiency in the replication process under free chlorine selective pressure, since it was only observed in the chlorine-treated population. In the capsid region, major changes at nucleotide number 7,280 were observed, which confirmed our previous study (Chapter 3). In the capsid region, major nucleotide changes occurred at nucleotide number 7,280, which was confirmed in our previous study (Chapter 3).

4.4 CONCLUSION

The impact of free chlorine selective pressure to the MNV population was described in this chapter. In terms of viral genetic diversity of chlorine-treated population, a decrease of diversity in cycle 10 were observed. dN/dS calculation suggested that chlorine selective pressure exerted positive selection towards MNV population. Furthermore, slower evolutionary rate for the chlorine-treated population compared to that for the control population suggested that selective pressure by chlorine might have delayed the evolution process of murine norovirus.

Chapter 5: CONCLUSIONS AND RECOMMENDATIONS

Water is a precious and crucial resource that is necessary for drinking, farming, industry, and recreational activities. Unfortunately, only one percent of all water on earth can be used by humans, which makes potable water a limited resource. Water availability and consumption amongst developing and developed counties is unequal; thus, water management is a difficult task due to the over-demand and limited supply. Another problem in drinking water management are the disease outbreaks due to contaminated water, caused by enteric pathogens. For these reasons, an equitable, safe, and affordable drinking water access for all, is an important goal for WHO to solve the global health and sanitation problem.

Wastewater reclamation is one of the solutions to balance the high demand of potable water and other needs, such as irrigation and recreational purpose. An important factor when using reclaimed wastewater, is the removal of pathogenic enteric viruses presents in the influent. A multiple barrier system including primary, secondary, and disinfection stages with a total of 6-7 $\log_{10}$ reduction of enteric viruses is recommended by WHO. In order to fulfil those requirements, a 3-4 $\log_{10}$ reduction of enteric viruses should be achieved during the disinfection process. In this study I proposed obtaining 0.19 – 6.25 and 100-1011 of CT values to obtain 2 $\log_{10}$ reduction of enteric viruses in the wastewater, using free chlorine and monochloramine as disinfectant agent. A 4 $\log_{10}$ reduction would require double the amount of CT values mentioned above. Different susceptibility of enteric viruses is described in detail, in the results. Thus, a continuous monitoring of new enteric viruses which can be resistance to free chlorine is important.

The different sensitivities to chlorine are observed in enteric viruses within the same genogroup/strain/serotype. It is expected, due to their genetically diverse strains and a high mutation rate. In this study I indicated that nucleotide changes at T7280C[VP2:F200S] may correlate with MNV' lower susceptibility to chlorine. One possible explanation for its lower susceptibility to free chlorine is that the VP2 protein increases the stability of the capsid by protecting VP1 proteins from disassembly, due to free chlorine exposure. This study showed that free chlorine disinfection acted as a selective pressure on an MNV population. Our PCoA analysis results displayed distinct sequence clusters between chlorine-treated and the control MNV population; which employed a 10,000-fold dilution - suggesting the genetic drift was exerted due

to the dilution treatment. A further chlorine sensitivity study involving a single desired mutation of MNV in the nt located at 7280, is important to clearly confirm the chlorine lower susceptibility phenomenon. If this is also the case for human noroviruses, it is important to use less susceptible strains in a water disinfection tests for estimating the virus' inactivation rate; otherwise the disinfection efficiency in an actual setting is not reaching the expected levels, and an infection risk is posed even with disinfected water. The possibilities of enteric viruses cross-resistance with other disinfectants needs to be further investigated in order to achieve safer usage of water that has been possibly contaminated by pathogenic viruses.

The effect of selective pressure on MNV populations is described in this study. The genetic diversity of MNV is decreasing from cycle 5 to 10, where the lower susceptibility population is present. Based on the dN/dS calculations, the selective pressure of free chlorine is a positive selection, whereas the lower susceptible MNV populations have appeared as a result of the evolutionary process. In addition, a lower number of evolutionary rates within chlorine treated populations compared with that of control populations, suggests that selective pressure by chlorine may delay the evolutionary process of MNV. If this evolutionary delay can also occur in the human norovirus population, it could prevent or further delay a new outbreak of norovirus, although further research is necessary.

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LIST OF RESEARCH ACCOMPLISHMENT

1. **Phd main publications**
   (1) **Rachmadi, A. T**.; Kitajima, M.; Watanabe, K.; Yaegashi, S.; Serrana, J.; Nakamura, A.; Nakagomi, T.; Nakagomi, O.; Katayama, K.; Okabe, S.; et al. Free chlorine disinfection as a selection pressure on norovirus. *Appl. Environ. Microbiol.* **2018**, AEM.00244-18.
   (2) **Rachmadi, A. T**.; Kitajima, M.; Watanabe, K.; Okabe, S.; Sano, D. Disinfection as a Selection Pressure on RNA Virus Evolution. Environ. Sci. Technol. 2018, acs.est.8b00590.

2. **Others publications**
   (1) Kitajima, M., **Rachmadi, A.T**., Iker, B.C., Haramoto, E., Gerba, C.P. Temporal variations in genotype distribution of human sapoviruses and Aichi virus 1 in wastewater in southern Arizona, United States. Journal of Applied Microbiology, 2018, 124(5), 1324-1332.
   (2) Kitajima, M., **Rachmadi, A.T**., Iker, B.C., Haramoto, E., Gerba, C.P. Genetically distinct genogroup IV norovirus strains identified in wastewater Archives of Virology, 161(12), 3521-3525.
   (3) **Rachmadi, A.T.**, Torrey, J.R., Kitajima, M. Human polyomavirus: Advantages and limitations as a human-specific viral marker in aquatic environments. Water Research, 2016, 105, 456-469.
   (4) **Rachmadi, A.T.**, Kitajima, M., Pepper, I.L., Gerba, C.P. Enteric and indicator virus removal by surface flow wetlands. Science of The Total Environment, 2016, 542(Part A): 976-982.

(5) Kitajima, M., **Rachmadi, A.T.**, Iker, B.C., Haramoto, E., Pepper, I.L., Gerba, C.P. Occurrence and genetic diversity of human cosavirus in raw and treated wastewater in Arizona. Archives of Virology, 2015, 160(7), 1775-1779.

(6) Kitajima, M., Iker, B.C., **Rachmadi, A.T.**, Haramoto, E., Gerba, C.P. Quantification and genetic analysis of salivirus/klassevirus in wastewater in Arizona, USA. Food and Environmental Virology, 2014, 6(3), 213-216.

(7) **Rachmadi, A.T.**, Mubarik, N.R., Prawasti, T.S. Screening of Proteolytic Bacteria Isolated from Tilapia (Oreochromis niloticus) in Inhibiting the Growth of Microcystis aeruginosa BT-02. Journal of International Environmental Application & Science, 7(2), 273-277.

3. Conferences

(1) Andri Taruna Rachmadi, Arata Nakamura, Masaaki Kitajima, Satoshi Okabe, Daisuke Sano. A Genetic Factor of Norovirus for Resistance to Free Chlorine Disinfection. Water and Environment Technology Conference (WET2017), July 22-23, 2017, Hokkaido University. Oral and poster presentation.

(2) Andri Rachmadi, Arata Nakamura, Kozo Watanabe, Sakiko Yaegashi, Satoshi Okabe, Toyoko Nakagomi, Osamu Nakagomi and Daisuke Sano. Genetic factors of murine norovirus for determining free-chlorine susceptibility. The 5th Food and Environmental Virology Conference. Kusatsu, Gunma. September 13-16, 2016.

CURRICULUM VITAE

Educational background

13/8/2003	Bogor Agricultural University Bogor, Indonesia	enrolled
20/2/2008	Bogor Agricultural University Bogor, Indonesia	graduated
17/8/2012	University of Arizona Tucson, United State of America	enrolled
30/5/2014	University of Arizona Tucson, United State of America	graduated
29/9/2015	Doctoral Program, Div of Environmental Engineering, Graduate school of Engineering, Hokkaido University	enrolled
29/9/2018	Doctoral Program, Div of Environmental Engineering, Graduate school of Engineering, Hokkaido University	coursework expected to complete

Professional background

1/3/2008	Agency for the assessment and application of technology	joined
30/12/2008	Agency for the assessment and application of technology	resigned
1/1/2009	The ministry of industry, Indonesia	joined
1/9/2014	Chemical engineering department, Lambung Mangkurat University, Indonesia	joined
1/6/2015	International training for trainer on integrated water Resource management, Wageningen University, The Netherlands	joined

Research background

20/2/2008	Department of Biology, Bogor Agricultural University, Screening of proteolytic bacteria isolated from Tilapia (Oreochromis niloticus) in inhibiting the growth of *Microcystis aeruginosa* BT-02 Involved during bachelor degree
1/6/2011	The ministry of Indonesia, The Using of fermented bamboo shoot as food supplement and modified flour (in Bahasa)
1/12/2011	The utilization of gemor bark (Alseodaphne sp.) and hazelnut (Aleurites Molucca) shell as natural mosquito coil (in Bahasa)
17/8/2012 ~	Soil, Water and Environmental Science, The University of Arizona Enteric and indicator virus removal by surface flow wetlands
30/5/2014	involved during Master's program
29/9/2015	Division of Environmental Engineering, Hokkaido University Population dynamics of enteric virus under a disinfectant pressure involved until now

Award/Prize

1/7/2013	First winner, Graduate division of Agriculture and Environmental Science Category Student Show Case 2013. President's Award, Student Show Case 2013. The University of Arizona "Assessment of pepper mild mottle virus (PMMoV) as a novel human fecal indicator in wetland wastewater treatment."
1/2/2014	Best research abstract award, 87th Arizona Water Association annual conference. "Virus reduction efficiency in wetland as an additional wastewater treatment in Arizona."
26/6/2014	Excellent presentation award, water and environment technology conference. Waseda University, Tokyo, Japan "Virus occurrence and attenuation in constructed wetland used for additional wastewater treatment in Arizona, United States"
1/8/2014	CAP award for research, 2015 AIPG and AHS National Conference

	"Virus reduction efficiency in wetland as an additional wastewater treatment in Arizona."
1/11/2014	Best poster presenter award, The 11th International symposium on southeast Asian water environment AIT, Bangkok, Thailand "Virus reduction in constructed wetland as an additional wastewater treatment for water reuse"
1/1/2015	Best young presentation award, 2nd international workshop, River basin environment and management, Hanoi, Vietnam "Virus reduction efficiency in wetland as an additional wastewater treatment in Arizona."

APPENDIX

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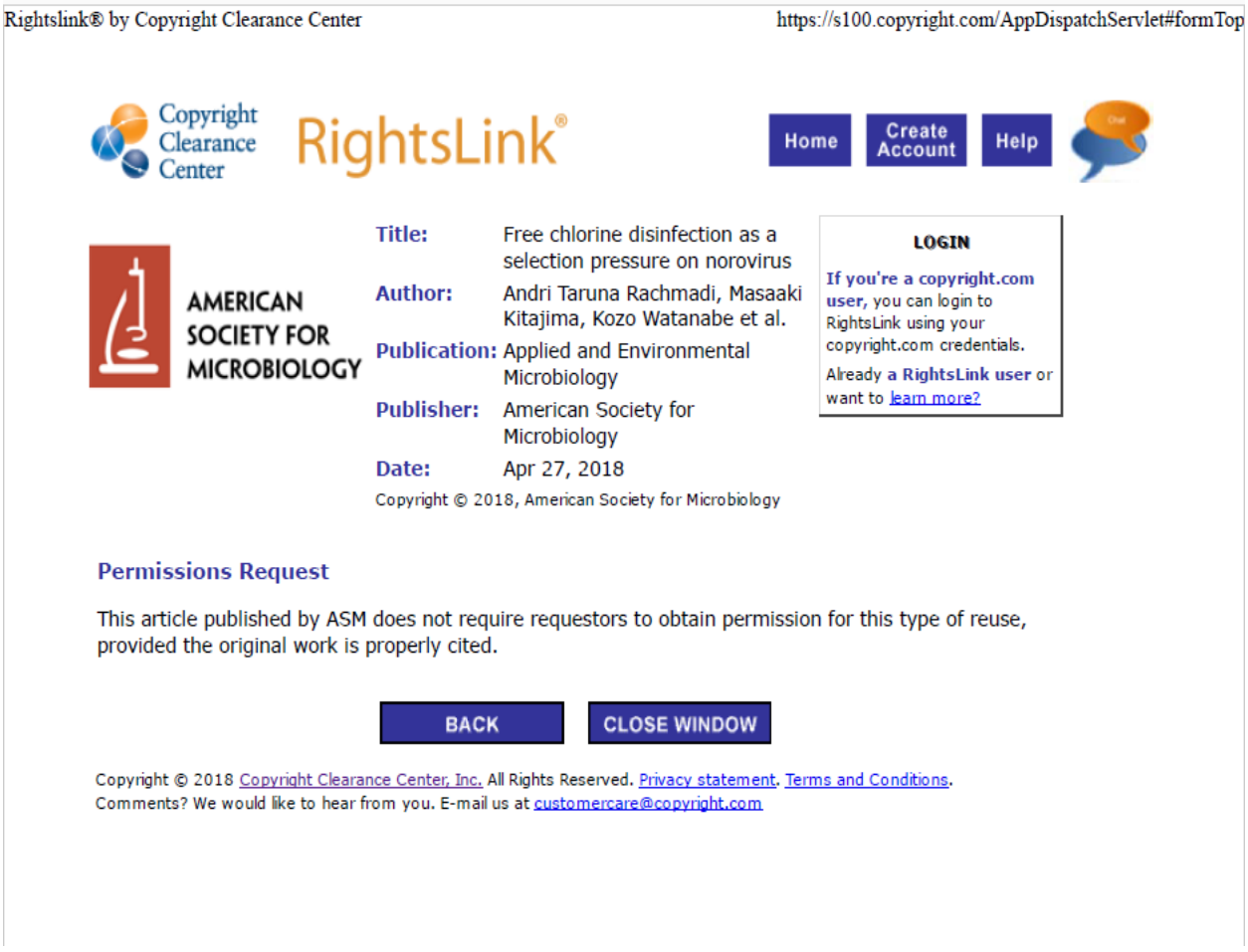

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